THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

PATTERN RECOGNITION AND INNATE IMMUNE SIGNALING IN HUMAN DENDRITIC CELLS

by

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DEBRECEN, 2012

ABBREVIATIONS

ALR	-	AIM2-like receptor
AP-1	-	Activator protein-1
APC	-	Antigen presenting cell
ATRA	-	All-trans retinoic acid
BIR	-	Baculovirus inhibitor repeat
Btk	-	Bruton's tyrosine kinase
CARD	-	Caspase recruitment domain
CCL	-	Chemokine ligand with "CC" motif
CCR	-	C-C chemokine receptor
CD	-	Cluster of differentiation antigen/marker
cDC	-	Classical/conventional dendritic cell
CLR	-	C-type lectin receptor
CTL	-	Cytotoxic/cytolytic T cells
CXCL	-	Chemokine ligand with "CXC" motif
CXCR	-	C-X-C chemokine receptor
DAMP	-	Damage-associated molecular patterns
DC	-	Dendritic cell
ELISA	-	Enzyme-linked immunosorbent assay
ELSIPOT	-	Enzyme-linked immunosorbent spot
FADD	-	Fas-Associated protein with Death Domain
Flt-3	-	FMS-like receptor tyrosine kinase-3
GM-CSF	-	Granulocyte-monocyte colony-stimulating factor
IFN	-	Interferon
IKK	-	inhibitor of NF-κB kinase
IL	-	Interleukin
IRAK	-	Interleukin-1 receptor-associated kinase
IRF	-	Interferon regulatory factor
ISRE	-	Interferon-stimulated response element
ISG15	-	Interferon-induced 17 kDa protein
LBP	-	LPS-binding protein
LC	-	Langerhans cell
LGP2	-	Laboratory of Genetics and Physiology 2
LPS	-	Lipopolysaccharide
LRR	-	Leucine-rich repeats
LN	-	Lymph node
MAPK	-	Mitogen-activated protein kinase
MAP3K7	-	MAPK kinase kinase 7 (synonym: TAK1)
MAVS	-	Mitochondrial antiviral signaling protein (synonym: IPS1, VISA)
MDA5	-	Melanoma differentiation-associated gene 5
MDP	-	Muramyl dipeptide
MHC	-	Major histocompatibility complex
MKK	-	MAPK kinase
moDC	-	Monocyte-derived dendritic cell
MMP	-	Matrix metalloproteinase
MyD88	-	Myeloid differentiation primary response gene 88
NEMO	-	NF-κB essential modulator
NF-κB	-	Nuclear factor kappa-B

NLR	-	NOD-like receptor
NOD	-	Nucleotide-binding oligomerization domain
NS	-	Nonstructural protein (viral)
PAMP	-	Pathogen associated molecular pattern
PBMC	-	Peripheral blood mononuclear cell
pDC	-	Plasmacytoid dendritic cell
pI:C	-	Polyinosinic:polycytidylic acid
PGN	-	Peptidoglycan (synonym: Murein)
PPAR	-	Peroxisome proliferator-activated receptor
PRR	-	Pattern recognition receptor
PYD	-	Pyrin domain
RIG-I	-	Retinoic acid-induced gene I
RIP	-	Receptor interacting protein
RLR	-	RIG-I-like receptor (synonym: RLH)
RLH	-	RIG-I-like helicase (synonym: RLR)
RNF125	-	Ring finger protein 125
SOCS1	-	suppressor of cytokine signaling 1
STING	-	Stimulator of IFN genes
TANK	-	TRAF-family-member-associated NF-κB activator
TBK1	-	TANK-binding kinase 1
Th	-	Helper T lymphocyte
TIR	-	Toll/IL-1 receptor domain
TIRAP	-	TIR domain-containing adapter (synonym: Mal)
TLR	-	Toll-like receptor
TNF	-	Tumor necrosis factor
TRAF	-	TNF receptor-associated factor
TRAM	-	TRIF-related adapter molecule
TRIF	-	TIR-domain-containing adapter-inducing interferon-β
TRIM25	-	Tripartite motif 25
Ubc	-	Ubiquitin-conjugating enzyme
Uev1A	-	Ubiquitin-conjugating enzyme E2 variant 1

CONTENTS

SUPPLEMENTARY 1

Tunde Fekete*, **Attila Szabo***, Luca Beltrame, Nancy Vivar, Andor Pivarcsi, Arpad Lanyi, Duccio Cavalieri, Eva Rajnavolgyi and Bence Rethi: Constraints for monocyte-derived dendritic cell functions under inflammatory conditions.

