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

by

Tünde Fekete

Supervisor: Prof. Dr. Éva Rajnavölgyi

UNIVERSITY OF DEBRECEN

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ABBREVIATONS

AP-1: activator protein 1
APC: antigen presenting cell
ATF3: activating transcription factor 3
BDCA: blood dendritic cell antigen
BSA: bovine serum albumin
CARD: caspase recruitment domain
CCL: chemokine ligand
CCR: chemokine receptor
CD: cluster of differentiation
CD40L: CD40 ligand
cDC: conventional/classical/myeloid dendritic cell
cDNA: complementary DNA
CDP: common dendritic cell progenitor
CTL: C-type lectin receptors
CBP: CREB-binding protein
CREB: cAMP response element binding protein
DAMP: damage-associated molecular pattern
DC-SIGN: dendritic cell specific ICAM-grabbing non-integrin
DEPTOR: DEP domain-containing mTOR-interacting protein
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dsRNA: double-stranded RNA
EBI3: Epstein-Barr virus induced gene 3
4EBP-1: eukaryotic initiation factor binding protein
ECL: enhanced chemiluminescence
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
ET: endotoxin tolerance
FBS: fetal bovine serum
Flt3: fms-like thyrosine kinase 3
FKBP12: FK506 binding protein 12
GM-CSF: granulocyte-macrophage colony-stimulating factor
HIV: human immunodeficiency virus
HLA-DQ: human leukocyte antigen-DQ
hIEC: human intestinal epithelial cells
HKSA: heat-killed Staphylococcus aureus
HMGB1: high mobility group box 1
IAD: IRF association domain
ICAM-3: intracellular adhesion molecule 3
IDO: indoleamine 2,3-dioxygenase
IFN: interferon
IKK: IκB kinase
IL: interleukin
ILT: Ig-like transcript inhibitory receptors
IPC: IFN producing cells
IPS-1: IFNβ promoter stimulator 1
IRAK: IL-1 receptor-associated kinase
IRF: interferon regulatory factor
ISG: interferon-stimulated gene
JNK: c-Jun amino-terminal kinase
LBP: LPS-binding protein
LC: Langerhans cell
LGP2: laboratory of genetics and physiology 2
LILRB: leukocyte immunoglobulin-like receptor subfamily B member
LPS: lipopolysaccharide
LRR: leucin reach repeat
MAPK: mitogen-activated protein kinase
MD2: myeloid differentiation factor 2
MDP: macrophage-DC progenitor
MDA5: melanome differentiation-associated gene 5
MHC: major histocompatibility complex
miRNA: microRNA
MK2: MAPK-activated protein kinase 2
MKK3/6: MAPK kinase kinase 3/6
mLST8: mammalian lethal with SEC13 protein 8
moDC: monocyte-derived dendritic cell
mTOR: mammalian target of rapamycin
mTORc: mTOR complex
NAP1: NFκB-activating kinase-associated protein 1
NBD: nucleotid-binding oligomerization domain
NEMO: NFκB essential modulator
NFκB: nuclear factor kappa B
NLR: NOD-like receptors
PAMP: pathogen-associated molecular patterns
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
pDC: plasmacytoid dendritic cell
PD-L1: programmed cell death 1 ligand 1
PI3K: phosphatidylinositol-3 kinase
PIKK: PI3K-related kinase
PKC: protein kinase C
polyI:C: polyinosinic-polycystidylic acid
PPARγ: proliferator-activated receptor γ
PRAS: proline-rich Akt substrate
PROTOR: protein observed with RICTOR-1
PRR: pattern recognition receptor
Q-PCR: real time quantitative polymerase chain reaction
RA: retinoic acid
RAPTOR: regulatory associated protein of mTOR
RE: relative expression
RHEB: RAS homologue enriched in brain
RHO: RAS homologus
RICTOR: rapamycin-insensitive companion of mTOR
RIG-I: retinoic acid-induced gene I
RIP1: receptor interacting protein
R-LPS: rough LPS

RLR: RIG-I like receptor
RNA: ribonucleic acid
RORγt: retinoid-related orphan receptor γ t
RSK2: p90 ribosomal protein S6 kinase
RSV: respiratory syncytial virus
S6K1: S6 kinase 1
SD: standard deviation
SDS-PAGE**:** sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD: standard error of mean
siRNA: small interfering RNA
SIRS: systemic inflammatory response syndrome
SLAM: signaling lymphocyte activation molecule
S-LPS: smooth LPS
SOCS1: suppressor of cytokine signaling 1
ssRNA: single-stranded RNA
STAT3: signal transducer and activator of transcription 3
TAB1: TAK-binding protein
TAK: TGFβ-activated kinase 1
TBK1: TANK-binding kinase 1
TBS: Tris-buffered saline
Tc: cytotoxic T-lymphocytes
TGFβ: transforming growth factor β
Th: helper T- lymphocytes
TIRAP: TIR domain containing adaptor protein
TIR domain: Toll/IL-1R domain
TLR: Toll-like receptor
Tollip: Toll interacting protein
TRAF3: TNF receptor associated factor 3
TRAM: TRIF-related adaptor molecule
Treg: regulatory T-cells
TRIF: TIR-domain-containing adapter-inducing interferon-β
TSC: tuberous sclerosis complex
Ubc13: ubiquitin conjugating enzyme 13
Uev1A: ubiquitin-conjugating enzyme variant 1A

CONTENTS

SUPPLEMENTARY 1

Fekete T*, Szabo A*, Beltrame L, Vivar N, Pivarcsi A, Lanyi A, Cavalieri D, Rajnavölgyi E, Rethi B. Constraints for monocyte-derived dendritic cell functions under inflammatory conditions.

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SUPPLEMENTARY 2

Fekete T, Pazmandi K, Szabo A, Bacsi A, Koncz G, Rajnavölgyi E. The antiviral immune response in human conventional dendritic cells is controlled by the mammalian target of rapamycin

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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 named for their tree-like or dendritic shape were discovered in 1973 by Ralph Steinman and his colleagues. He dedicated his life to the characterization of DCs and identified their primary role as the priming of adaptive immune responses; therefore, he was awarded the Nobel Prize in Physiology or Medicine in 2011 [1].

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 [2].

Since the discovery of DCs several subtypes have been identified based on the origin and functional properties and major progresses have been accomplished in the understanding of differentiation and function of diverse DC lineages [1]. This process has tremendously been accelerated by the observation in the middle of 1990s that beside the common DC progenitors monocytes and hematopoetic stem cells can also serve as progenitors of DC differentiation. Thus, functionally competent DCs become available within a few days of differentiation in the presence of appropriate cytokines *in vitro* [3].

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 [4]. Recently, it has also been raised that DCs could be used as a tool to induce transplantation tolerance or to treat autoimmune disorders [5]. 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.

2. THEORETICAL BACKGROUND

2.1. 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 [2].

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) [6]. 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 [3].

Bone marrow-derived hematopoetic stem cells expressing the PU.1 transcription factor can differentiate into the macrophage-DC progenitor (MDP) that is the common precursor of monocytes, macrophages and DCs. In the presence of fms-like thyrosine kinase 3 (Flt3)-ligand (Flt3L) MDP can differentiate to the common dendritic cell progenitor (CDP), which exlusively give rise to DCs but not to monocytes or macrophages [6]. Furthermore, CDPs might generate plasmacytoid DCs, which accomplish their development in the bone marrow and the so called pre-DCs, the direct precursors of convetional DCs, that can travel to both lymphoid and non-lymphoid tissues and undergo terminal differentiation in the periphery (**Figure 2.1.)** [3].

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 [7]. 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 [6].

A functional heterogeneity within the DC population present in the lymphoid and non-lymphoid tissues has been described. There are two populations of resident DCs present in the lymph nodes, spleen and tonsil that correspond to the two blood subsets: pDCs and CD11c expressing cDCs. $CD141^+$ DCs have been identified in the human skin, lung and

liver in addition to $CD1c^+$ and $CD14^+$ DCs [6]. These cells exhibit enhanced ability to cross-present antigens to $CD8^+$ T-cells; therefore, $CD141^+$ DCs are regarded as the functional equivalent of $CD8^+/CD103^+$ mouse DCs. The $CD14^+$ DCs found in lymph nodes and skin may arise from classical monocytes and are usually referred to as the third subset of cDC. These cells express the dendritic cell specific ICAM-grabbing non-integrin (DC-SIGN) and also macrophage markers but lack the typical cDC markers such as CD1c or CD141. Thus, they are considered rather as monocyte- or macrophage-like cells than as DCs [8]. Beside $CD14^+$ DCs Langerhans cells (LC) can be found in the skin, where they form a network and express high levels of the C-type lectin langerin and CD1a, which has the capacity to present lipid antigens to T-cells. LCs are important in maintaining tolerance to commensals in the steady state via induction of regulatory T-cells and are also able to respond to selected pathogens under inflammatory conditions [8]. $CD1c^+$ DCs also express CD1a and display a high potential to present glycolipid antigens via CD1a and CD1c. These cells represent an important source of inflammatory cytokines and are potent stimulators of $CD4^+$ T-cells upon encounter with infectious agents.

In the intestinal tract lamina propria DCs consist of $CD103^+CX3CR1^-$ DCs derived from CDPs and $CD103^-CX3CR1^+$ DCs originating from monocytes [6]. Via producing transforming growth factor β (TGFβ) and retinoic acid (RA) human intestinal epithelial cells (hIEC) drive the generation of $CD103^+$ tolerogenic DCs, which under steady-state conditions promote the induction of regulatory T-cells (Treg). Contrary to their tolerogenic function, $CD103^+$ DCs can also initiate immune responses against pathogenic bacteria along with the $CD103^-$ DC subset [6]. pDCs are not present in quiescent tissues abundantly but under pathological conditions can rapidly be recruited to the sites of inflammation. This cell type is mainly characterised by the rapid production of high levels of type I interferons (IFN) in response to viral infection due to their feature pDCs are named natural type I IFN producing cells (IPC). It has also been reported that pDCs might be able to induce regulatory T-cells or tolerance, which could be connected to their ability to sense DNA released from apoptotic cells [8].

However, monocyte-derived or inflammatory DCs have been found first during inflammation, several recent papers have indicated their existence at very low numbers under steady state conditions. During infection moDCs are mainly involved in ameliorating

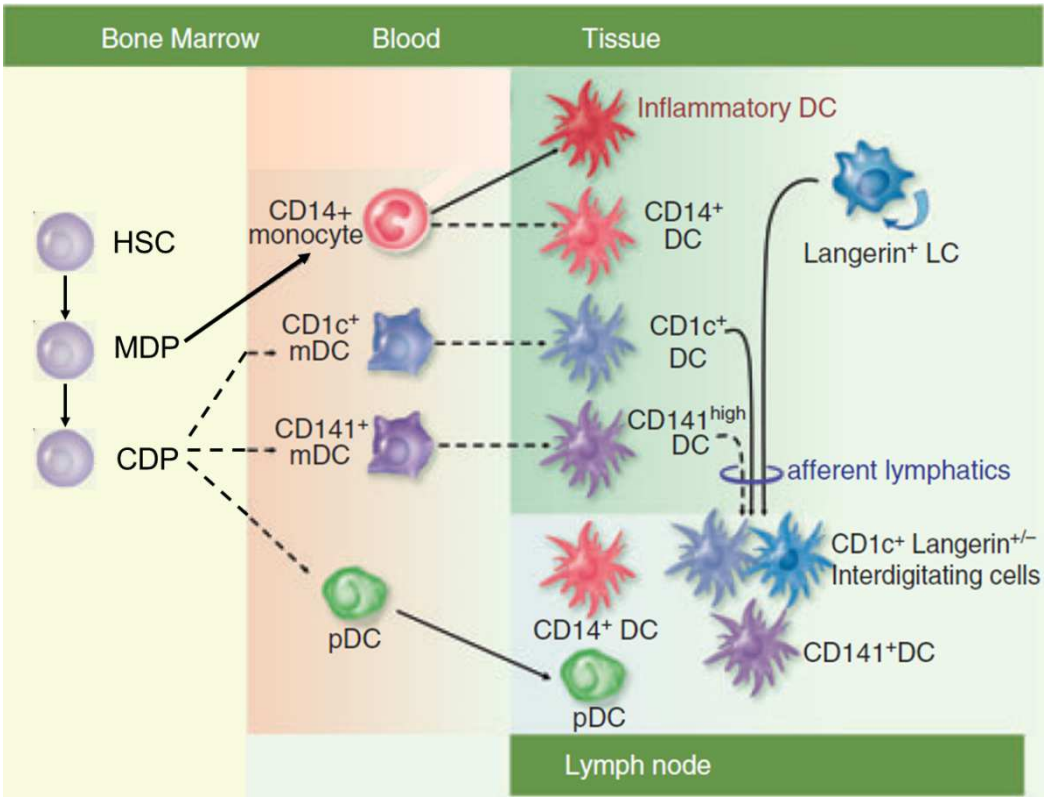

Figure 2.1. Origin and distribution of the major human dendritic cell subsets. *(modified from Matthew Collin et al. Immunology,* ***140****, 22-30, 2013)*

Human DCs originate from CDPs arising from HSC-derived MDPs. In peripheral tissues blood monocytes differentiate to CD14$^+$ DCs and inflammatory DCs, while circulating mDCs and pDCs are the precursors of their counterparts in the lymph nodes and tissues. Tissue mDCs and Langerhans cells can also arise from CD1c$^+$ interdigitating cells found in the T-cell area of lymph nodes. Dotted lines represent relationships requiring further confirmation in humans.

innate defense mechanisms and T-cell activation whereas cDCs appear to be critical for maintaining self tolerance [3]. Both cDCs and pDCs might migrate to the thymus to maintain central tolerance, where the two DC populations cooperate in the negative selection of self-reactive T-cells and positive selection of Tregs.

In the steady-state different peripheral tolerogenic mechanisms may operate in which DCs take an important part via producing anti-inflammatory cyoktines such as IL-10 and TGFβ and by expressing tolerogenic molecules such as indoleamine 2,3-dioxygenase (IDO) or Ig-like transcript inhibitory receptors (ILTs). IL-10 secreted by tolerogenic DCs can modulate the activation of neighbouring cDCs directly via downregulating the expression of costimulatory molecules and inhibiting the production of pro-inflammatory cytokines.

Furthermore, monocyte differentiation in the presence of IL-10 results in the generation of a DC population with high IL-10 producing and potent Treg inducing capacity. In addition TGFβ and the immunoregulatory enzyme IDO, both of which are also expressed by pDCs, can directly inhibit T-cell responses and proliferation, respectively [9]. Nevertheless, high amounts of pro-inflammatory cytokines produced during inflammation can lead to the down-regulation of IL-10 production and to the impairment of tolerogenic DC function. These counter-regulatory processes can alter the balance of tolerogenic and immunogenic DCs and thus may result in constitutive activation of self-reactive T-cells leading to chronic inflammation or autoimmunity [9]. Thus, DCs act 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.

2.2. Phenotypic and functional properties of *in vitro* generated DCs

DCs are extremely efficient in antigen uptake, processing and presentation to T-cells via the major histocompatibility complex (MHC) class I and II molecules. Further, DCs provide additional signals via upregulating costimulatory molecules and by secreting polarizing cytokines, the type of which highly determines the fate of T-cells (**Figure 2.2.**). The secretion of IL-12 during the priming of $CD4^+$ or $CD8^+$ T-cells results in the induction of T helper type 1 (Th1) cells that produce high levels of IFNγ, express the transcription factor T-bet and provide protection primarily against intracellular infections [10]. The IL-27 cytokine acts in synergy with IL-12 and drives antiviral immunity by amplifying IFNγ production and cytotoxicity in $CD8^+$ T-cells [11]. In contrast, IL-4 supports the generation of $CD4^+$ T helper type 2 (Th2) cells that express the transcription factor GATA-3 and produce cytokines, which enhance humoral immunity and protection against helminths and are also responsible for allergic reactions [12]. IL-1β, IL-6 and IL-23 can polarize T-cells into RORγt (retinoic acid-related orphan receptor γ) expressing and IL-17 producing T helper type 17 (Th17) cells that respond preferentially to extracellular bacteria and fungi and are also associated with several autoimmune diseases [13]. Cytokines such as IL-10, TGFβ and the recently identified IL-35 [14] promote the development of foxp3 expressing

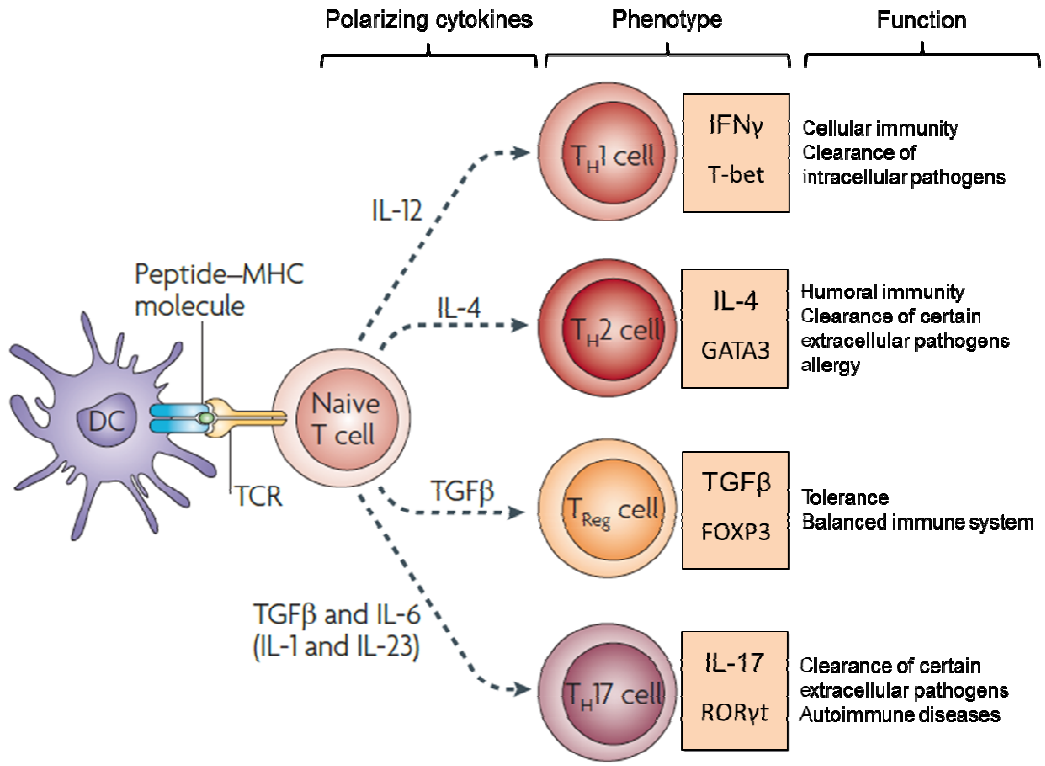

Figure 2.2. Dendritic cells control T-cell polarization. *(modified from Weiping Zou & Nicholas P. Restifo, Nature Reviews Immunology,* ***10****, 248-256)*

Antigen presenting cells such as dendritic cells can polarize T-cells to differentiate into diverse effector T-cell subsets. This differentiation is driven by the the local cytokine milieu and by a distinct set of transcription factors.