Eur J Immunol 2012 42(2):458-469. **shared first authors*

SUPPLEMENTARY 2

Attila Szabo, Krisztian Bene, Peter Gogolak, Bence Rethi, Arpad Lanyi, Istvan Jankovich, Balazs Dezso and Eva Rajnavolgyi: RLR-mediated production of interferon-β by a human dendritic cell subset and its role in virus-specific immunity.

J Leukoc Biol 2012 92(1):159-169.

1. INTRODUCTION

Dendritic cells (DCs) involve a heterogeneous population of hematopoietic cells that co-evolved with the adaptive immune system. They are efficient orchestrators of innate and adaptive immune responses by their ability to sense environmental changes, internalize and process proteins, and present their peptide fragments to T lymphocytes. Their activity has been shown to be critical in the induction and maintenance of self tolerance and initiation of immune responses.

The 2011 Nobel Prize in Medicine awarded to Bruce Beutler, Jules Hoffmann and Ralph Steinman further supported the admission of the field of innate immunity and its impact on modern medical sciences. The Prize was shared for the discoveries of the role of Toll-like receptors (TLRs) in fruit fly immunity, TLR activities in mammals and the identification and characterization of DCs as initiators of the adaptive immunity.

The innate immune system is an ancient host defense mechanism thought to be appeared in early arthropods about 400 million years ago. Representing the very first barrier against invading pathogens, it is known about its capability to distinguish between innocuous and dangerous, as well as self and non-self structures by means of the so-called pattern recognition receptors (PRRs). These ancient receptors are able to recognize evolutionally conserved motifs associated to microbes, and subsequently trigger cellular immune responses leading to inflammation and the production of antimicrobial molecules.

Membrane-bound TLRs and the cytoplasmic RIG-I-like helicases (RLHs) are two important families of innate sensors. Upon activation both receptor types mediate pro-inflammatory and interferon responses, two crucial branches of early-phase host defenses. In the past decade novel vaccination strategies based on the specific activation or co-stimulation of these receptors have also been developed to increase the efficacy of protective immune responses to combat virus infections.

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

2. THEORETICAL BACKGROUND

2.1 Dendritic cells and their functions

The description of DCs by Steinman and Cohn (Steinman and Cohn 1973) and the functional characterization of their antigen-presenting capability (Steinman 1991) was a major advance in immunology. The finding that epidermal Langerhans cells (LCs) are functionally identical to splenic DCs led to a unified model of DC ontogeny (Matzinger 1994). DCs are part of the hematopoietic system and have a relatively short *in vivo* turnover time in both mice and humans (Kamath et al. 2000; O'Keeffe et al. 2002; Collin et al. 2011). Based on their origin, tissue localization and functional properties, human DCs can be classified into two major types: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). As cDCs and pDCs found in peripheral tissues are non-dividing cells, they must be continuously replenished from bone marrow-derived hematopoietic stem cells (HSCs) (Figure 1). The majority of human DCs derive from myeloid precursors and give rise to various subsets, such as LCs, interstitial (dermal or tissue) DCs, and monocyte-derived DCs (moDCs) (Shortman and Liu 2002). The coordination of their lineage commitment and subsequent functional versatility has been shown to be defined by the unique cooperation of transcription factors (Belz and Nutt 2012). The origin of pDCs is controversial, as their lineage may be rooted to both myeloid and lymphoid progenitors that express the FMS-like receptor tyrosine kinase-3 (Flt-3) showing that DC developmental programs can likely be induced in both precursors due to the flexibility of DC generation (Chicha et al. 2004; Benko et al. 2008). Unlike cDCs that are localized to the periphery, circulating pre-pDCs require activation signal(s) to enter lymphoid tissues and to obtain DC morphology. Migratory DCs originate from blood-circulating monocytes, LCs from inflammatory $CD14^{high}$ monocytes (Schaerli et al. 2005), and interstitial DCs from non-inflammatory $CD16^{+}$ monocytes (Qu et al. 2004; Gordon and Taylor 2005). Granulocyte-macrophage colony stimulatory factor (GM-CSF)-induced transformation of monocytes to DCs is considered to be a standard *in vitro* model of human inflammatory DC development, and Flt-3 ligand was found to be critical for the steady state development of both pDC and cDC subtypes (Shortman and Naik 2007). cDCs are be found in all peripheral tissues and act as professional sentinels that constantly sample their surrounding microenvironment (Steinman 1991). The internalization of physiological tissue-derived

materials by cDCs induces steady state migration to draining lymph nodes (LNs), where cDCs in turn interact with T-lymphocytes. This direct DC-T cell communication results in tolerance induction through different negative regulators of the immune response that inhibit inflammatory signaling pathways. The heterogeneous population of DCs, acting as antigen presenting cells (APC), interacts with many leukocyte types in both lymphoid and non-lymphoid tissues (Shortman and Naik 2007).

Circulating pre-pDCs differentiate and become activated in lymphoid tissues and specialized for the production of type I interferons (IFN) (Swiecki and Colonna 2010). Classical steady state DCs derive from a common bone marrow monocyte/dendritic cell progenitor, migrate to lymphoid and peripheral tissues, and are replenished from circulating and/or local precursors (Geissmann et al. 2003) (Figure 1). Under inflammatory conditions, circulating monocytes are recruited to inflamed tissues where they develop into macrophages or monocyte-derived DC with phagocytic activity, and as a result of activation they express co-stimulatory molecules and produce cytokines and chemokines (Ginhoux et al. 2006). Recent studies have revealed the origin and developmental pathways of multiples DC subsets in both mice and humans (reviewed by Geissmann et al. 2010 & Yona and Jung 2010); these studies have shown DCs to exhibit specialized functions, such as high migratory potential, the ability to secrete unique combinations of cytokines, and the capability to cross prime $CD8\alpha^{+}$ cytolytic T cells (CTL) (Kurts et al. 2010).

During pathogenic invasion or inflammation, resting DCs become activated resulting in their transition to a mature cell type with altered chemokine receptor expression. This process ensures the rapid migration of activated DCs through the lymphatics to draining LNs. Here, activated DCs carrying their accumulated antigenic content act as highly potent APCs and instruct T lymphocytes to differentiate into effector and memory cells (Figure 2). Microbial compounds are strong activators of resting DCs and mediate stimulatory signals through conserved pattern recognition receptors (PRRs) (Benko et al. 2008). In response to these stimuli, steady state tissue resident DCs exit inflamed tissues and transport peripheral antigens to secondary lymphoid organs where these cells can initiate the adaptive immune response by triggering naïve T cell activation. At the same time, monocytes enter the inflamed tissues and give rise to phagocytic APCs including DCs, thereby counterbalancing the rapid egress of DCs (Eidsmo et al. 2009; Randolph et al. 1999; Ginhoux et al. 2006).