Tregs essential for controlling peripheral tolerance and maintaining immune homeostasis [15]. DCs are key regulators of T-cell polarization, thus have an impact on both the innate and adaptive arms of the immune system. These result lead to study the use of *ex vivo* generated DCs for the treatment of various diseases such as cancer or autoimmune disorders.

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 for finding more abundant progenitors [16]. It was demonstrated in 1994 that DCs derived from blood mononuclear cells possess the typical features of immature DCs developed *in vivo* [17]. 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. Monocyte-derived DCs represent overlapping biological activities, have specialized features and show functional differences that highlight the flexibility of *in vitro* generated DC populations. DCs generated in the presence of IL-15 produce high levels of Th17 differentiation-promoting cytokines and are potent inducers of antigen-specific $CD8^+$ T-cells upon TLR stimuli [18]. Type I IFNs such as IFNα1 and IFNβ also promote the transition of blood monocytes into DCs that efficiently take up apoptotic bodies and induce cross-priming of $CD8^+$ T-cells against certain antigens [19]. Monocytes can be converted to $CD14^+CD1a^{low}$ adherent cells by utilizing TNFα that are poor stimulators of T-cells; however, upon stimulation by lipopolysaccharide (LPS) they may differentiate to mature DCs, which control both Th1 and Th17 responses [20]. IL-4 is the most widely used cytokine applied in combination with GM-CSF in the course of monocyte to DC transition as it exerts an inhibitory effect on macrophage differentiation and promote DC development [21].

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 [22, 23]. 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 (**Figure 2.3.**) [22]. An other 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 [24]. During their *in vitro* differentiation moDCs downregulate the monocytic marker CD14 and upregulate the DC-specific 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 (**Figure 2.3.**). 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 Th1 and cytotoxic T-lymphocytes (Tc) [16]. On the other hand addition of TGFβ, IL-10 or corticosteroids results in the induction of tolerogenic DCs that can induce both $CD4^+$ and $CD8^+$ Tregs.

It has been described in our laboratory that monocytes cultured in the presence of GM-CSF and IL-4 can differentiate into phenotypically and functionally distinct DC subsets: $CD14^+CD1a^-$ or $CD14^-CD1a^+$ moDCs that are often represented in different ratios in the same cultures [25]. These DC subsets have the same migratory potential however, they differ in their functional properties. $CD1a^+$ DCs can produce high levels of IL-12, a cytokine required for polarizing the immune response towards strong cell mediated toxicity whereas $CD1a^-$ DCs mainly produce IL-10, an anti-inflammatory cytokine and possess strong phago/endocytic activities. The ratio of $CD1a^+$ and $CD1a^-$ cells is strongly affected by the presence of lipoproteins in the culture of differentiating DCs, namely by the ligands of peroxisome proliferator-activated receptor γ (PPARγ) that skews moDC differentiation toward the generation of $CD1a^-$ cells by inhibiting the development of $CD1a^+$ cells [25].

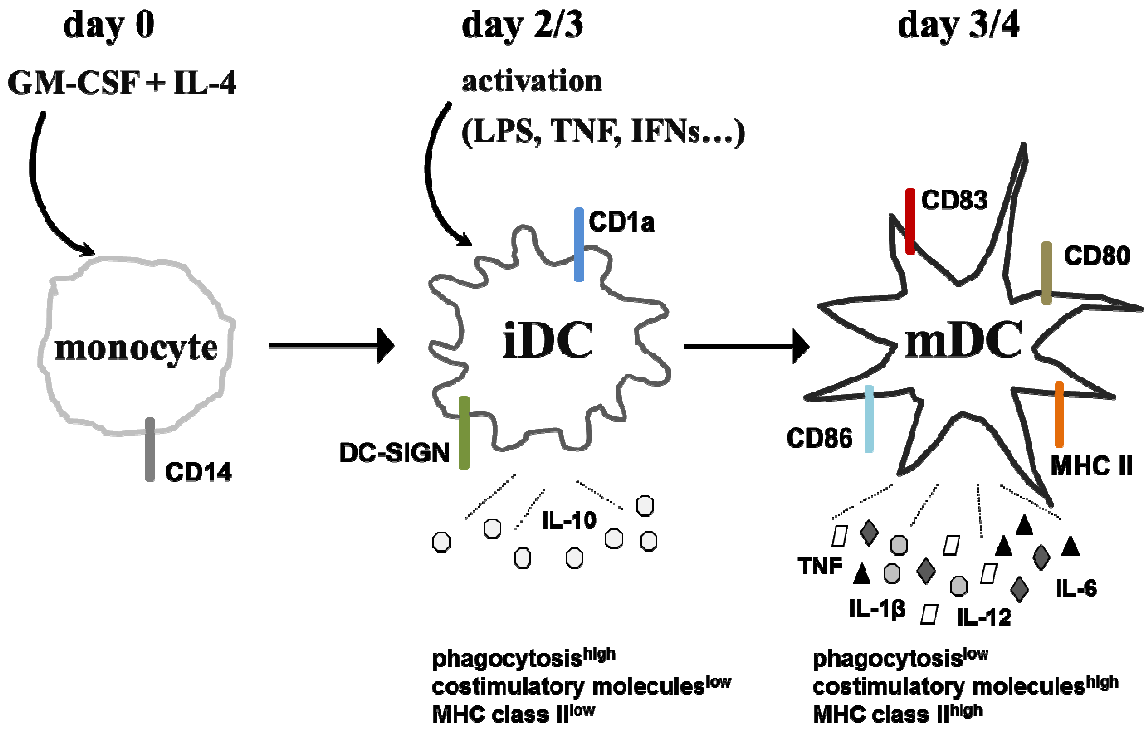

Figure 2.3. Generation and maturation of „fast" dendritic cells.

In the presence of IL-4 and GM-CSF isolated monocytes can differentiate into immature dendritic cells within 2-3 days. Fully mature DCs might be acquired upon stimulation with microbial ligands or inflammatory cytokines.

We initiated experiments to clarify the factors responsible for the observed differences and found an extremely strong influence of cell culture density on moDC phenotype and functionality. Higher density led to higher ratios of $CD14^{+}CD1a^{-}$ DCs that produced IL-10, a suppressor cytokine but no IL-12 upon stimulation by microbial ligands. Decreasing cell densities resulted in increasing ratio of $CD14^{-}CD1a^{+}$ DCs and cell dilutions far beyond the widely used culture densities eliminated IL-10 production and primed the cells to produce very high levels of IL-12, IL-23 and TNF. Further, we found that lactic acid, a side product of glycolytic metabolism, accumulated in dense cultures is the responsible factor for the density-dependent DC regulation [26]. These results suggest that metabolic pathways might be efficiently manipulated to modulate the immunogenicity or tolerogenicity of *in vitro* generated DCs.

2.3. 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. The definition of PAMPs for molecular products recognized by PRRs was proposed by Charles Janeway in the late 1980s. His innovative hypothesis has been supported 10 years later by a study demonstrating the essential role of Toll receptors to initiate immunity against fungal infection in *Drosophila melanogaster*. In 1996 the homologue of the Toll was identified in human cells that is known today as Toll-like receptor 4 (TLR4) and its ability to induce innate responses was also demonstrated subsequently [27]. Later on Polly Matzinger indicated the Danger Model suggesting that PRRs can also detect endogeneous molecules derived from damaged cells, referred to as damage-associated molecular patterns (DAMPs) [28, 29]. PAMPs and DAMPs can initiate similar signaling pathways by inducing the transcription of genes involved in innate inflammatory immune responses (**Figure 2.4.**). 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) (**Table 1**.) [30].

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 [30]. 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 [27]. 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.

PRRs	Localization	Ligand	Origin of the ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	bacteria
TLR2	Plasma membrane	Lipoprotein	bacteria, viruses, parasite
TLR3	Endolysosome	dsRNA	viruses
TLR4	Plasma membrane	LPS	bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	bacteria, viruses
TLR7	Endolysosome	ssRNA	viruses, bacteria, self
TLR8	Endolysosome	ssRNA	viruses, bacteria, self
TLR9	Endolysosome	CpG-DNA	viruses, bacteria, protozoa, self
TLR10	Plasma membrane	unknown	fungi, bacteria, viruses
RLR			
RIG-I	Cytoplasm	short dsRNA, 5' triphosphate dsRNA	viruses
MDA5	Cytoplasm	long dsRNA	viruses
LGP2	Cytoplasm	unknown	viruses
NLR			
NOD1	Cytoplasm	diaminopimelic acid	bacteria
NOD2	Cytoplasm	muramyl dipeptide	bacteria
CLR			
Dectin-1/2	Plasma membrane	beta-glucan	fungi
DC-SIGN	Plasma membrane	mannose	fungi, bacteria, viruses

Table 1. Selected human pattern recognition receptors and their ligands

TLR3, TLR7, TLR8 and TLR9 are localized to intracellular vesicular compartments and detect nucleic acids such as single-stranded or double-stranded ribonucleic acid (ssRNA or dsRNA) and deoxyribonucleic acid (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 [31]. TLR10 has also been identified as an innate sensor of viral infections; however, its specific ligand is still under investigation [32]. Ligand binding of TLRs results in the recruitment of one or more adaptor molecules and 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 responses [33].

The various subsets of DCs differ significanlty in the expression of TLRs. To detect pathogenic structures human cDCs use various TLRs localized to both the cell membrane and the endosomal compartments [34]. cDCs express TLR1, 2 and 6, which upon formation of heterodimers, show a high capacity to bind a wide range of bacterial and fungal ligands leading to DC maturation and secretion of pro-inflammatory cytokines. Upon encounter with the bacterial cell wall component LPS cDCs induce the production of IL-12 confirming the expression of TLR4 in this DC subset. cDCs also express TLR5 on their surface that mainly recognizes and responds to bacterial flagellin by upregulating pro-inflammatory cytokines. The expression of TLR3 (specific for dsRNA) and TLR8 (specific for ssRNA) is also evident in cDCs; however, the presence of TLR7 that also binds viral ligands is still questionable. In contrast, pDCs abundantly express TLR7 and uniquely express TLR9 in their endosomal compartments. Triggering of these receptors leads to the secretion of high levels of type I IFNs but not of pro-inflammatory cytokines. Other receptors such as TLR1 or TLR10 are not or minimally expressed by pDCs and their function is yet to be identified [33]. In conclusion, these data suggest that cDCs express all intracellular TLRs except TLR9 and pDCs respond only to TLR7 and TLR9 stimuli by secreting high amounts of type I IFNs.

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 [28]. 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 [35]. The dendritic cell specific ICAM-grabbing non-integrin (DC-SIGN, also known as CD209) is broadly expressed on the surface of professional antigen presenting cells and binds with high affinity to intracellular adhesion molecule-3 (ICAM-3). DC-SIGN also acts as a receptor for several viruses such as human immunodeficiency virus type 1 (HIV-1), thus porposed to enhance the transmission of the virus to host T-cells [36]. However, the precise role of DC-SIGN in *in vivo* HIV dissemination by DCs remained to be elucidated.

NLRs are cytoplasmic pathogen sensors with multidomain structure composed of a central nucleotid-binding oligomerization domain (NBD) and a C-terminal LRR sensor domain [37]. 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 [28]. 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 [38].

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 [27]. Intriguingly, beside detecting viral RNA genomes RLRs also act as sensors for cytoplasmic DNA [39] and collaborate with TLRs and other signaling networks to modulate innate and adaptive immune responses [40].

The members of the RLR family are DexD/H box RNA helicases organized into three functional domains. There is a central DexD/H box RNA helicase domain that catalysis ATP hydrolysis and binding/unwinding RNA and a C-terminal regulatory domain that is involved in the autoregulation processes of RIG-I [41]. RIG-I and MDA5 have two caspase recruitment domains (CARD) at the N-terminal region required for initiating the downstream signaling pathway. As LGP2 lacks the N-terminal CARD domains it is unable to transmit signals in the absence of the other receptors and rather acts as a regulator in the RIG-I- and MDA5-mediated signaling [40]. Knockout studies revealead the essential role of LGP2 in MDA5-induced type I IFN production whereas it was proved to be indispensable in RIG-I-mediated signaling [42].

RIG-I and MDA5 possess different ligand specificity, although some viruses might be detected by both of them. The ligands of RIG-I and MDA5 have extensively been studied during the previous years and it has been observed that the size and form of RNA as well as the level of phosphorylation determines the RNA sensor engaged to viral recognition. MDA5 is specialized in the recognition of dsRNA. Initially, RIG-I was characterized as a sensor for dsRNA that triggers type I IFN production in response to viruses or the synthetic dsRNA polyinosinic-polycystidylic acid (polyI:C). Later on, studies revealed that the presence of RNA sequences marked with 5’triphosphorylated (5’ppp) ends can greatly enhance the type I IFN producing capacity of RIG-I [43]. It has been concluded that RIG-I can recognize ssRNA with at least one phosphate at the 5’ end of RNA but 5’ppp is required to trigger optimal signaling. Moreover, results comparing the interaction of RIG-I and MDA5 with synthetic dsRNA have suggested that RIG-I preferentially detects short

dsRNA, while MDA5 shows a preference for long dsRNA [44]. Thus according to our present knowledge MDA5 is the key sensor for longer viral dsRNA fragments, while RIG-I detects ssRNA or short dsRNA genom. Host RNAs are single-stranded and are capped by methylation at the 5' end, thus are prevented from recognition by cytosolic RNA sensors [45].

However, RIG-I and MDA5 exhibit different ligand preference, share common signaling features. Once RIG-I and MDA5 is activated by the detection of viral PAMPs, they are recruited to the mitochondrial outer membrane by the adaptor protein IFNβ promoter

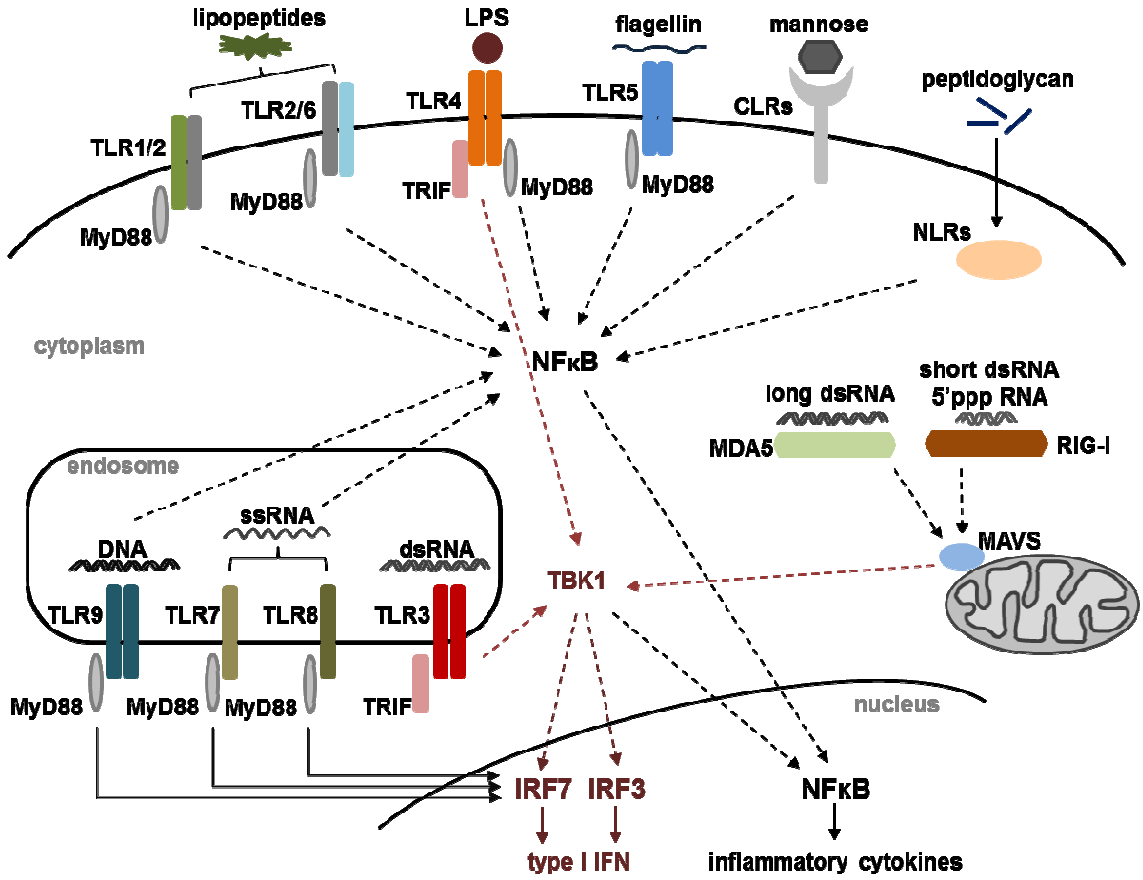

Figure 2.4. Distribution and cooperation of PRRs in dendritic cells.

TLRs, NLRs, RLRs and CLRs are expressed on the cell surface, in the endosomes, or in the cytosol of various types of immune cells. Activation by their specific ligands induces the NFκB and IRF3/IRF7 transcription factors, thus resulting in the production of inflammatory cytokines and type I IFNs, respectively.

stimulator 1 (IPS-1, also known as MAVS). This assotiation results in the activation of TRAF family member-associated NF-kappa-B activation (TANK)-binding kinase 1 (TBK1) and the non-canonical IκB kinase ε (IKKε) and signals IRF3/IRF7 and NFκB responsive genes, such as type I IFNs and inflammatory cytokines, respectively [41].

It is important to note that both RIG-I and MDA5 are absent or expressed at low levels in resting moDCs; however, upon induction by various stimuli such as LPS or polyI:C their expression can be highly upregulated [46]. Studies performed with pDCs indicated that RIG-I/MDA5 signaling is dispensable to the production of type I IFNs, since these cells rely predominantly on the TLR7 pathway to induce antiviral defense mechanisms [45]. Recent *in vivo* studies with mice infected by West Nile virus indicated that RLRs are the primary sensors of virus infection, which upon triggering the production of IFNs enhance TLR expression and potentiate the TLR-coupled signaling pathway [41]. Thus, the crosstalk between the TLR and RLR signaling cascades serves to induce optimal production of antiviral cytokines and to enhance effector mechanisms such as controlling viral replication or suppressing viral dissemination and further infection of tissues [41].

2.4. The TLR3 signaling pathway and its role in antiviral immune responses

TLR3 is a major inducer of antiviral responses as it responds to double stranded RNA (dsRNA) that is the genetic material of a group of viruses and also a by-product of viral replication. However, it is still unclear whether the dsRNA produced during viral infection is able to stimulate TLR3 [47]. While human moDCs and $CD11c^+$ blood DCs express TLR3 in the cytoplasm only, it may also localize to both the cell membrane and the endosome in epithelial cells and fibroblast [48]. It has also been demonstrated that cell-surface TLR3 co-localizes with CD14, that binds polyI:C directly and mediates its uptake to intracellular compartments, thus enhancing TLR3-mediated signaling in murine bone marrow-derived macrophages [49]. As human DCs do not express TLR3 and CD14 on the cell surface, they internalize dsRNA delivered to the cell membrane via clathrin-mediated endocytosis [50]. Addition of type I IFNs or dsRNA to human DCs upregulates the expression of TLR3 and induces the redistribution of the receptor from the endoplasmic reticulum (ER) to endosomes [50]. Once dsRNA is recognized TLR3 becomes phosphorylated at the cytoplasmic tail that induces a cascade of downstream signaling events resulting in the activation of IRF3, NFκB and activator protein-1 (AP-1) (**Figure 2.5.**) [51].

TLR3-mediated signaling occures via recruitment to the adaptor protein TRIF followed by the formation of a complex composed of several adaptors and kinases [52]. TNF receptor associated factor 3 (TRAF3) has been proposed to act as a bridging molecule between TRIF and TBK1 that together with IκB kinase-related kinase ε (IKKε, also known as IKKi) is responsible for the phosphorylation of IRF3 and IRF7 [51]. TBK1 deficent mice show reduced activation whereas TBK1 and IKKε double-deficient embrionic fibroblasts show a complete loss of IRF3 activation and IFNβ production upon stimulation with TLR3 and TLR4 ligands, which findings indicate the essential role of these kinases in initiating type I IFN responses [53]. In addition to the TRAF3 adaptor protein, the NFκB-activating kinase-associated protein 1 (NAP1) has also been implicated in the interaction of TRIF and IRF3 [54]. It has also been reported that phosphatidylinositol-3 kinase (PI3K) recruitment to specific tyrosine residues in the cytoplasmic domain of TLR3 is also necessary to full phosphorylation and activation of IRF3 [47]. In this experiment performed with HEK293 cells mutation of TLR3 at residues Tyr759 and Tyr858 or the functional blockade of PI3K resulted in the inhibition of the interferon stimulated gene 56 (ISG56) expression induced by dsRNA [47]. This observation was further supported by the finding that the PI3K/Akt signaling pathway is involved in the regulation of immune responses to dsRNA and influenza A virus infection in lung epithelial cells [55]. Thus, IRF3 activation is proposed to rely on both the TBK1- and the PI3K-dependent pathways. Phosphorylated IRF3 molecules associate by dimer formation via the IRF association domain (IAD) then translocate to the nucleus and bind to specific DNA sequences with the aid of the co-activator CREB-binding protein (CBP) to induce the production of type I IFNs, which are important in antiviral innate immune responses [51].

Several reports demonstrated that type I IFNs such as IFNα and IFNβ can be induced both at mRNA [56] and protein level [57] in moDCs upon stimulation with polyI:C. It has also been indicated that IFNα/β genes are induced in a biphasic manner: in the early phase of virus infection the IRF3-dependent IFNβ production results in the strong induction of IRF7 whereas in the late phase IRF3 cooperates with IRF7 to potentiate the induction of IFNα/β genes [58]. It has also been shown in mouse cDCs that the IFN feedback response requires the contribution of IRF8 for the induction of a second phase transcription of IFN genes [59]. In addition to type I IFN secretion cDCs are proposed to be the main producers of type III IFNs as well (IFNλ1-3) [56, 60]. Even though IFNλs differ genetically from type I IFNs, a similar mode of regulation has been suggested for both cytokines. Similarly to IFNβ, the expression of IFNλ1 is regulated by IRF3 and IRF7 whereas IFNλ2/3 is mainly

controlled by IRF7, thus resembeling IFNα genes [61]. Type I and III IFNs activate overlapping signaling routes such as the Januse kinase/signal transducer and activator of transcription (JAK/STAT) pathway and induce the upregulation of common IFN-stimulated genes (ISG) to promote potent antiviral immune responses [60].

In addition to protection against viruses, type I and III IFNs exert a strong effect on cell survival, proliferation and differentiation [62] and may induce antitumor responses [60]. Even though IFNα/β receptors are widely distributed on various cell types, only a narrow fraction of cells acquired the ability to respond to IFNλs such as pDCs and epithelial cells [60, 62]. In pDCs IFNλ1 synergizes with IFNα to upregulate the expression of CD80, CD83 and modulates the expression of chemokine receptor 7 (CCR7), CD62 ligand homing molecules all contributing to pDC activation [63]. Interestingly, moDCs acquire tolerogenic properties in response to IFNλ and induce proliferation of foxp3 expressing $CD4^+CD25^+$ T-cells in an IL-2-dependent manner [64].

Beside type I and III IFNs the production of IL-27, a pleiotropic cytokine having both anti-inflammatory and antiviral activity also requires the TRIF-dependent activation of IRF3 [65]. Based on its structure and functions IL-27, composed of the EBI3 and p28 subunits, belongs to the IL-12 family cytokines [11]. $CD1c^+$ DCs produce high amounts of IL-12 and IL-27 upon induction with viral dsRNA. Whereas IL-12 effectively upregulates the expression of inflammatory cyoktines such as IFNγ, IL-27 induces the proliferation and cytotoxicity of naive $CD8^+$ T-cells [11]. In human macrophages the TLR-dependent induction of IL-27 relies on the induction of IFNα posing a direct autoregulatory mechanism for the transcriptional activation of p28 [66]. However, further studies are required to clarify the exact regulatory role of IL-27, especially under *in vivo* conditions. Nevertheless, these recent data favour the concept of a functional relationship between IL-27 and type I/III IFNs in the fight against viral infections.

Activation of the NFκB pathway by TLR3 stimulation can be driven by two distinct adaptor molecules, TRAF6 and receptor interacting protein 1 (RIP1). Currently there is no evidence for the involvement of RIP1 in TLR3-mediated signaling in DCs [51]. Association of TRAF6 to TRIF results in the recruitment of TGFβ-activated kinase 1 (TAK1) and TAK-binding protein 1, 2 and 3 (TAB1/2/3). TAK1 then becomes phosphorylated and activates the IKK complex composed of IKKα, IKKβ and NFκB essential modulator (NEMO or IKKγ). IKKβ then phosphorylates the NFκB inhibitor IκB leading to its degradation, thus allowing the release and nuclear translocation of NFκB. In human DCs two main NFκB pathway exist: the canonical/classical and the non-

canonical/alternative signaling cascades. TLR ligation in most cases results in the induction of the canonical pathway that depends on IKKβ and NEMO and leads to the nuclear translocation of heterodimers composed of p65 (RelA) and p50 [67]. Other signals, such as lymphotoxin-β, TNF or CD40 ligand (CD40L) activate the alternative pathway, which requires IKKα that via phosphorylation of p100 leads to the generation of RelB and p52 complexes [68]. Activation of this alternate pathway results in the production of chemokines, whereas the classical pathway is responsible for the induction of inflammatory cytokines. Inhibition of the NFκB pathway impairs the expression of T-cell stimulatory molecules such as CD80 and CD86, indicating a link between this signaling route and DC maturation [69]. Down-modulated expression of TRIF and TRAF6 in human $CD1c^+$ DCs derived from HCV patients was found to be accompanied with decreased production of TLR3-mediated IFNβ, TNF and IL-12. These data indicate the importance of the TRAF6-regulated NFκB pathway in the production of innate cytokines [70].

TAK1 also has the capability to induce the downstream activation of MAPK kinase kinase 3 and 6 (MKK3/6) leading to the phosphorylation of p38, Jun kinases (JNK) and cAMP response element binding protein (CREB) culminating in the activation of AP-1 [52]. It has been shown that AP-1 activation occuring upon TLR3 stimulation of human moDCs requires the control of the MAPK signaling cascade [48]. In response to dsRNA the transcription factor c-Jun and the proto-oncogene c-fos are phosphorylated and form the AP-1 heterodimer. This process is accelerated by JNK that phosphorylates c-Jun thus potentiating the transcription of its target genes [71, 72]. The activation of NFκB and AP-1 contributes to the induction of pro-inflammatory cytokines such as IL-6, IL-12, TNF and chemokines such as chemokine ligand 3 (CCL3) [52]. In addition, several members of the MAPK family such as JNK, MKK3 and p38 can also contribute to the induction of type I IFNs. Studies using $MKK3^{-/-}$ mouse embryonic fibroblasts defined the essential role of MKK3 in the generation of type I IFN responses [73]. Inhibitory studies also revealed that activation of p38 together with JNK contributes to IFNβ gene expression [74]. Furthermore, it has recently been reported that TLR3-elicited activation of p38 and MAPK-activated protein kinase 2 (MK2) is required for the stabilization of IFNβ mRNA, that may be important in the initial phase of antiviral responses [75]. In addition to the above described outcomes TLR3 mediates the induction of several other cytokines such as IL-10, IL-15 [76], chemokines such as CCL4 and CCL5 [76] and receptors such as RIG-I and MDA5 [46]. The versatile role of TLR3 to transmit high numbers of different signals upon recognition of dsRNA highlights the functional complexicity of this receptor.

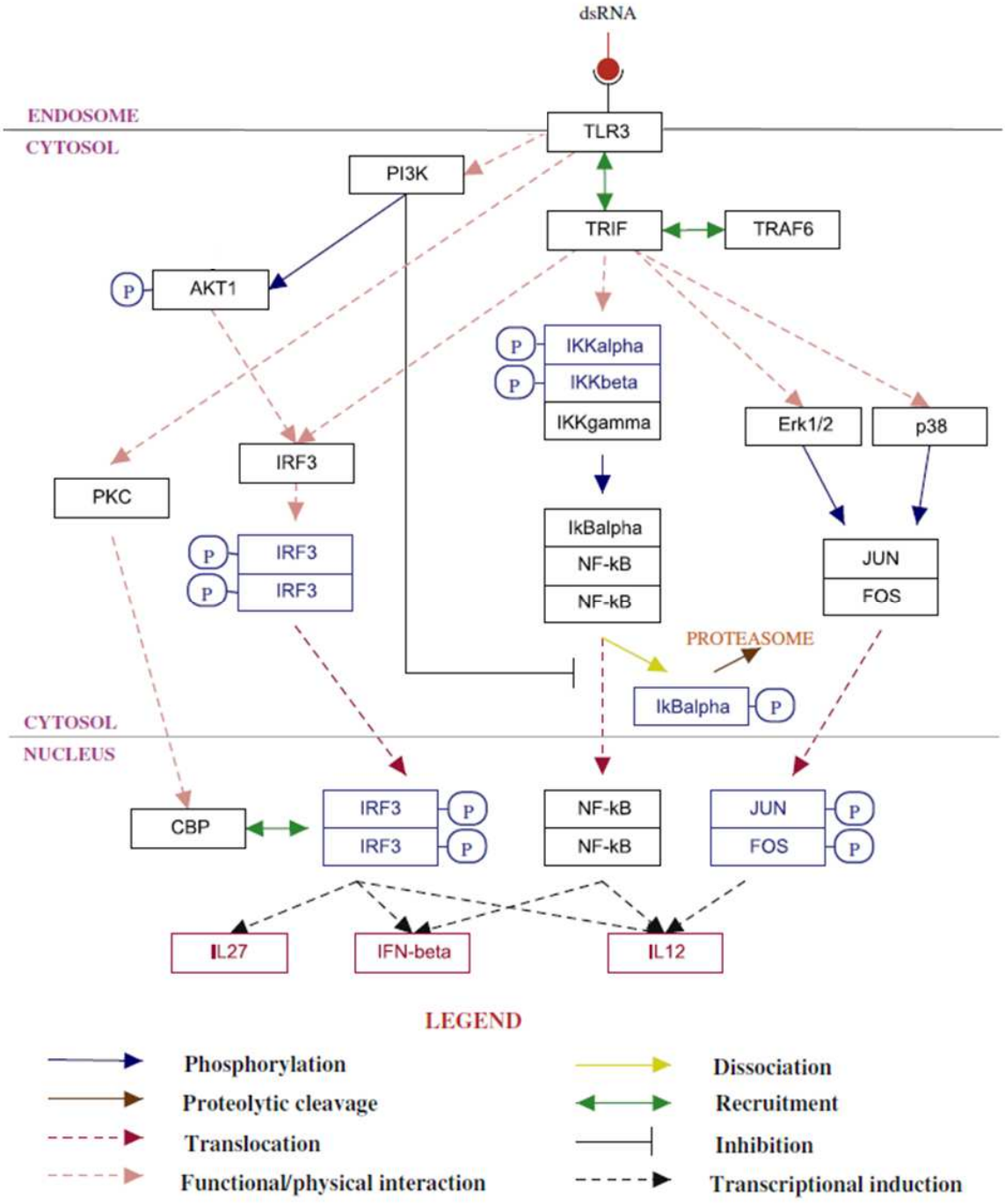

Figure 2.5. Schematic representation of the TLR3-signaling pathway in DCs. *(M. Gauzzi et al. Immunobiology,* ***215****, 713-723, 2010)*

In response to dsRNA TLR3 activates the TRIF-dependent signaling pathway resulting in the activation of the IRF3, NFκB and AP-1 transcription factors that promote the expression of type I IFNs and other pro-inflammatory cytokines.

2.5. The role of MyD88-dependent TLR4 signaling pathway in the induction of inflammatory responses

TLR4 is essential fo the detection of LPS (endotoxin), a major component of the outer membrane of Gram-negative bacteria. Recognition of LPS can result in systemic inflammation and sepsis if excessive signal occures. Smooth LPS (S-LPS) produced by most Gram-negative bacteria consists of three regions: the core oligosaccaharide, the O-specific chain and the lipid A tail, which is the main PAMP responsible for the endotoxic activity of the molecule [27]. Rough-mutant Gram-negative bacteria produce R-form LPS (R-LPS) that lacks the O-specific chain. This structural difference modifies the stimulatory capacity of LPS, since R-LPS readily activates the TLR4 pathway, while S-LPS requires the help of additional factors such as CD14 and LPS-binding protein (LBP) [77]. LPS from wild type bacteria represents a heterogeneous mixture of the two forms usually containing a higher proportion of S-LPS. Intriguingly, some Gram-negative WT bacteria such as *Clamydia* and *Neisseria* species produce LPS with reduced sugar residues, thus resembeling the R-form LPS. In addition to Gram-negative bacteria TLR4 is implicated in the recognition of a wide range of ligands such as heat-shock proteins, the fusion protein of respiratory syncytial virus (RSV) and cell wall components of fungi or protozoan parasites [78].

Since TLR4 is not able to bind LPS directly its activation requires the participation of several molecules. Upon association with myeloid differentiation factor 2 (MD2) TLR4 forms the surface receptor complex required for the recognition of the lipid A compartment of LPS. The presentation of LPS to the receptor complex is facilitated by LBP and membrane-bound or soluble form of CD14 [79]. LBP acts as a shuttle protein via directly binding LPS and facilitating its association with CD14 that transfers LPS to the TLR4/MD2 complex [80]. MD2 binds directly to LPS and causes conformational changes in TLR4 leading to its dimerization or oligomerization and subsequent activation of diverse downstream signaling pathways [80]. TLR4 then recruits various TIR-domain containing adaptor proteins, thus mediating the induction of two major signaling pathways, one that depends on the adaptor protein MyD88 and the other requiring the recruitment of TRIF (**Figure 2.6.**). The MyD88-dependent pathway was shown to be resposible for the pro-inflammatory cytokine production, whereas the TRIF-dependent pathway mainly regulates the induction of type I IFNs and ISGs [27].