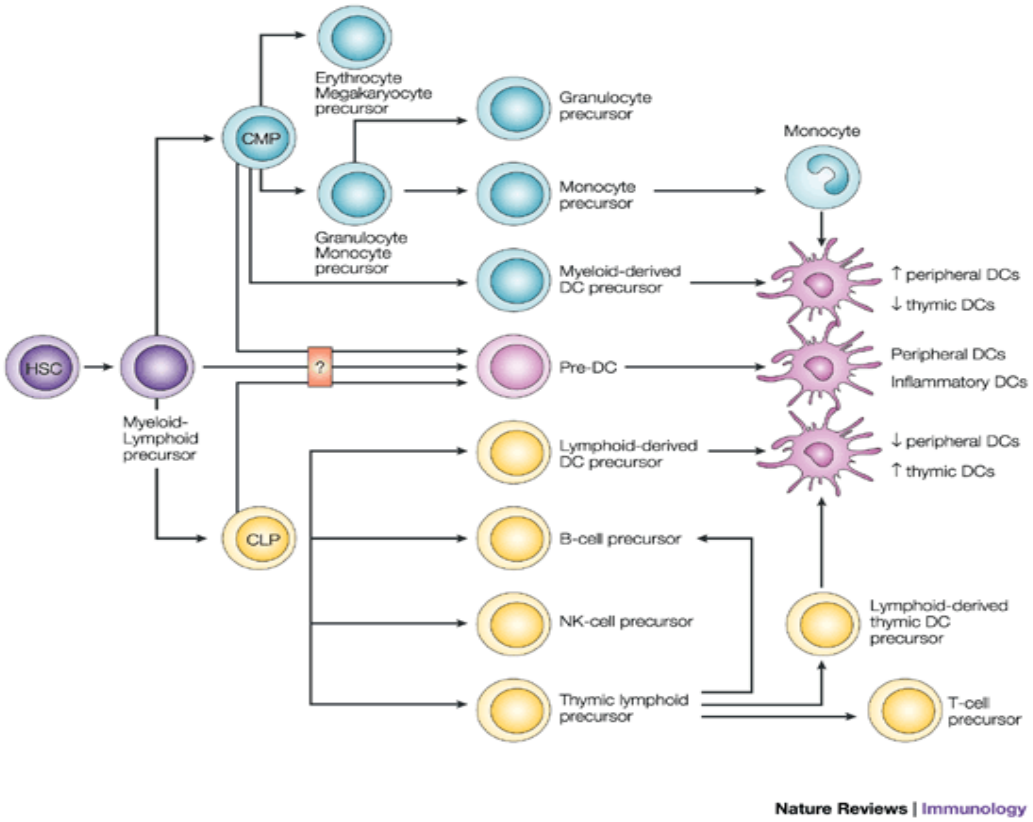

Figure 1. *Ardavín, C. (2003) Nat Rev Immunol* ***3****:582-591.*

The newly differentiated monocyte-derived dendritic cells (moDCs) may function as resting tissue resident APCs or as sources of inflammatory cytokines (McGill et al. 2008; Wakim et al. 2008). In addition, these cells may migrate to peripheral lymphoid organs and maintain the activation of naïve T lymphocytes (Randolph et al. 1999; Cheong et al. 2010).

In vitro generated human moDCs provide a rich source of cells with remarkable plasticity upon responding to various signals and thus emerged as promising candidates for various immunotherapies and have been utilized for targeting vaccines against cancers and viruses (Banchereau and Palucka 2005; Palucka et al. 2010). DCs express a wide array of PRRs that are distributed to various cellular compartments. C-type lectin receptors (CLRs), Fc- and complement receptors, and Toll-like receptors (TLRs) are integrated to plasma- or vesicular membranes, whereas nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-induced gene I (RIG-I)-like receptors or helicases (RLRs/RLHs) are cytosolic molecular sensors of foreign and modified nucleotides (Benko et al. 2008) (see also parts *2.2.1* and *2.2.2*).

Conventional DCs display membrane TLR4 and intracellular TLR3, TLR7, and TLR8 (Barton and Kagan 2009), and also express cytosolic RLR and NLR (Deretic 2011). The cell type specific expression and the intracellular compartmentalization of PRR determine the synergistic or inhibitory cross-talk of these receptors, the interplay of the coupled signaling pathways they trigger, and the effector molecules they produce (Medzhitov and Horng 2009).

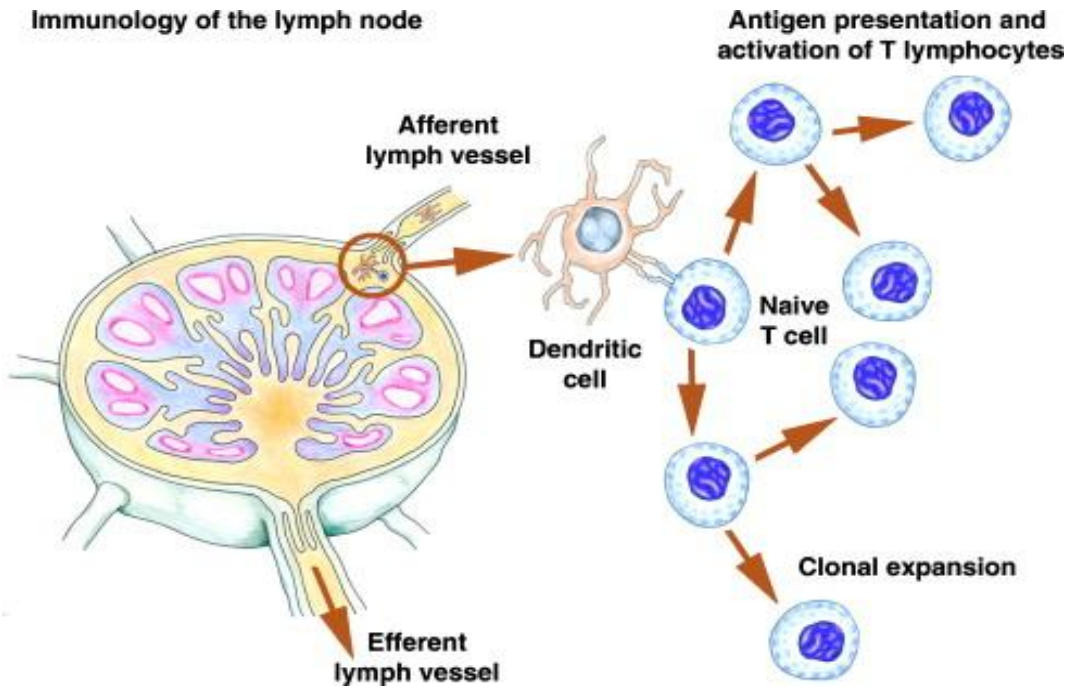

Figure 2. Antigen-presentation by DCs in the lymph node
Sherif, A. et al. (2010) Eur Urol ***58****:105-11.*