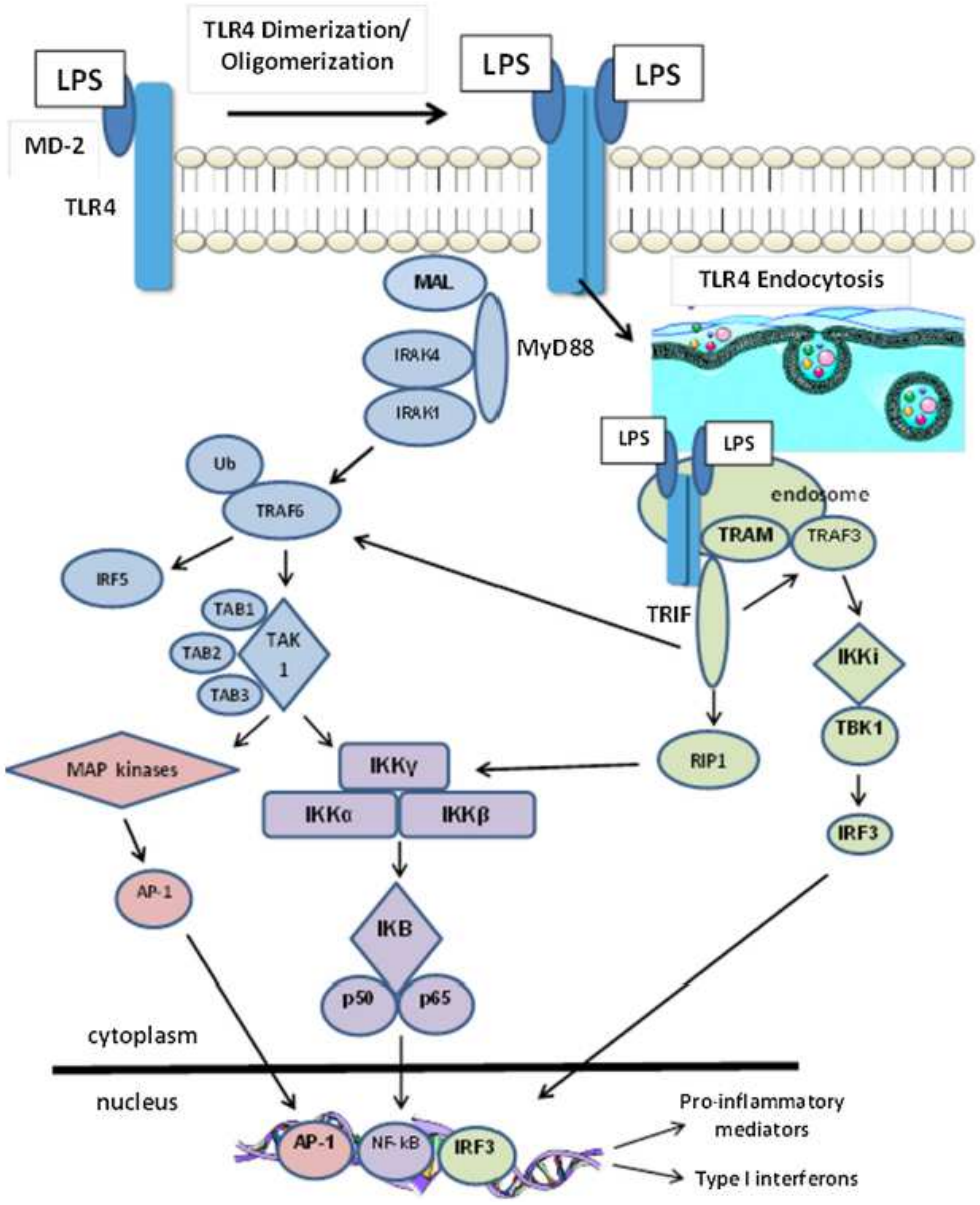

Figure 2.6. TLR4 signaling pathway. *(Bohannon et al. SHOCK,* ***40****, 451-462, 2013)*
The TLR4/MD2 complex expressed on the cell surface recognizes LPS leading to the activation of both the MyD88-dependent and TRIF-dependent signaling pathways. The MyD88-dependent pathway is responsible for the activation of the NFκB and MAPK pathway, whereas the TRIF-dependent pathway activates IRF3 resulting in the induction of type I IFNs.

The MyD88-dependent pathway requires the recruitment of the TIR domain containing adaptor protein (TIRAP) to the TLR4/MD2 complex, that facilitates the association between MyD88 and the cytoplasmic domain of TLR4. MyD88 recruits and activates the IL-1 receptor-associated kinase 4 (IRAK-4), which is involved in the signaling of several

TLRs as shown by the almost complete abrogation of TLR2-, TLR3-, TLR4- and TLR9-induced responses in IRAK-4 knock-out mice [81]. IRAK-4 is responsible for the subsequent recruitment and activation of IRAK-1, that together with IRAK-2 and IRAK-M (IRAK-3) belongs to the IRAK family. Initially, IRAK-1 was thought to be crucial for the activation of the TLR4 pathway; however, its downregulation inhibited only partially the LPS-induced pro-inflammatory cytokine production suggesting the involvement of other molecules in the downstream signaling of TLR4 [82]. IRAK-2 is also proposed to play a positive role in LPS-triggered TLR4 signaling whereas IRAK-M together with toll interacting protein (Tollip) block the IRAK-1 and IRAK-4 interaction and the phosphorylation and activation of IRAK-1 [83]. The activation of IRAK-1 results in TRAF6 activation, which forms a complex with the E2 ubiquitin complex Ubc13 (ubiquitin conjugateing enzyme 13) and Uev1A (Ubiquitin-conjugating Enzyme Variant 1A) to promote the activation of TAK1 [80]. TAK1 subsequently induces the activation of the NFκB and MAPK pathways as indicated previously.

The TRIF-dependent pathway of TLR4 requires the interaction with the TRIF-related adaptor molecule (TRAM) responsible for TRIF activation [28]. Recently, it has been found that following receptor internalization TRAM drives the LPS-induced translocation of TLR4 to the endosome that is essential for the activation of endosomal TRIF-TRAF3 [84]. Further, the authors suggested a sequential activation of the two signaling pathways. TLR4 first activates TIRAP-dependent MyD88 signaling at the cell surface, then following endocytosis it induces TRAM-TRIF signaling from the early endosomes. Similarly to TLR3, TLR4-mediated TRIF-dependent signaling cascade leads to the activation of the NFκB and IRF3 pathways resulting in the induction of pro-inflammatory cytokine and type I IFN genes [80].

2.6. Molecular mechanisms associated with endotoxin tolerance

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 renders innate immune cells such as DCs and macrophages hyporesponsive to subsequent challenges with LPS [85]. 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 [79]. Due to these effects ET is considered to be a protective mechanism against repeated exposure to Gram-negative infections; however, its incidence is associated with an elevated risk of secondary infections and mortality [85]. Intriguingly, hyporesponsiveness to LPS can also be induced by pre-exposure to other TLR ligands causing cross-tolerance or heterotolerance. For example Pam3Cys, a synthetic TLR2 ligand renders macrophages and DCs tolerant to subsequent challenges with LPS or Pam3Cys by this mechanism [86, 87]. Pre-stimulation of murine bone-marrow derived mast cells with LPS displays tolerance to stimulation by TLR2, TLR4 and FcεR1 that draws our attention to the fundamental role of mast cells in the modulation of immune responses associated with sepsis [88].

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 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.

SOCS1 is strongly induced by cytokines such as IFNγ, IL-4, IL-6 and inhibits signal transduction by suppressing the JAK/STAT-mediated signaling cascade. SOCS1 also acts as a negative regulator of the TLR4-induced MyD88-dependent and MyD88-independent pathways by interacting with several of the downstream signaling molecules [89]. The adaptor protein TIRAP is a SOCS1 target and the association of the two molecules leads to the polyubiquitination and degradation of TIRAP. It has been suggested that upon LPS stimulation SOCS1 can form a complex with phospho-IRAK-1 (pIRAK-1) resulting in its proteasomal degradation, thereby preventing further signaling. It has also been demonstrated that SOCS1 directly targets p65 enhancing its ubiqutin-mediated proteolysis, thus resulting in the downregulation of NFκB activity. Importantly, SOCS1 deficient mice do not develop endotoxin tolerance in response to repeated exposure to LPS pointing to the essential negative regulatory role of SOCS1 in LPS-induced responses [90]. Another SOCS family member, SOCS3 is induced in LPS-stimulated macrophages to induce a

negative feedback loop for cytokine signaling. Murine macrophages transfected with SOCS3 produce reduced levels of IL-6, IL-12 and TNF and the extent of inhibition is similar to that elicited by IL-10 or LPS treatment [91]. In pathological situations, where IL-6-related cytokines play an important role SOCS3 suppresses inflammatory reactions via inhibiting STAT3 activation indicating that it can act as a negative regulator of inflammation [92]. SOCS2 is also a direct target of TLR signaling; however, compared to SOCS1 and SOCS3 shows a delayed induction in both human and murine DCs. SOCS2 silencing leads to augmented STAT3 activation and increased IL-10 and IL-1β cytokine production underlying its negative regulatory potential; however, its molecular targets remained elusive [93].

More than a decade ago STAT3, a cytoplasmic transcription factor and a key mediator of cytokine signaling has emerged as a negative regulator of inflammatory responses. Disruption of STAT3 signaling in macrophages leads to overactivation and abundant production of inflammatory cytokines in response to LPS [94]. The lack of functional STAT3 in macrophages and bone-marrow derived DCs enhanced the antigen presenting ability and restored the responsiveness of anergic $CD4^+$ T-cells *in vivo* indicating the essential regulatory role of STAT3 in inducing antigen specific T-cell tolerance [95].

S100A8 and S100A9 (also reffered to as MRP8 and MRP14) belonging to the S100 family are readily secreted as heterodimers during inflammatory processes. S100A8 can be induced by a variety of stimulants including LPS or TNF and has a protective role in the resolution of inflammation [96]. S100A9 may inhibit DC differentiation and contribute to the accumulation of myeloid-derived suppressor cells in tumor tissues [97]. It has also been suggested that the S100A8/A9 complex suppresses acute inflammation by modulating the activity of pro-inflammatory cytokines produced upon injection of LPS into rats [98].

The signaling lymphocyte activation molecule (SLAM/CD150) is an activation-induced self-ligand receptor expressed on the surface of T- and B-lymphocytes, monocytes, macrophages and moDCs. It has been described in our laboratory that SLAM/SLAM engagement inhibits the production of IL-6, IL-12 and TNFα of CD40L-induced DCs but not of LPS-stimulated cells [99]. Futhermore, SLAM/SLAM interaction could impair the ability of DCs to induce IFNγ-producing T-lymphocyte differentiation [99]. These results indicate that SLAM/SLAM dimerization mediates DC functionality in a complex manner via downregulating CD40L-induced inflammatory signals while not affecting LPS-stimulated IL-12 production.

Leukocyte immunoglobulin-like receptor subfamily B member 2 (LILRB2/ILT4) belongs to the LILR family of innate immune receptors that are predominantly expressed on the surface of antigen presenting cells and are divided into activating and inhibitory receptors [100]. Their most broadly characterized ligands involve MHC class I and MHC-I-like molecules such as CD1d that binds LILRB2. Inhibitory LILRs may exhibit their function alone or in association with activating receptors such as TLRs. In response to Salmonella infection LILRB2 and LILRB4 has been shown to be upregulated in *in vitro*-generated macrophages and DCs. The two inhibitory receptors exert different effects: LILRB2 inhibits APC effector functions via downregulating costimulatory molecules such as CD86 whereas LILRB4 increases IL-10 production and decreases IL-8 secretion [101]. LILRB2 and LILRB4 play an important role in dampening immune responses during infection that poses their possible regulatory role in the induction of endotoxin tolerance.

The activating transcription factor 3 (ATF3) is a nuclear factor that can be rapidly induced by TLR stimulation and acts as a transcriptional repressor [102]. By presumably inducing a negative feedback loop ATF3 negatively regulates the transcription of pro-inflammatory genes such as IL-6 and IL-12. It can rapidly be induced by LPS stimulation in mouse macrophages as well as in plasmacytoid and myeloid human DC subsets. ATF3 exerts its inhibitory effect via reducing the expression of high mobility group box 1 (HMGB1) that acts as a transcription factor in the nucleus and as a pro-inflammatory cytokine when released resulting in the secretion of other pro-inflammatory cytokines [103]. Clinical reports revealed that the level of HMGB1 is increased in patient with sepsis, suggesting that the suppression of LPS-induced inflammation via inducing ATF3 might be a feasible strategy for the treatment of sepsis.

MicroRNAs (miRNAs) are small non-coding RNAs that represent a new class of gene regulators that are implicated in innate immunity by regulating TLR signaling and subsequent cytokine responses [104]. MiRNA profiling of human monocytes revealed that miR-146a/b, miR-155 and miR-132 can be highly upregulated in response to LPS [105]. miR-146a was identified as an NFκB-dependent gene that negatively regulates the expression of TNFα via repressing TRAF6 and IRAK-1 kinase activity. Beside LPS other TLR agonists and cytokines such as IL-1β and TNFα were shown to increase the expression of miR-146a [106]. Importantly, the expression of miR-146a is induced upon activation of TLR2, TLR4 or TLR5 but not by endosomal TLR3, TLR7 and TLR9 indicating that miR-146a plays a role in regulating immune responses predominantly to bacterial and fungal components. Further studies also demonstrated that miR-146a is

critical for the induction of endotoxin tolerance in the human monocytic THP-1 cell line as the transfection of inhibitors specific for miR-146a abolished LPS-induced tolerance significantly [107]. In contrast to miR-146a, miR-155 could be upregulated even by TLR3 and TLR9 triggering in murine macrophages and exerted both positive and negative effects on the NFκB signaling pathway [104]. In RAW267.4 macrophages a strong but transient upregulation of miR-155 was reported upon challenge with LPS. Furthermore, miR-155 transgenic mice produced higher levels of TNFα and were more sensitive to LPS-induced septic shock than wild type mice [108]. All these results point to the critical role of miRNAs in the regulation of innate immune responses induced by TLR stimuli, thus modulating the activity of these miRNAs might be a promising therapeutic target against various inflammatory diseases.

The above discussed findings point to the multi-level regulation of endotoxin tolerance. To reveal the molecular mechanisms behind endotoxin tolerance necessitates the integration of several regulatory components including signaling molecules, negative regulators and miRNAs into a complex network.

It is important to note that the induction of endotoxin tolerance in donor mice can prolong heart allograft survival via inhibiting alloimmune responses [109]. The development of non-toxic TLR4 ligands or negative regulators of TLR signaling might provide alternative means to inhibit allograft rejection in human organ recipients. In addition, the adoptive transfer of LPS-pre-treated pDCs was shown to prevent the development of renal functional and histological injury in mice with chronic kidney disease [110]. The mechanism of protection might be connected to the ability of pDCs to induce foxp3^{+} Tregs in the kidney and in the lymph nodes and to suppress pro-inflammatory cytokine production by endogenous renal macrophages. Thus, the tolerogenic potential of LPS-pre-treated DCs may offer novel therapeutic approaches to treat autoimmune or chronic inflammatory diseases.

2.7. 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 [111]. 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 (**Figure 2.7.**). The rapamycin-sensitive mTORc1 contains mTOR, regulatory associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (mLST8), proline-rich Akt substrate of 40 kDa (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR) whereas the rapamycin-resistant mTORc2 encompasses mTOR, rapamycin-insensitive companion of mTOR (RICTOR), mLST8, mSIN1 (mammalian stress-activated protein kinase), DEPTOR and protein observed with RICTOR-1 (PROTOR) [111]. Rapamycin is a pharmacological inhibitor of mTOR that forms a complex with the FK506 binding protein 12 (FKBP12). The rapamycin-FKBP12 complex binds to the kinase region of mTOR and blocks its kinase activity efficiently, probably through disrupting the interaction between mTOR and RAPTOR. Intriguingly, mTORc2 is insensitive to the direct inhibition by rapamycin; however, a prolonged exposure to rapamycin can disrupt its assembly in several cell types [112].

mTORc1 is able to integrate signals from both extracellular and intracellular stimuli such as nutrients, growth factors, cytokines, antigens or Toll-like receptor ligands [111]. These factors activate mTOR via the PI3K-Akt axis resulting in the inhibitory phosphorylation of tuberous sclerosis complex 2 (TSC2), which in complex with TSC1 is the main negative regulator of mTORc1 activity. Repression of TSC2 activity abrogates its inhibitory effect on RAS homologue enriched in brain (RHEB) that is an essential stimulator of mTOR activity. mTOR then phosphorylates S6 kinase 1 (S6K1) and the eukaryotic initiation factor binding protein 1 (4EBP-1), thus enhancing protein synthesis and cell growth [112].

Our knowledge on the functional activity of mTORc2 is less defined due to the lack of specific inhibitors. Targeting RICTOR activity revelead that mTORc2 activated by PI3K is important for the phosphorylation of Akt. Besides, mTORc2 mediates actin cytoskeleton rearrangement through the small GTPase RAS homologue (RHO) and protein kinase C (PKC). It has also been found that knockdown of RICTOR does not influence the activation of S6K1 indicating that mTORc2 does not stimulate mTORc1 [111]. 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. It has also been demonstrated that mTOR mediates the Flt3L-driven maturation and homeostasis of both conventional and plasmacytoid DCs in mice [113].

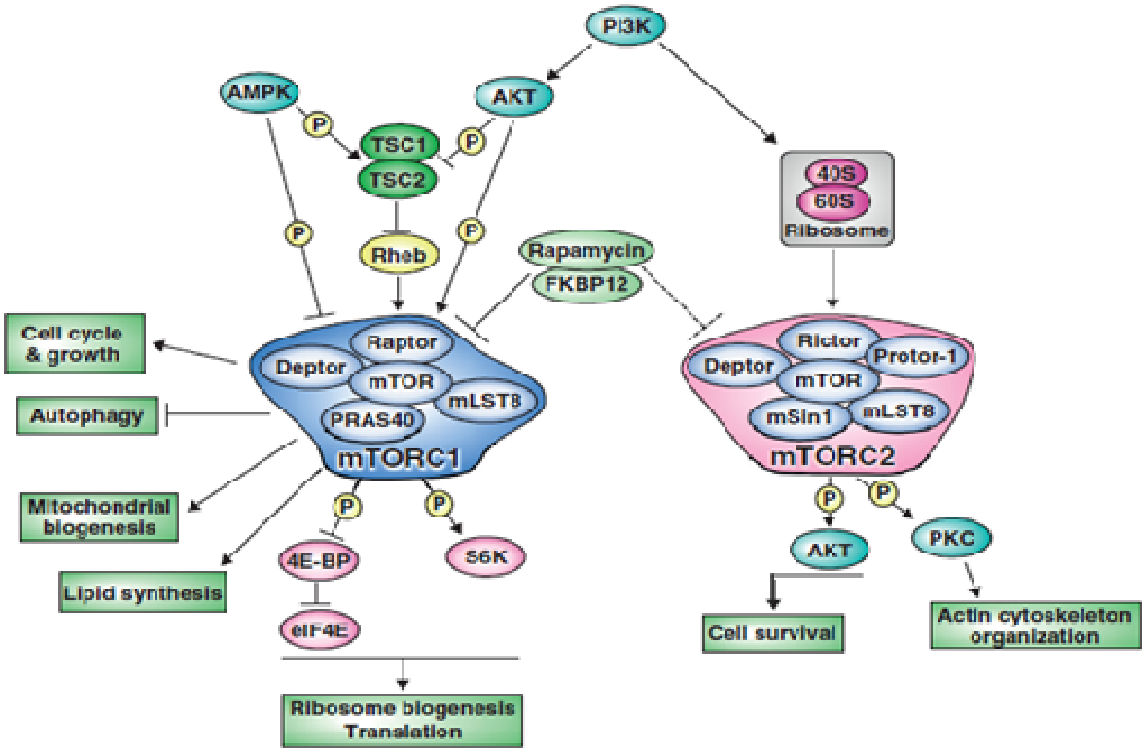

Figure 2.7. The mTOR signaling pathway*. (Araki et al. Current Opinion in Cell Biology,* ***23****, 707-715, 2011)*

mTOR controls many cellular processes through two different complexes. Rapamycin can inhibit the induction of mTORc1 while not affects mTORc2. However, a long-term treatment with rapamycin can also decrease the activity of mTORc2.

Deletion of Pten, an inhibitor of the PI3K/Akt/mTOR axis promotes DC development providing further support for the regulatory role of mTOR in the Flt3L-induced DC maturation [113]. In the presence of PI3K or mTOR inhibitors the development of human $CD34^+$ stem cell-derived DCs was decreased [114]. Further, blocking mTOR activity by rapamycin during moDC differentiation decreased cell survival and detained its immunostimulatory functions leading to the generation of a tolerance promoting DC phenotype [115]. 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 (**Figure 2.8.**). Most importantly, rapamycin treatment of pDCs impaired the TLR9-induced production of IFNα/β via the suppression of IRF7 activity significantly [116]. It has been demonstrated that the production of type I

IFNs and pro-inflammatory cytokines is also suppressed in TLR7-activated human pDCs. Interestingly, these cells failed to induce IFNγ and IL-10 producing effector T-cells while enhanced the proliferation of naive and memory helper T-cells and foxp3-expressing $CD4^+$ Tregs upon rapamycin treatment [117]. Another report demonstrated suppressed functional activity of moDCs following rapamycin treatment. Similarly to pDCs, rapamycin decreased the production of both pro- and anti-inflammatory cytokines in LPS-stimulated moDCs and affected their allogenic T-cell stimulatory capacity negatively [115]. In contrast, rapamycin enhanced the secretion of IL-12 in $CD1c^+$ DCs upon stimulation with various TLR ligands and potentiated the ability of LPS-stimulated $CD1c^+$ DCs to induce T-cell proliferation [115]. These data suggests that mTOR may exert both immunostimulatory and inhibitory effects depending on the DC subtype.

Beside controlling DC development and maturation mTOR directly drives the differentiation and function of T-cells. Upon deletion of Rheb, a positive regulator of mTORc1 $CD4^+$ T-lymphocytes were unable to differentiate into Th1 and Th17 cells but preserved the ability to differentiate into Th2 cells [111]. When RICTOR, the integral component of mTORc2, was deleted $CD4^+$ T-cells failed to differentiate into either Th1 or Th2 cells. In contrast, mTOR inhibition by rapamycin promoted the differentiation and expansion of $foxp3^+$ regulatory T-cells [111] and the generation of $CD8^+$ memory T-cells *in vivo* [118]. These findings indicate the fundamental role of mTOR in the induction of T-cell proliferation and maturation.

In human oral keratinocytes rapamycin was shown to impede polyI:C-stimulated upregulation of TNFα and IL-1β whereas enhanced the secretion of bioactive IL-12p70 [119]. 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 polyI:C-induced 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 polyI:C. These results indicate that via regulating the activity of JNK mTOR collaborates with the 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.

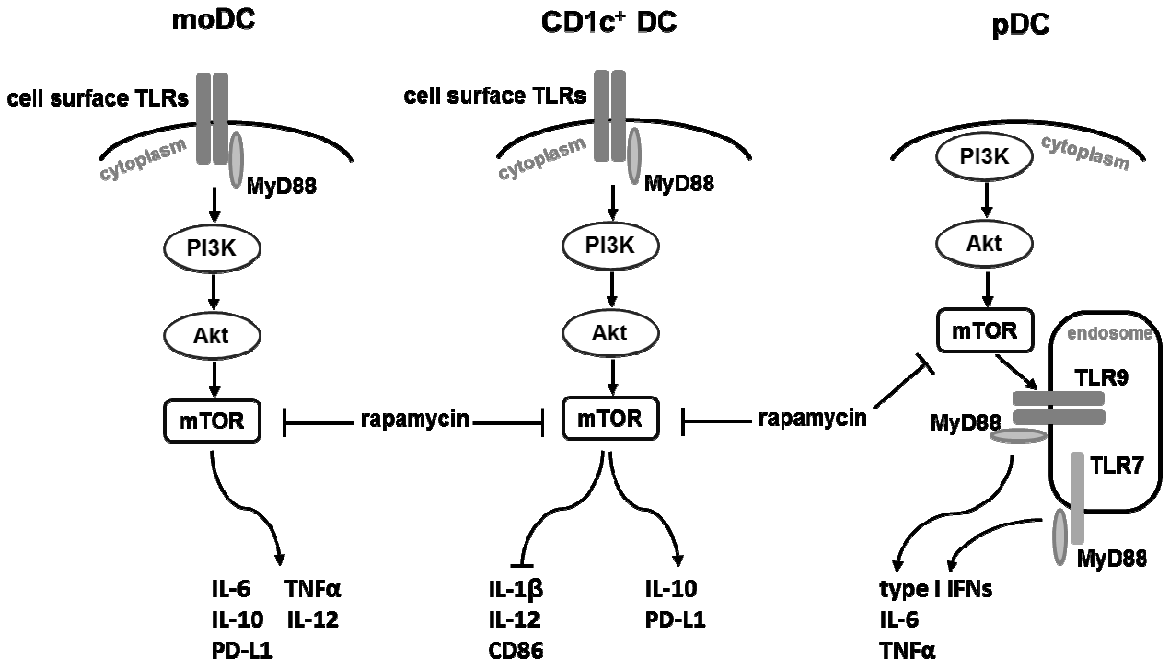

Figure 2.8. Model of mTOR-mediated signaling in different human DC subtypes.
Interruption of mTORc1 signaling in human moDCs during TLR ligation results in decreased production of both pro- and anti- inflammatory cytokines indicating the crucial role of mTOR in the functional activity of moDCs. In CD1c$^+$ DCs mTORc1 signaling inhibits the induction of IL-1β, IL-12 and CD86 whereas promotes the upregulation of IL-10 and PD-L1. In human pDCs intact mTORc1 signaling is needed to drive the production of type I IFNs and pro-inflammatory cytokines in response to TLR7 and TLR9 stimuli.

Rapamycin used alone or in combination with other immunosuppressants is a strong anti-rejection agent in renal transplantation due to its ability to induce foxp3$^+$ Tregs [112]. When utilized together with IL-10 or CXCR3-specific monoclonal antibody rapamycin is also able to induce tolerance in pancreatic islet cell transplantation or in heart allografts, respectively [112]. Intriguingly, rapamycin applied at a lower concentration than that required for eliciting immunosuppression is able to exert antiviral activities against viruses, such as HIV, cytomegalovirus or several herpesviruses via different mechanisms [120, 121]. Furthermore, e*x vivo* rapamycin conditioning of DCs (RAPA-DCs) offers a potential therapeutic tool to generate tolerogenic DCs for preventing graft rejection or treating autoimmune disorders. Murine RAPA-DCs possess the ability to migrate to secondary lymphoid tissues where they induce Treg proliferation [122]. It has been found in rodent models that prior to transplantation the administration of recipient-derived RAPA-DCs

pulsed with donor antigen could prolong donor heart allograft survival. It is important to note that human and rodent DCs might be affected differently when generated in the presence of clinically relevant concentration of rapamycin. Both murine and human RAPA-DCs are phenotypically immature with low expression of costimulatory molecules; however, only murine cells preserve their immature phenotype when exposed to inflammatory stimuli [123]. Both rodent and human macrophages are able to promote Treg proliferation despite exhibiting a paradoxical decrease in the expression of PD-L1 and IL-10 [115, 122]. As compared to rodent cells human RAPA-DCs display augmented migration to CCL21 due to the upregulation of CCR7 [124]. Furthermore, human RAPA-DCs show increased IL-12 production that is associated with enhaced Th1/Th2- polarizing capacity upon stimulation with LPS [122]. These results point to the need for further studies to better understand the functionality of mTOR in human DCs, which may facilitate the development of efficient and reliable DC vaccines.

2.8. The aims of the study

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 isolated 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 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 used this relatively low LPS concentration as it consistently induced a desensitization of developing moDCs to further LPS-mediated activation (**Fig. 4.1.1.A**). We analyzed moDC activation following a short, 2-day culture, to better represent an in vivo situation when monocyte precursors enter inflamed tissues and differentiate into DCs in the presence of activation signals that readily induce effector functions. 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 (**Fig. 4.1.1.B**).

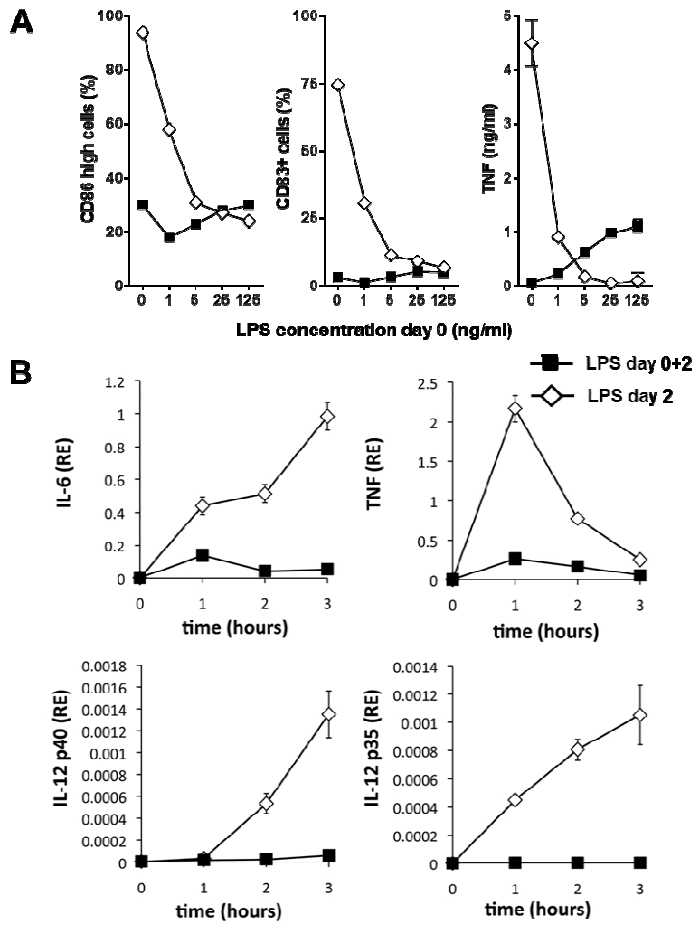

Figure 4.1.1. Early stimulation of developing moDCs with LPS induces tolerance to further activation with endotoxin. *(A) MoDCs were cultured in the presence of various LPS concentrations or in the absence of LPS. At day 2, the expression of CD86 and CD83 on the cell surface and the TNF concentration in the culture supernatants were analyzed (black symbols). Alternatively, the cells were washed and reactivated using 100 ng/ml LPS and the levels of CD86, CD83 and TNF were analyzed 1 day later (white symbols). (B) MoDCs cultured in the absence (◊) or presence (■) of 5 ng/ml LPS were treated with 100 ng/ml LPS on day 2 and the kinetics of IL-6, TNF, IL-12 p40 and p35 gene expressions were studied using real-time PCR. Mean values ± SD were calculated from three replicates used for each sample. Representative results of at least three independent experiments are shown.*

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 (**Fig. 4.1.2.A**). 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 (**Fig. 4.1.2.B**). 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. Contrary to other activation signals that we applied, polyI:C did not tolerize moDCs to LPS-induced activation and the pretreatment with IFNγ synergized strongly with a later LPS signal (**Fig. 4.1.2.B**).

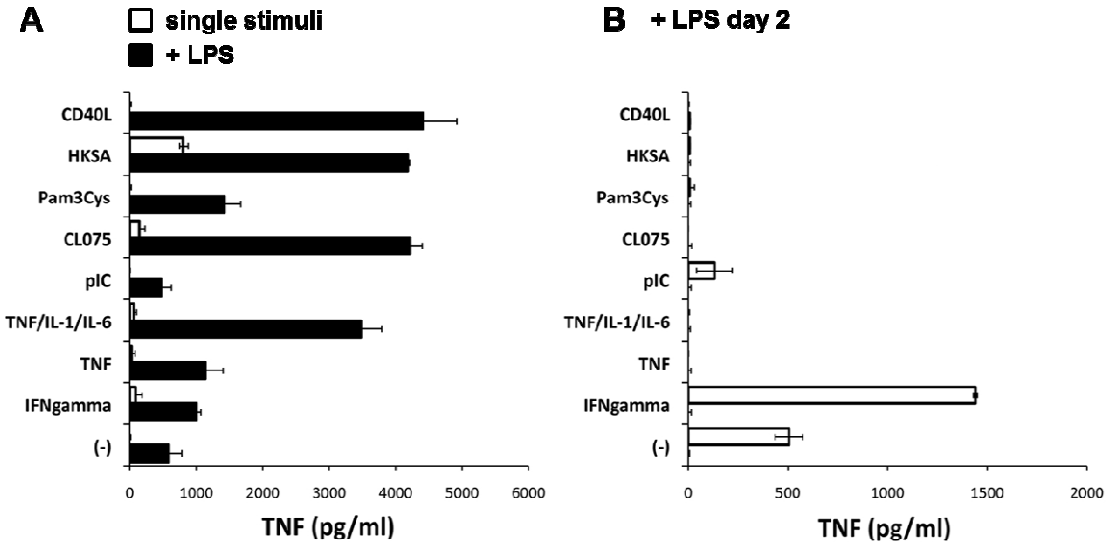

Figure 4.1.2. Early stimulation of developing moDCs induces tolerance to further activation with LPS. *(A) MoDC cultures were established in the presence of various activation signals applied alone (open bars) or combined with 5 ng/mL LPS (black bars) and TNF concentration was measured in the supernatants at day 2. (B) On day 2 moDCs treated as in (A) were washed and activated with 100 ng/ml LPS. After 24-hour activation TNF concentration was measured in the supernatants. Mean values ± SD were calculated from three replicates used for each sample. Representative results of at least three independent experiments are shown.*

The inability of early-stage moDCs developing in the presence of various activation signals to respond to further TLR stimuli is in line with previous data obtained with macrophages or DCs [85]. Here we showed that synergistic activation signals do not rescue the cells from functional exhaustion. In addition, we observed the complete lack of inflammatory cytokine gene expression in LPS-tolerized moDCs in response to further stimuli.

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 (**Fig. 4.1.3.A**). 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 was detected in moDCs cultured for 2 days in the presence of LPS by ELISA (**Fig. 4.1.3.B**). In line with previous findings the expression of miR-146a and miR-155 were upregulated by LPS added at day 2 to moDCs (**Fig. 4.1.3.C**) [105, 108]. However, miR-146a levels were only minimally elevated and miR-155 was not affected in moDCs cultured for 2 days in the presence of LPS as compared with non-treated cells, suggesting a time-limited functionality of these microRNAs in LPS-activated DCs. 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. As shown by **Fig. 4.1.3.D**, the expression of all studied DC modulatory factors, namely, SOCS1, SOCS2, SOCS3, IRAK-M, ATF3, S100A8 and S100A9, STAT3, LILRB2, IκBα, IκBβ 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 levels by day 2 in LPS-treated DCs as compared to control cultures, supporting the microarray data that indicated minimally altered expressions of most genes at day 2 in response to LPS. These results indicated a time-limited effect of the studied molecules in DC functions rather than a role in persistent DC inactivation.