Human monocytes obtain DC-like features when cultured for 5-8 days in the presence of GM-CSF and IL-4 or other cytokines (Sallusto and Lanzavecchia 1994; Zou and Tam 2002). During their differentiation, moDCs downregulate CD14, upregulate CD1a and DC-SIGN, and obtain the ability to express CCR7, which is required for migration to lymphoid tissues. On the other hand, such differentiation of immature moDCs is unlikely to occur in inflamed tissues where the developing cells constantly receive stimulatory signals from microbial compounds, inflammatory mediators, and tissue damage. It has been extensively documented that long term activation leads to functional exhaustion of macrophages and DCs (Biswas and Lopez-Collazo 2009).

Therefore, DC inactivation by constant stimulatory signals counteracts the development of potent monocyte-derived APCs in inflamed tissues.

There are several molecular mechanisms implicated in macrophage and DC exhaustion (Biswas and Lopez-Collazo 2009; Liew et al. 2005). These include increased or decreased expression of signaling components, the release of soluble mediators that interfere with DC functions, and the altered expression of molecules that regulate gene expression. Bacterial lipopolysaccharide (LPS) increases SOCS1 (suppressor of cytokine signaling 1) expression in developing moDCs, and can inhibit nuclear factor kappa-B (NF-κB) activation (Bartz et al. 2006; Yoshimura et al. 2007) and GM-CSF signaling, thereby interfering with moDC survival and differentiation (Bartz et al. 2006). Chronic stimulation of moDCs through NOD2 molecules has been linked to the upregulation of interleukin-1 receptor-associated kinase M (IRAK-M, also known as IRAK-3), an inhibitor of IRAK-1 activation (Hedl et al. 2007), and IRAK-M induction has been detected in monocytes of septic patients (Escoll et al. 2003). LPS induced microRNAs have been shown to downmodulate TLR signaling components, TNF receptor associated factor 6 (TRAF6), and IRAK-1 via the microRNA miR146a (Taganov et al. 2006), IKKε via miR-155 (Tili et al. 2007) in macrophages, and TAB2 via miR155 in moDCs (Ceppi et al. 2009). TLR4 expression is decreased in LPS-treated macrophages (Nomura et al. 2000) and the degradation of IRAK-1 has been linked to impaired TLR signaling in both macrophages and DCs (Li et al. 2000; Albrecht et al. 2008). LPS-induced IL-10 was shown to prime IRAK-1, IRAK-4, and TRAF6 for proteasomal degradation in murine DCs (Chang et al. 2009), and IL-10 also contributed to decreased IL-12 production via STAT3 (Hoentjen et al. 2005). Although several pathways have been implicated in the functional exhaustion of long-term activated macrophages and DCs (Biswas and Lopez-Collazo 2009; Liew et al. 2005), the relative contribution of these pathways is not fully understood. It is yet to be clarified whether these pathways cooperate or operate within different conditions and/or time frames, or multiple inhibitory mechanisms act in a redundant manner.

2.1.1 Control of LPS-induced moDC activation

In our studies we analyzed the effects of a variety of activation-induced inhibitory factors on the cytokine production of moDCs receiving TLR4 stimulation during their early differentiation. Among these factors, we associated CD150, STAT3, SOCS1, miR146, and IL-10 molecules with LPS-induced short term inhibitory effects

on DC activation and identified the down modulation of IRAK-1 as a mechanism that can contribute to persistent DC inactivation. We found that early LPS treatment inactivated the myeloid differentiation primary response gene 88 (MyD88)-dependent TLR pathway in developing moDCs whereas TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent IFNβ induction remained intact. In order to determine whether an inflammatory environment could allow the differentiation of migratory moDCs to instruct T cell responses, we also studied the effects of early activation of developing moDCs on their functional activities Strong activation of early stage moDCs led to inflammatory cytokine production that surprisingly was not followed by the characteristic changes in chemokine receptor expression that allow mature DCs to migrate into peripheral lymphoid tissues. Thus activities of newly developing inflammatory moDCs might be limited to peripheral tissues due to the inability of these cells to modulate chemokine receptor expression.