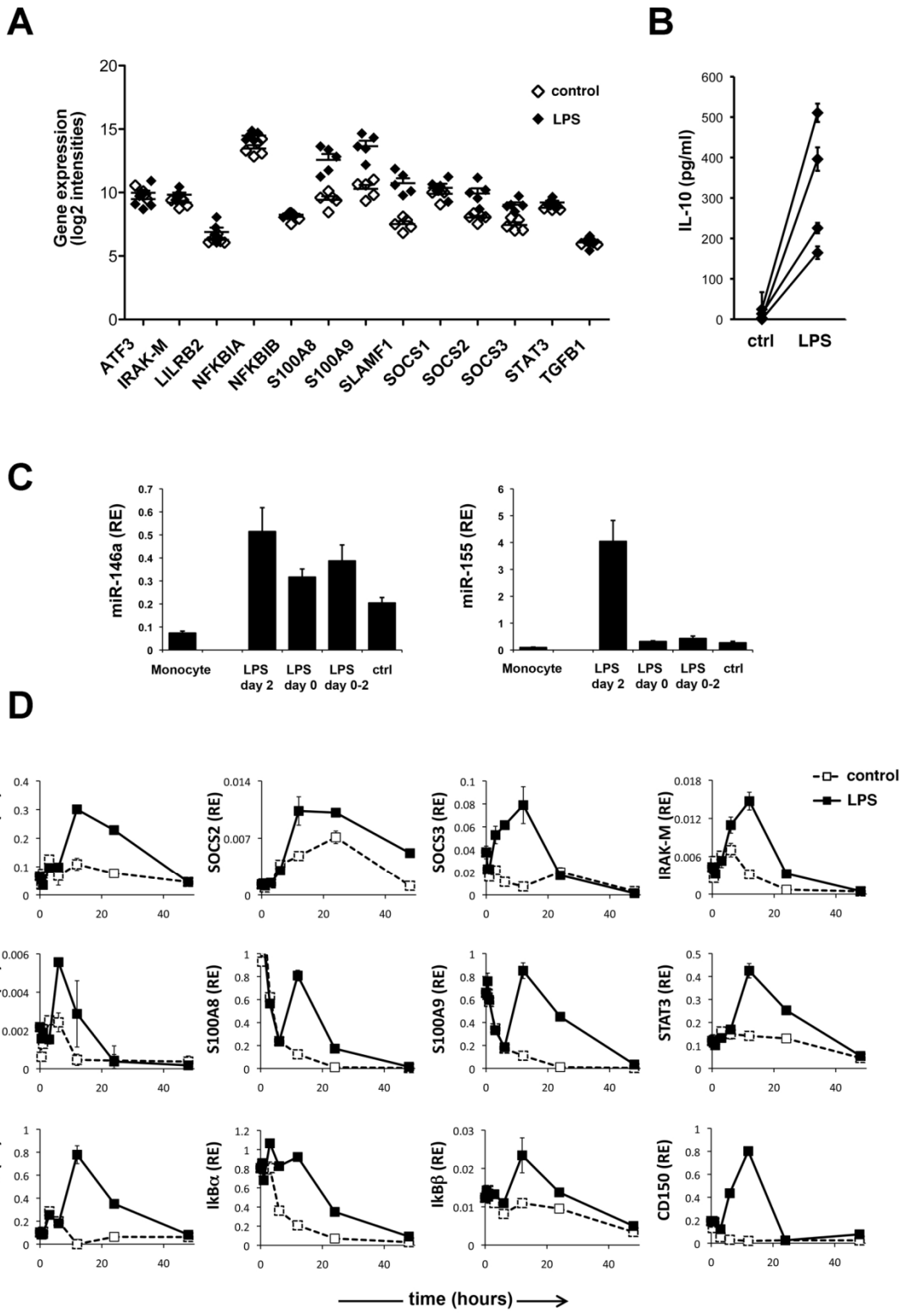

Figure 4.1.3. LPS-induced inhibitory mechanisms in early stages of moDC differentiation. *(A) Microarray analysis of potential DC inhibitory factors in moDCs cultured with or without 5 ng/ml LPS for 2 days. (B) IL-10 concentrations are shown in supernatants of 2-day moDCs cultured in the presence or absence of LPS. (C) The expression of miR-146a and miR-155 was measured by real-time PCR in monocytes, moDCs that were activated with 100 ng/ml LPS on day 2 for 24h (LPS day 2), in moDCs cultured for 3 days in the presence of 5 ng/ml LPS (LPS day 0), in moDCs that were cultured for 2 days with 5ng/ml LPS and then activated with 100 ng/ml LPS (LPS day 0–2) and in moDCs cultured without LPS (ctrl). (D) Gene expression of potential DC inhibitory molecules was analyzed using real-time PCR in moDCs cultured with (■) or without (□) 5 ng/ml LPS. (B–D) Data are shown as mean ± SD calculated from three replicate measurements. Representative results of at least three independent experiments are shown.*

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 (**Fig. 4.1.4.**). 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 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 we measured IL-12 production. As shown on Fig. 4.1.4. , 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. These inhibitory factors, when induced during moDC activation, may act as immediate negative regulators that might help to terminate gene expression in activated DCs. 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 (**Fig. 4.1.5.**). In line with the data obtained with miR-146a-specific siRNAs, transfection of developing moDCs with miR-146a led to decreased IL-12 and TNF production in response to all tested activation signals. Transfection with miR-155 inhibitor led to decreased IL-12 producing ability (**Fig. 4.1.4.**) and, similarly, transfection of moDCs with miR-155 led to a mild, but consistent, decrease of IL-12 and TNF production (**Fig. 4.1.5.**). These results possibly reflect multiple, often counteracting, effects of miR-155 on DC activation pathways that is also indicated by previously described effects of this miRNA, both stimulatory or inhibitory, on macrophage and DC functions [108, 125, 126]. 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 (**Fig. 4.1.4.**). Importantly, inhibition of none of the tested DC modulatory molecules had an impact on the strong inhibitory effect of the LPS pre-treatment on IL-12

production triggered by a second activation signal (**Fig. 4.1.4.**). Thus, moDC activation during early differentiation may lead to functional exhaustion independently of the tested regulatory factors.

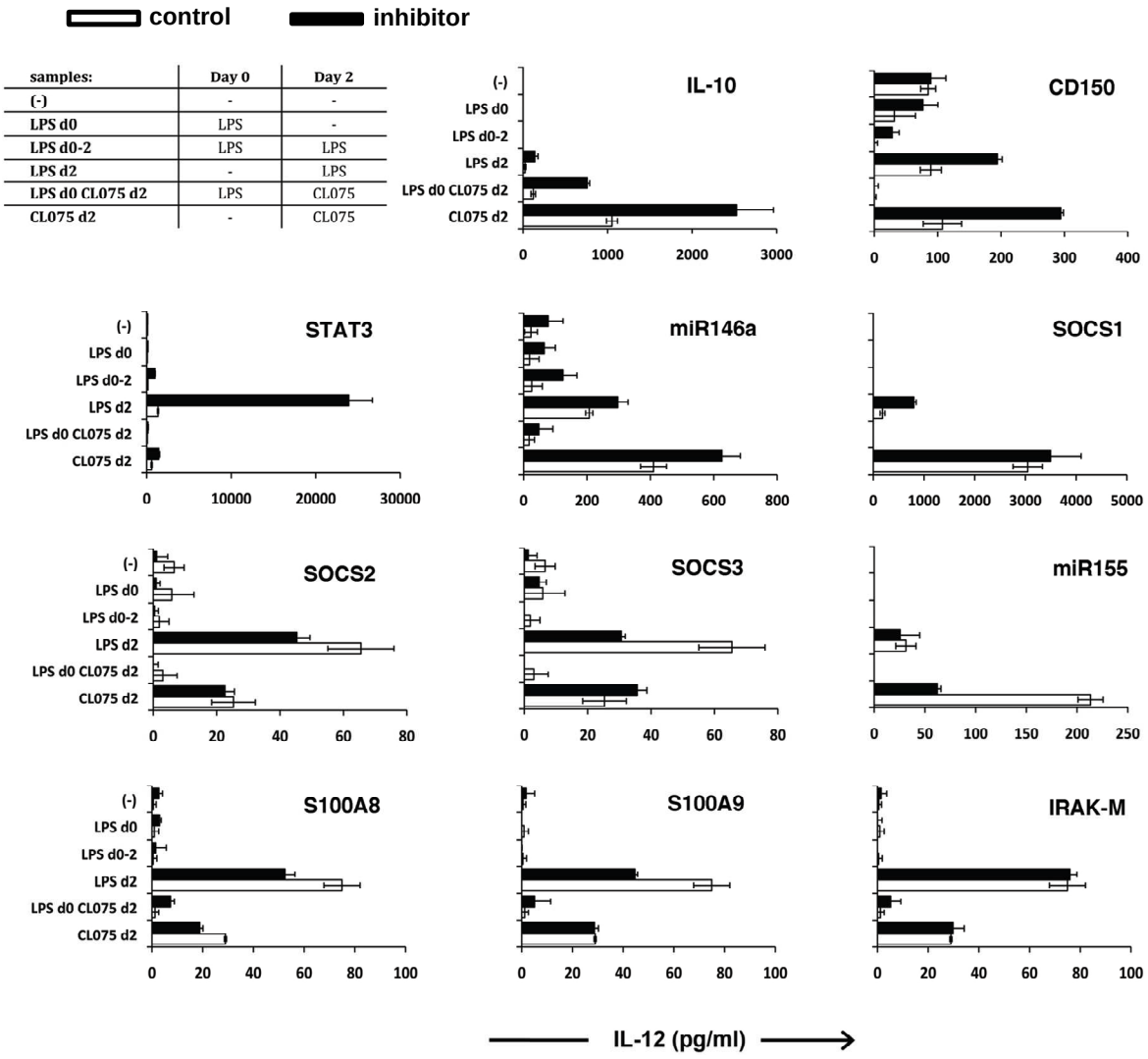

Figure 4.1.4. The effect of LPS-inducible inhibitory factors on moDC activation. *(A) The LPS-induced IL-12 production of moDCs pre-cultured in the absence (LPS day 2) or presence of 5 ng/mL LPS (LPS day 0–2) for 2 days is shown. The tolerizing ability of an early LPS stimulus on a heterologous activation signal was tested via comparing the CL075-induced IL-12 production of moDCs pre-cultured in the absence (CL075 day 2) or presence of 5 ng/mL LPS (LPS day 0-CL075 day 2). Alternatively, moDCs pre-cultured or not with 5ng/mL LPS were left without further activation (LPS day 0 or (-), respectively). The effect of IL-10 on DC activation was tested using 10 μg/ml neutralizing anti-IL10 or isotype control antibodies added at days 0 and 2. The effect of STAT3, SOCS1, SOCS2, SOCS3, S100A8, S100A9, IRAK-M and CD150 molecules was tested by transfecting the monocyte precursors with siRNA molecules targeting mRNA of the individual molecules. The miR-146a and miR-155 effects on IL-12 production were analyzed by transfecting the monocytes with specific locked nucleic acid (LNA) miRNA inhibitors or with LNA miRNA control inhibitor. Data are presented as mean±SD calculated from three replicate measurements. Representative results of at least three independent experiments are shown.*

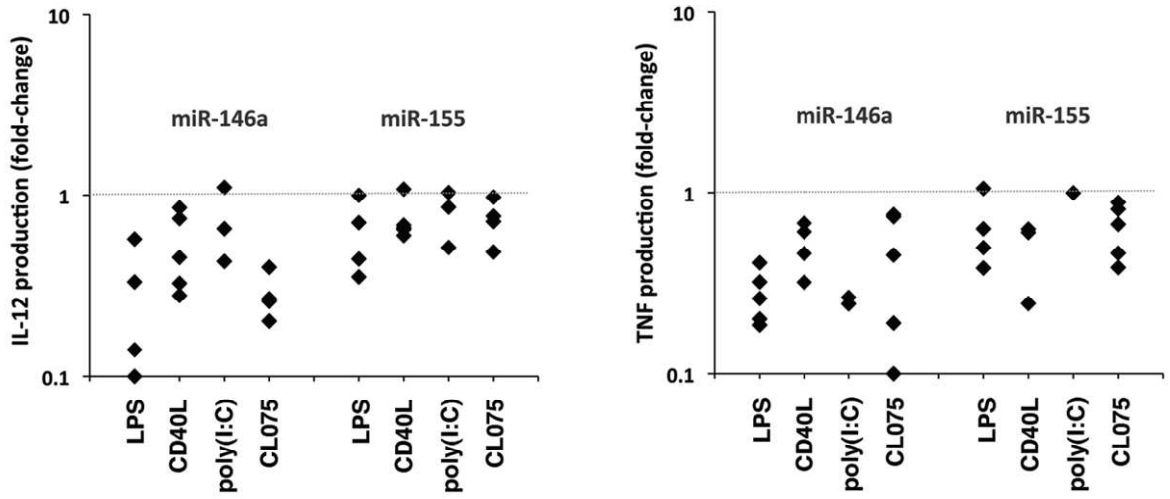

Figure 4.1.5. The effect of miR-146a and miR-155 on moDC activation. *Monocytes were transfected with miR-146a or miR-155 precursor molecules or with control miRNA. Thereafter moDC cultures were established and maintained for 2 days. The cells were activated using LPS (100 ng/ml), CD40L-expressing L cells (1:10 L cell:DC ratio), polyI:C (20 μg/ml) or CL075 (1 μg/ml) for 24 h. The effect of miRNA molecules on cytokine production is compared with control miRNA-transfected samples. Cytokine production induced by the different activation signals was tested in three to five different experiments.*

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 (**Fig. 4.2.1.A**). mTOR is also expressed in circulating $CD1c^+$ DCs at levels comparable to those found in moDCs (**Fig. 4.2.1.B**). 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 (**Fig. 4.2.1.C**). We have also found that TLR3 is expressed at comparable levels in fast moDCs and circulating $CD1c^+$ DCs **(Fig. 4.2.1.D)**.

Several *in vitro* studies [23], including our previous results [127] 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 [115], we decided to use 3-day moDC cultures in our further experiments.

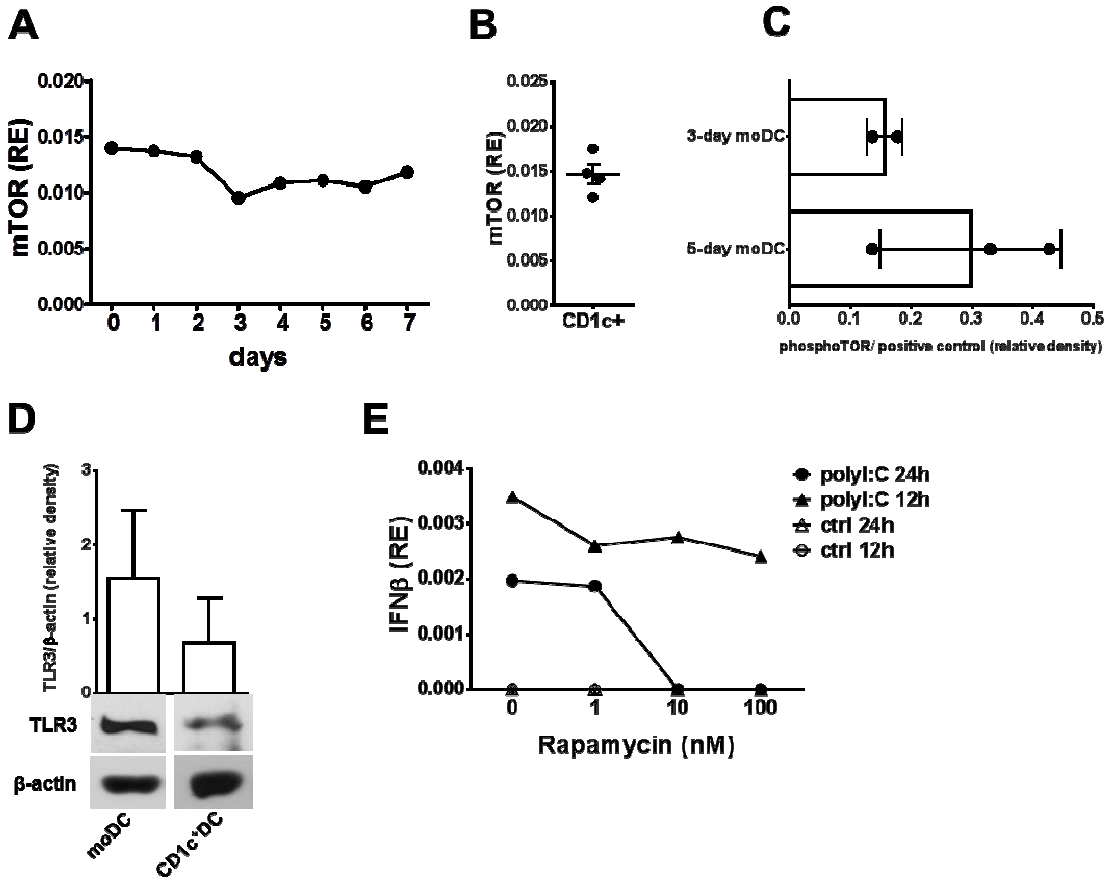

Figure 4.2.1. Expression of mTOR and TLR3 in moDCs and CD1c$^+$ DCs, and dose responses of moDCs to various rapamycin concentrations

(A) Freshly isolated monocytes were cultured in the presence of GM-CSF and IL-4 for 7 days. Every other day half of the culture medium was removed and replaced by fresh, cytokine-supplemented medium. Samples were collected every day and real-time Q-PCR was performed to assess the mRNA expression of mTOR during moDC differentiation. Relative expression of mTOR was calculated following normalization to the expression of the cyclophilin housekeeping gene. A representative experiment out of 3 is shown. (B) Expression of mTOR in CD1c$^+$ DCs was measured by real-time Q-PCR. Each point indicates an individual experiment; the horizontal line shows the mean and error bars denote the SD. (C) Bar graphs show the relative density values of phosphorylated TOR in resting, 3-day and 5 day moDCs. Data were collected from the results of 5 MAPK arrays and each dot represents an individual experiment. (D) Bar graphs (Mean ± SD, 3 independent experiments) and the representative blots show TLR3 expression in 4-day moDCs and CD1c$^+$ DCs measured by Western blotting. (E) To determine the most effective dose of rapamycin a concentration-dependent experiment was performed. Cells were harvested at 12 and 24 hours following stimulation with polyI:C and the relative expresssion of IFNβ was determined by normalizing with cyclophilin.

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 [16]. To test the effects of short-term treatment of DC with rapamycin (100nM) the expression of various membrane-

expressed co-stimulatory markers and HLA-DQ was analyzed by flow cytometry after 24 hours of stimulation by the TLR3 ligand polyI:C. The dose of rapamycin was selected on the basis of clinically achievable concentrations in treated individuals [128], based on previous *in vitro* studies [115, 129] and also on our concentration-dependent dose response curve obtained by using a wide range of rapamycin doses (**Fig. 4.2.1.E**). A significant decrease in the fluorescence intensity of human leukocyte antigen-DQ (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 (**Fig. 4.2.2.A**). We also tested the expression of programmed cell death 1 ligand 1 (PD-L1), an inhibitory molecule known to promote regulatory T-cell development [130]. Although the upregulation of PD-L1 has been shown in various cell types upon encounter with rapamycin [131, 132] we could not detect any alteration in the levels of PD-L1 in resting moDCs (**Fig. 4.2.2.A**).

Moreover, as a result of polyI:C-mediated moDC activation rapamycin pre-treatment prevented the upregulation of PD-L1 in moDCs (**Fig. 4.2.2.A**). Similar results were obtained when the expression of CD40, CD80 and PDL-1 was measured in $CD1c^+$ DCs (**Fig. 4.2.2.B**). However, in contrast to moDCs we observed that rapamycin did not affect the expression of HLA-DQ while enhanced the percentage of $CD83^+$ $CD1c^+$ DCs significantly (**Fig. 4.2.2.B**). These results indicate that mTOR controls the expression of various cell surface molecules on both moDCs and $CD1c^+$ DCs differently.

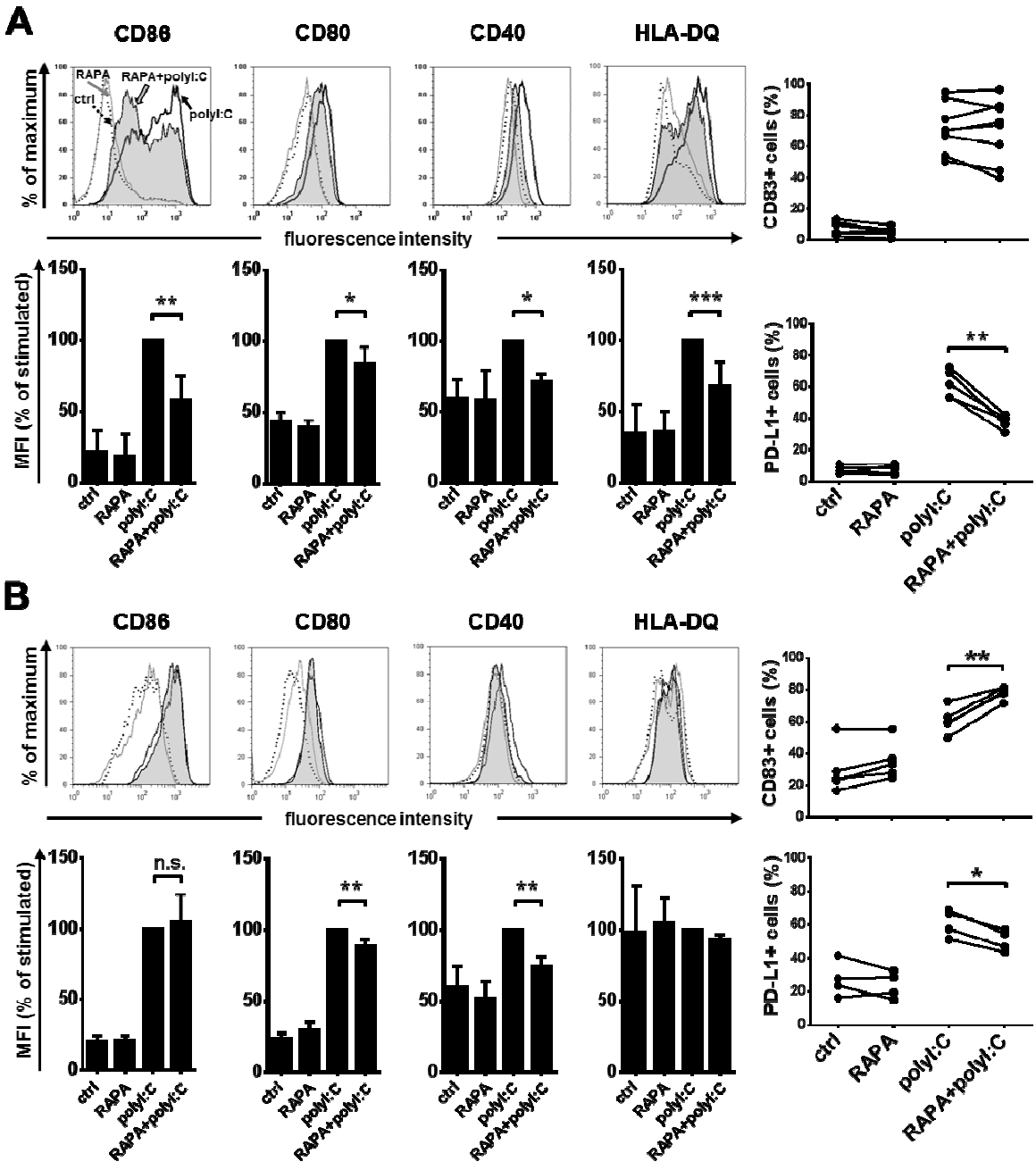

Figure 4.2.2. mTOR modulates the expression of cell surface molecules in moDCs and CD1c$^+$ DCs. *MoDCs (A) or CD1c$^+$ DCs (B) were pre-treated with rapamycin or DMSO (as control) for 2 hours that was followed by a 24-hour incubation with polyI:C. Alterations in the expression levels of cell surface molecules were analyzed by flow cytometry. Histogram overlays of one representative experiment are shown, where dotted lines represent untreated cells, grey lines show rapamycin pre-treated cells, black lines correspond to polyI:C-activated and filled histograms to rapamycin pre-treated and polyI:C-activated cells. Bars represent the mean ± SD of MFI (median fluorescence intensity) from 4 independent experiments. The values are expressed as the percentage of stimulated cells. Graphs show the percentage of CD83 and PD-L1 positive cells from at least 5 independent experiments.*

4.2.3. Rapamycin pre-conditioning impairs 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 (**Fig. 4.2.3.A upper panel**). 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 (**Fig. 4.2.3.A lower panel**). 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 (**Fig. 4.2.3.B**). 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 [11]. Interestingly, rapamycin pre-treatment diminished the expression of the p28 subunit that was accompanied by the enhanced expression of the EBI subunit (**Fig. 4.2.3.C**), which in association with the p35 subunit forms the recently identified IL-35 cytokine with broad suppressive activity [15]. 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 (**Fig. 4.2.3.D**). Due to the limited numbers of both $CD1c^+$ blood DCs and naive $CD8^+$ T-cells in PBMC we performed co-culture studies with moDCs, only. 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 (**Fig. 4.2.3.D**). Remarkably, none of the treatment protocols resulted in the induction of $CD8^+foxp3^+$ T-cells (data not shown). 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 [133].

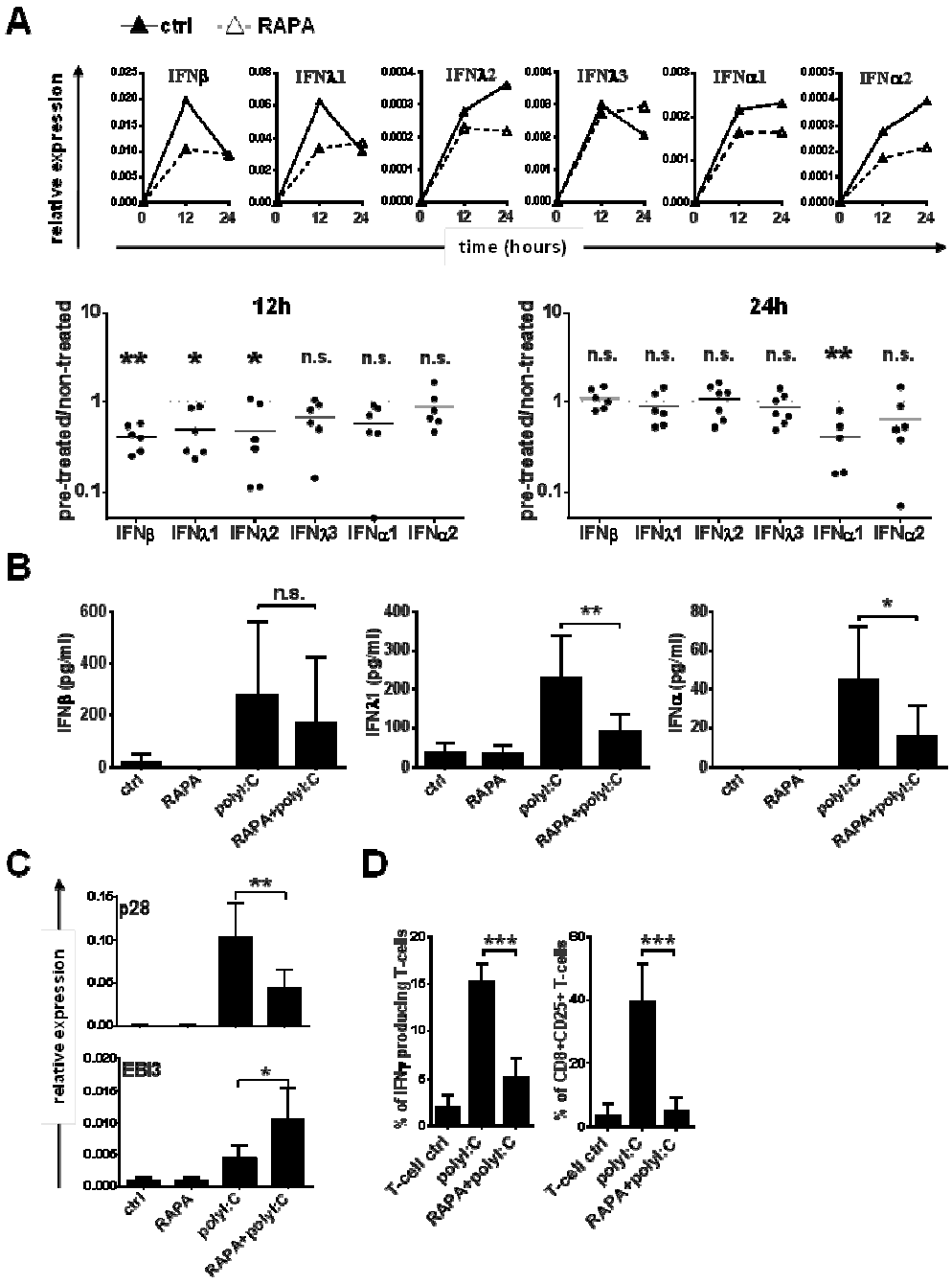

Figure 4.2.3. Rapamycin pre-conditioning decreases the antiviral potential of moDCs. *MoDCs were pre-exposed or not to rapamycin for 2 hours then stimulated with polyI:C. (A) The upper panel represents the kinetics of type I and III IFN gene expression. The lower panel shows gene expression levels in rapamycin pre-treated/non-treated cells. Each point indicates an individual experiment; the horizontal line corresponds to the mean. (B) Bar graphs represent the cytokine concentrations of at least 3 independent experiments determined by ELISA after 24 hours of activation. (C) Bar graphs show relative gene expression levels of p28 and EBI3 (Mean ± SD, 4 independent experiments) measured 12 hours after activation by Q-PCR (D). After 24-hour induction by polyI:C moDCs were co-cultured with naive $CD8^+$ T-cells. On day 5 the percentage of IFNγ-producing and $CD25^+CD8^+$ T-lymphocytes was measured by flow cytometry (Mean ± SD, 4 independent experiments).*

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 and the p28 subunit of IL-27 while the expression of EBI3 was upregulated as a result of polyI:C stimulation (**Fig. 4.2.4.A**). 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 (**Fig. 4.2.4.B**). Moreover, polyI:C-induced CD1c$^+$ DCs were able to produce detectable levels of IL-27 protein that could be inhibited by rapamycin pre-treatment (**Fig. 4.2.4.B**). 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 IRF3, the master regulator of interferon production in moDCs, we first assessed the expression level of ISG56 that is a sensitive indicator of IRF3 transcriptional activity [134]. 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. After 6-hour incubation the cells were harvested and total RNA extracts were prepared to measure the expression of ISG56 by Q-PCR (**Fig. 4.2.5.A**). 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 (**Fig. 4.2.5.A**). These data altogether suggest the pivotal role of mTOR signaling in the regulation of IRF3 activity.

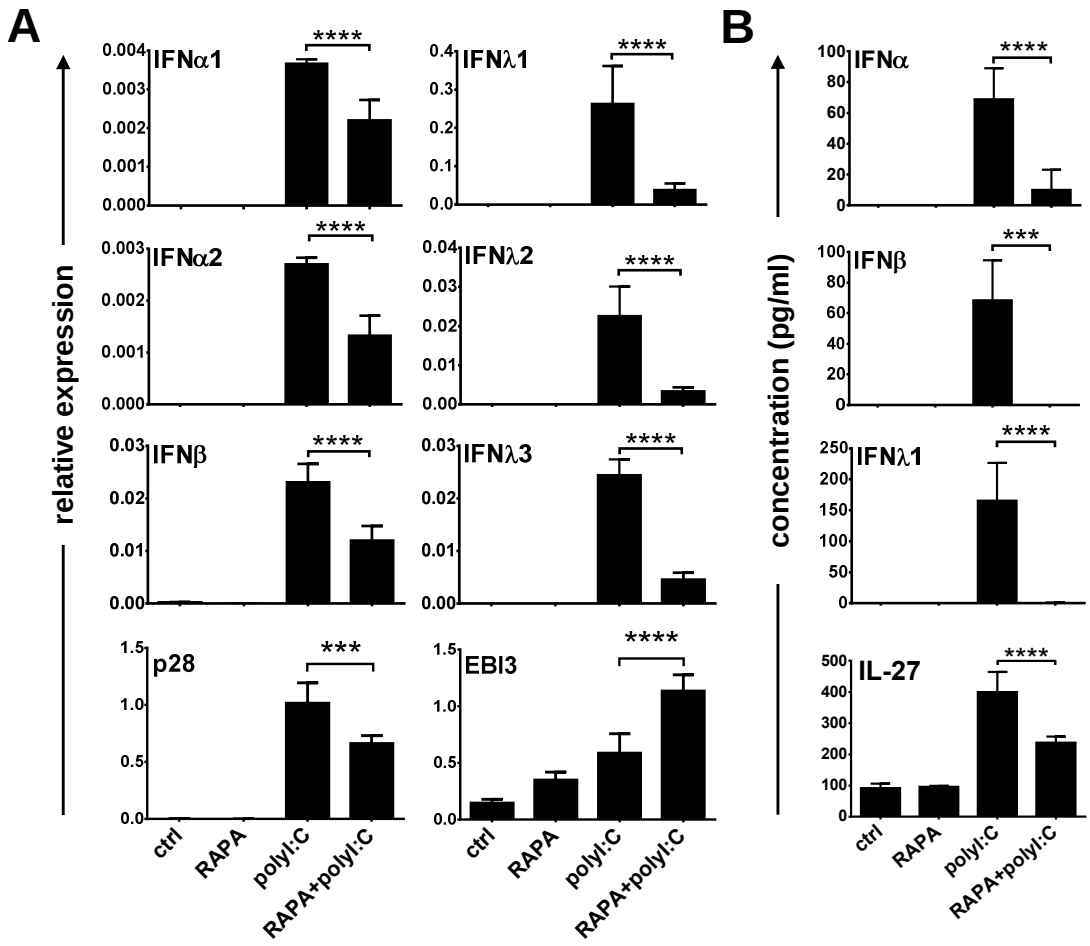

Figure 4.2.4. Rapamycin pre-treatment inhibits TLR3-induced antiviral cytokine production in CD1c$^+$ DCs

CD1c$^+$ blood circulating DCs were pre-treated or not with rapamycin for 2 hours then stimulated by polyI:C. (A) After 12 hours the expression of cytokine genes was measured by Q-PCR. (B) Following 24-hour activation cell culture supernatants were collected and the concentration of cytokines was measured by ELISA. Values represent the Mean ± SD of 4 independent experiments for (A) and (B).

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 [135-138]. 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, p38γ (**Fig.4.2.5.B**) and also JNK1, JNK2, RSK2, p38α (data not shown). PolyI:C stimulation enhanced the activity of several kinases, which was retained in case of Akt (pan), JNK3 and p38γ (**Fig.4.2.5.B**) and remained unaffected in case of Akt1, JNK2 and RSK2 by rapamycin pre-conditioning (data not shown). These data suggest that rapamycin treatment affects the activity of MAPKs differently in resting and polyI:C-activated moDCs.

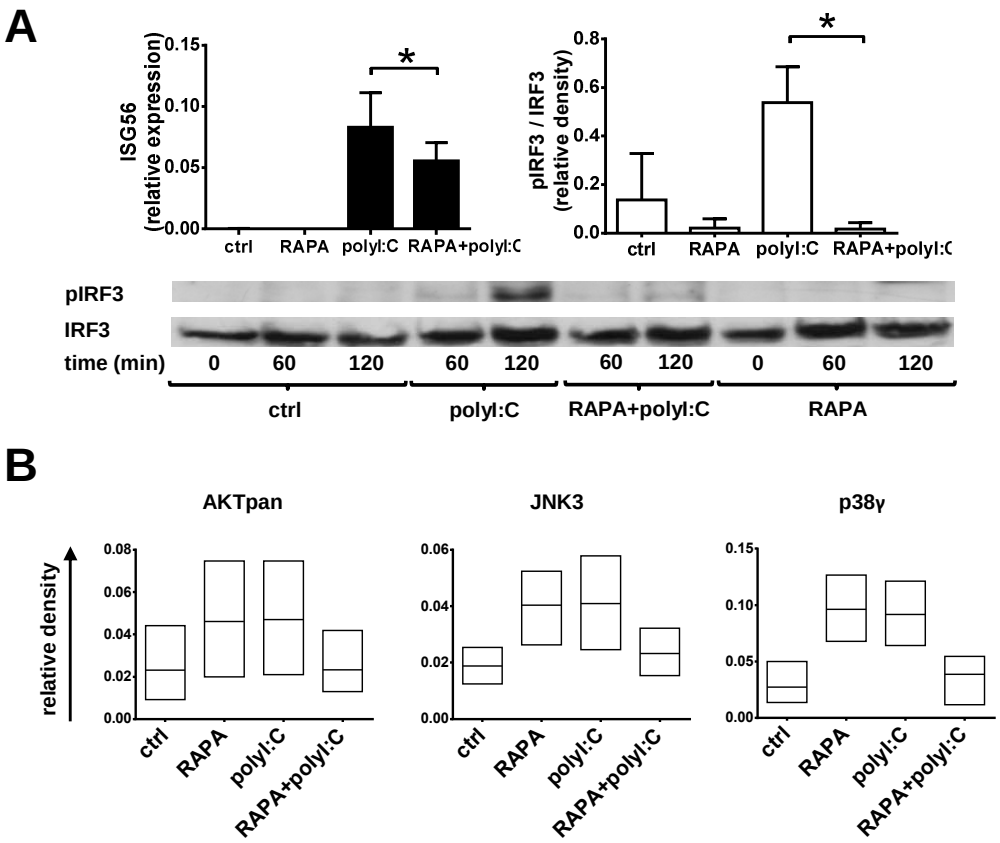

Figure 4.2.5. Rapamycin downmodulates the polyI:C-induced activity of IRF3 and MAPKs
MoDCs were incubated or not with rapamycin for 2 hours then stimulated with polyI:C. (A) Bar graphs on the left show ISG56 mRNA expression measured by Q-PCR 6 hour after induction (Mean ± SD, 6 independent experiments). Kinetics of IRF3 phosphorylation was determined by Western blotting. Bar graphs on the right show pIRF3/IRF3 ratios measured at 2 hours of stimulation (Mean ± SD, 3 independent experiments). (B) 20 minutes after activation the cells were lysed and the phosphorylation of MAPKs was detected and analyzed as described in the Materials and Methods. Values represent the Mean of 4 data points derived from 2 independent experiments.

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 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 (**Fig. 4.2.6.A**). 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 [112]. In another set of experiments, 1-day moDCs were transfected by siRNA specific for TBK1 or scrambled siRNA as control and 48 hours post-transfection the efficacy of gene silencing was verified by Western blot analysis of whole cell lysates. This procedure resulted in the depletion ~90% of TBK1 protein (**Fig. 4.2.6.C**). After stimulation by polyI:C, 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 (**Fig. 4.2.6.B**). These data suggest that TBK1 and mTOR cooperate to regulate the induction of type I IFNs. We also assessed the expression of ISG56 6 hours after stimulation but surprisingly neither LY-294002 treatment nor TBK1 silencing could decrease the transcript levels of ISG56 (data not shown), while rapamycin exerted its inhibitory effect both alone and in combination with the other blocking agents. These findings indicate complex, presumably network-like mechanisms in the regulation of moDC activities, where maximal ISG56 induction requires the contribution of mTOR but seems to be independent on TBK1 and PI3K signaling. 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 (**Fig. 4.2.7**).

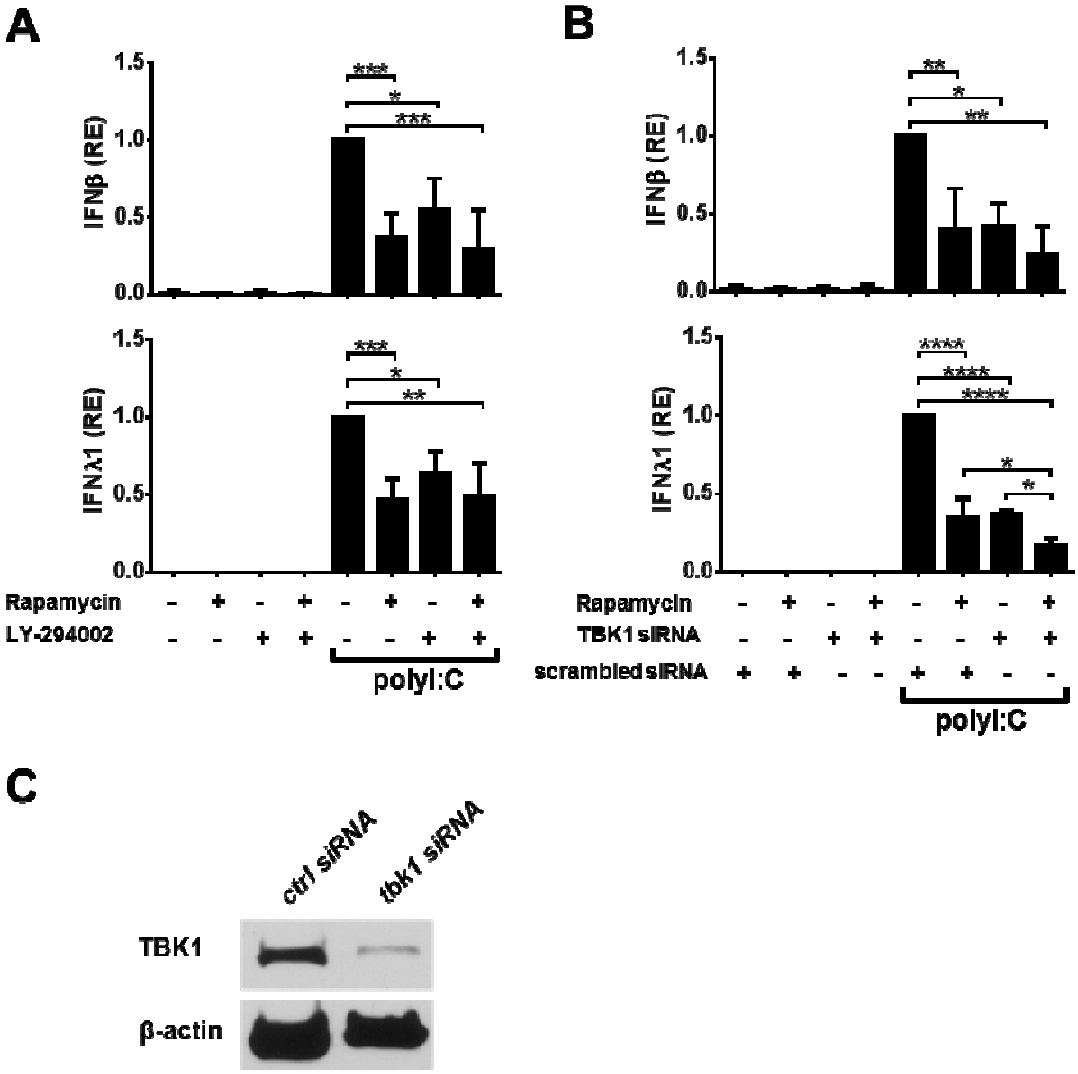

Figure 4.2.6. The PI3K/mTOR signaling pathway collaborates with TBK1 to induce IFNβ cytokine expression. *(A) MoDCs were incubated with rapamycin, LY-294002 or with the combination of the two inhibitors for 2 hours and subsequently were activated by polyI:C or left untreated for 12 hours. The expression of IFNβ and IFNλ1 was assessed by Q-PCR and is shown as relative expression levels compared to maximum values achieved by polyI:C treatment. Results represent the Mean ± SD of 4 independent experiments. (B) On day 1 moDCs were transfected with TBK1-specific or scrambled siRNAs and on day 3 were pre-conditioned or not with rapamycin that followed a 12-hour induction with polyI:C. Expression of IFNβ and IFNλ1 was measured and is shown as in (A). Results represent the Mean ± SD of 3 independent experiments. (C)One-day moDCs were transfected with TBK1-specific or scrambled (negative control) siRNAs and after 48 hours the cells were subjected to Western blot analysis to verify the efficacy of siRNA silencing on the expression of TBK1. One representative result is shown.*

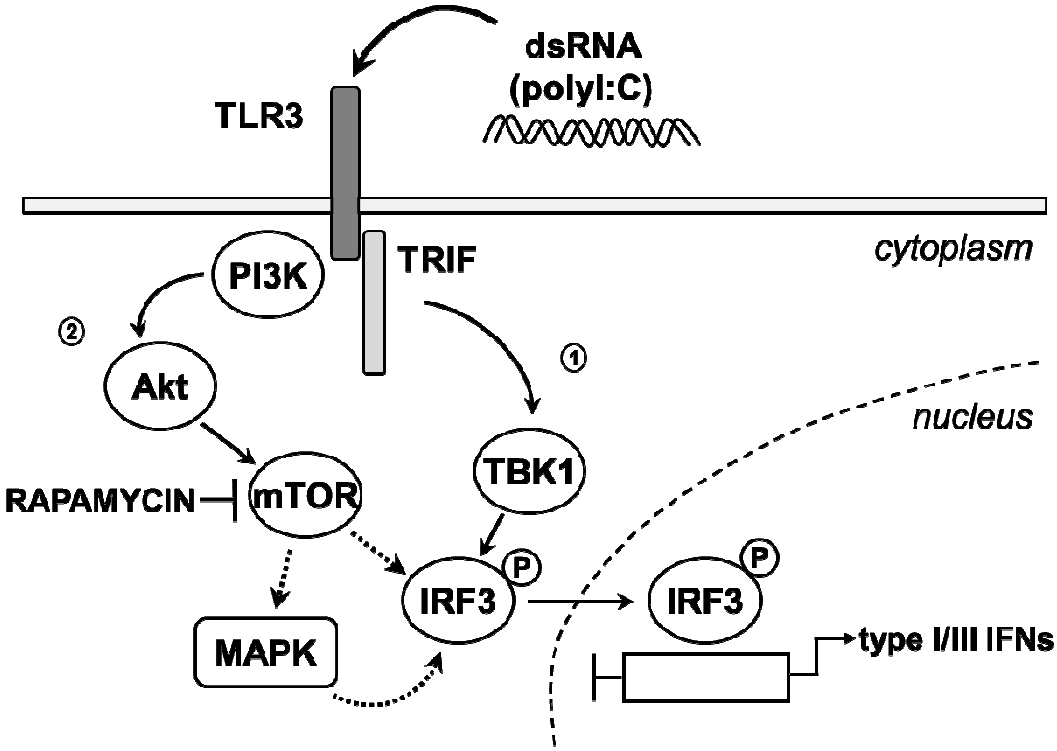

Figure 4.2.7. Proposed mechanism of mTOR-mediated induction of type I and III IFNs in cDCs *Upon stimulation with polyI:C the TLR3-TRIF pathway activates TBK1 that via phosphorylating IRF3 results in the induction of type I and III IFNs (49). Several members of the MAPK family are also activated by TLR3 triggering and support the production of type I IFNs (54). These pathways can be inhibited by rapamycin suggesting the regulatory role of mTOR in the TLR3-mediated induction of type I/III IFN responses. In another pathway TLR3 recruits PI3K, which via the Akt-mTOR signaling pathway can induce IRF3 phosphorylation (49). The simultaneous blockade of mTOR and TBK1 in cDCs indicates that the PI3K/mTOR pathway collaborates with the TRIF/TBK1 signaling cascade to induce optimal expression of type I and III IFNs. Alternatively polyI:C might be recognized by RIG/MDA5 in a cell type-specific manner. Solid lines represent known interactions, dotted lines indicate unknown associations.*

5. DISCUSSION

Dendritic cells are promising therapeutic targets for immune-based therapies and vaccines due to their central role in regulating the immune system [2]. They are capable of recognizing a wide range of pathogens and danger signals and bridging the innate and adaptive arms of the immune system via communicating with various immune cells. Their functional properties can be dramatically dependent and affected by the actual microenvironment of the cells, prompting them to diverge into immunogenic or tolerogenic DCs [9]. A large number of molecules controlling DC functionality have already been discovered; however, the complexity of their regulatory potential needs further clarifications [4]. 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 [85]. 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 [85]. An early study indicated the downregulated cell surface expression of TLR4 as a major cause of LPS tolerance in mouse macrophages [139]. On the contrary, human LPS-tolerized monocytes show unaltered TLR4/MD2 expression [140]. In line with that we did not find any significant difference comparing the TLR4 expression of LPS-pre-treated and non-treated moDCs [127]. 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 [141]. 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 [127]. Recently it has been observed that endotoxin tolerization of human monocytes compromises LPS-inducible K63-linked polyubiquitination of IRAK-1 while not affecting the expression of unmodified IRAK-1[142]. K63-linked polyubiquitination of IRAK-1 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.

Previously, SOCS1 has been implicated in the establishment of tolerance in moDCs developing in the presence of TLR2, TLR3 or TLR4 ligands via inhibiting GM-CSF receptor signaling and thereby preventing DC differentiation [143]. Other studies also indicated the blockade of DC differentiation pathway as a consequence of TLR stimulation of monocyte precursors, in human moDCs *in vitro* [144] and in monocytes entering the skin in response to Gram-negative bacteria [145]. On the other hand, several studies indicated impaired TLR pathways in persistently activated macrophages and DCs as the underlying mechanism for their decreased functionality. SOCS1 upregulation represents a potent negative feedback mechanism that can decrease DC activation, as demonstrated by our results showing higher IL-12 production in LPS-activated DCs following SOCS1 downregulation and also by the increased Th1-type T-cell responses induced by DCs of $SOCS1^{-/-}$ mice [146]. SOCS1 might directly interfere with NFκB activation [147] or it can contribute to the degradation of the adapter protein Mal, associated to TLR4 and TLR2 [148]. Mal modulation might explain why SOCS1 downregulation could increase TLR4-mediated activation but did not affect IL-12 production triggered by a ligand for TRL7/8 that do not utilize Mal. Nevertheless, our results showed no effect of SOCS1

downregulation on the permanent inactivation of moDCs that developed in the presence of continuous TLR ligation, indicating that the LPS-induced SOCS1 molecules, similarly to STAT3, SLAM, miR-146a and IL-10 act only as short-term inhibitory factors.

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 [149]. 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 [150]. Whereas a single exposure to LPS results in the high-level production of IL-6 promoting inflammation and the proteolysis of IDO, mouse cDCs stimulated twice with LPS upregulate the expression of IDO and TGFβ. Futher, it has been found that the adoptive transfer of LPS-primed cDCs can provide protection against a lethal dose of LPS only if the transferred cDCs are competent for IDO and the host is able to produce TGFβ. 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 [151]. Further studies suggested a potential role for the alternative NFκB pathway member p100 (NFκB2) in the control and development of ET in human monocytes [152, 153]. 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 [153].

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 [7]. However, TLR3 is predominantly expressed by $CD141^+$ DCs representing a minor cell population with specialized functions such as being the major producer of IFNλs upon polyI:C stimulation. Here we describe that both 3-day moDCs and $CD1c^+$ DC express TLR3 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. These results called our attention to the indispensable regulatory role of mTOR to the induction of type I and III IFNs in circulating $CD1c^+$ DCs. 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. This observation is in line with previous reports showing that mTOR inhibition promotes the activation of the MAPK pathway in endothelial cells [154] and in cancer patients [155], whereas rapamycin can enhance the activity of Akt by inhibiting the negative feedback loop regulated by mTOR in different cell types [156, 157]. More importantly, 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 [74]. In addition, it has recently been reported that TLR3-elicited activation of p38 is required for the stabilization of IFNβ mRNA that is important at the initial phase of antiviral responses [75]. 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.

It has previously been reported that rapamycin can modulate the phenotype and the subsequent T-cell stimulatory capacity of LPS-stimulated moDCs and $CD1c^+$ DCs differently [115]. Our phenotypic analysis revealed that rapamycin did not interfere with polyI:C-induced maturation of moDCs; however, the decreased expression of both inhibitory and co-stimulatory molecules indicates complex regulation of phenotypic changes in these DC subpopulations. 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 [122, 158, 159]. 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 [160], IFNβ [161] and IL-27 [162] 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. Moreover, a rapamycin-insensitive and rictor-independent mTOR pathway has recently been described, which interferes with the ability of murine DCs to induce foxp3$^+$ T-cells via inhibiting STAT3-induced PD-L1 expression [133]. Based on these results we hypothesize that decreased PD-L1 expression and the lack of a foxp3$^+$ regulatory T-cell expansion might be the result of incomplete rapamycin-induced mTOR inhibition. We propose that mTOR inhibitors targeting the ATP-competitive active site could further clarify the role of mTOR in modulating the ability of polyI:C activated cDCs to promote CD8$^+$ T-cell responses.

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 [163]. 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 [163]. In addition, mTOR initiates a switch from oxidative phosphorylation to glycolysis that is a hallmark of T-cell activation and proliferation [164]. 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 [165]. Besides, mTORc1 promoting the HIF-1α-dependent glycolytic pathway induces the differentiation of Th17 cells and suppresses Treg cell development [165]. 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.

ÖSSZEFOGLALÓ

Az emberi szervezet szinte minden szervében és szövetében fellelhető dendritikus sejtek (DS) hatékony antigénprezentáló sejtekként működnek. Megváltozott saját vagy testidegen struktúrákat felismerve aktiválódnak és antigén-specifikus T-sejt választ váltanak ki. E különleges képességüknek köszönhetően a DS-ek sokrétű szabályozó funkcióval rendelkeznek és immunterápiás eljárások fejlesztésére is alkalmasak. A DS-alapú vakcinák alkalmazási lehetőségeit vírus fertőzések és tumorok kezelésére széles körben vizsgálják, de a terápiás vakcinák megfelelő hatékonyságának fokozása a DS-ek összetett működésének mélyebb szintű megismerését igényli. A jelen tanulmány a DS funkciók két eltérő, a klinikai alkalmazás szempontjából kiemelten fontos vonatkozására, az endotoxin tolerancia és az mTOR általi gátló folyamatok mechanizmusának felderítésére összpontosít. Kísérleteink azt igazolták, hogy a bakteriális lipopolyszachariddal történő rövid (48 óra) előkezelés a moDS funkciók gátlásához vezet. Ezt a hatást az aktivációs jelek széles skálája képes kiváltani és az így válaszképtelenné tett DS-ek funkcióit az együttesen alkalmazott aktivációs jelek sem képesek felfüggeszteni. Mivel a LPS-dal történő ismételt stimuláció az endotoxinnal tolarizált moDS-ekben nem képes kiváltani a gyulladásos citokin gének kifejeződését, a DS-ek aktivációjához vezető jelátviteli útvonalak is gátlódnak. Eredményeink szerint az LPS előkezelés-indukálta SOCS1, STAT3, SLAM, miR-146 és IL-10 molekulák rövidtávú gátló hatás révén csökkentik a monocita-eredetű DS-ek IL-12 citokin termelését, azonban egyik vizsgált molekula sem játszik meghatározó szerepet az endotoxin tolerancia hosszú távú kialakításában.

További vizsgálataink a PI3K/mTOR jelátviteli pálya jelentőségét igazolták a polyI:C-vel aktivált moDS-ek I. és III. típusú interferon termelésének szabályozásában. Eredményeink szerint az mTOR funkciók rapamicinnel törtő gátlása csökkenti az IRF3 és a MAPK útvonal bizonyos tagjainak foszforilációját, ami arra utal, hogy virális eredetű antigének jelenlétében az mTOR számos jelátviteli folyamat elindításához járul hozzá. Emellett a rapamicinnel kezelt moDS-ek kevésbé hatékonyak a $CD8^+$ T-sejtek általi IFNγ termelés kiváltásában. Végezetül úgy találtuk, hogy az mTOR általi szabályozás, hasonlóan a moDS-ekhez, a perifériás vérben keringő $CD1c^+$ DS-ek I. és III. típusú IFN termeléséhez is szükséges. Ezek a kutatási eredmények átfogó képet adnak a DS funkciók szabályozásában résztvevő molekulák szerepéről a mikrobiális stimuláció hatására aktiválódó jelátvitel pályák működése során.

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7.2. Publication list prepared by the Kenézy Life Sciences library

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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.
DOI: http://dx.doi.org/10.1002/eji.201141924
IF:4.97

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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IF:3.365

Total IF of journals (all publications): 18.001
Total IF of journals (publications related to the dissertation): 9.274

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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8. KEYWORDS

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

TÁRGYSZAVAK

dendritikus sejt, Toll-szerű receptor, jelátviteli útvonalak, endotoxin tolerancia, gyulladási citokinek, mammalian target of rapamycin, interferon

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