2.1.2 Dendritic cell subtypes and subsets

The first analyses of lymphoid tissue-isolated DCs performed by flow cytometry revealed a vast heterogeneity in the expression of surface markers such as CD4, CD8, Fc receptors, and integrins (Crowley et al. 1989). These markers were originally used to distinguish DC subsets and had a quite low impact on the understanding of their functions. However, subsequent studies showed significant differences in the immunological properties of these DC subtypes, leading to the concept of functionally specialized DC subsets. These subpopulations were considered as products of separate branches of DC development (Shortman and Caux 1997; Manz 2006; Shortman and Villadangos 2006). The recent classification is based on functional properties, turnover, and kinetics of DC generation, and on the dependence on certain transcription factors for the development of the given DC subset. However, as several studies point out, a potential hazard may arise in distinguishing DC subtypes based on surface markers because of the propensity of DCs to acquire surface antigens from associated T cells. For instance, thymic DCs acquire CD8αβ, CD4, and Thy1 from thymocytes (though they do not seem to obtain the pan-T cell marker CD3 this way) (Vremec et al. 2000). The probability and level of cytometric staining of these pickup-antigens is usually moderate, and there are several options to test whether the detected staining represents authentic expression by DCs. The most commonly used technique is the co-measurement of the appropriate mRNA together with protein detection of the surface

marker. Another approach is the establishment of bone-marrow chimeras of wild-type and the selected marker-deficient mice, where any staining for the marker on the deficient cells proves its origin from other cells (Vremec et al. 2000; Shortman and Villadangos 2006).

The distribution of different DC subtypes in the body goes together with strict surface marker patterns. For example, mouse spleen DCs can be divided into three smaller groups, but all of them express the "spleen DC-specific" composition of CD8α, CD4, CD11b (Mac-1), and CD205 (DEC-205). Other markers, like CD24 and Sirp-α, can be a basis of further sub-classification. Likewise, lymph node (LN) DCs are very similar to spleen DCs as far as the surface marker composition is concerned, but they almost completely lack CD4 in mice (Henri et al. 2001). The direct comparison of human and mouse blood DC subpopulations showed the presence of similar pDC and early cDC populations (O'Keeffe et al. 2003) but in some cases functionally identical cells may express different surface markers, for instance CD8α, a key phenotypic marker of mouse cDC subpopulations is not expressed on human cDCs, although their function overlap significantly. Another example is TLR9, which is exclusively expressed by pDCs in humans, but is expressed by several mouse DC subtypes (Iwasaki and Medzhitov 2004; Shortman and Villadangos 2006). The evolutionary advantage of this versatile and manifold network of DC subtypes might lie in the differences of their functional potential.

Human CD1 is a family of surface glycoproteins expressed by thymocytes, epidermal LCs, and a subpopulation of B cells (Figure 3). The family consists of four homologous proteins: CD1a, CD1b, CD1c, and CD1d, and the CD1 gene locus on human chromosome 1 contains five potential CD1 genes (Amiot et al. 1986; Calabi and Milstein 1986). The main function of CD1is to present self- or pathogen-derived lipids to activate CD1-restricted T lymphocytes (Felio et al. 2009; Moody and Porcelli 2003; Vincent et al. 2003) (Figure 3). Unlike MHC class II molecules, the membrane expression of CD1a does not depend on DC maturation, but little is known about the transcriptional and/or ligand-dependent regulation of this process (Cao et al. 2002).

2.1.3 CD1 expression distinguishes phenotypically and functionally distinct DC subsets

Previously, two human moDC subsets were identified that differed in their phenotypic and functional characteristics (Gogolak et al. 2007; Szatmari et al. 2004).

The CD14^{low}DC-SIGN^{+}PPARγ^{high}CD1a^{-} moDC subset, referred to as CD1a^{-} DC, has been identified throughout lymph nodes and was characterized by efficient phagocytic activity to engulf bacteria and apoptotic cells (Majai et al. 2010). Depending on environmental cues, these cells develop into CD14^{-}DC-SIGN^{+}PPARγ^{low}CD1a^{+} inflammatory cells with high cytokine secretion and membrane expression of CD40 and E-cadherin. This CD1a^{+} DC subset is detectable in the interfollicular areas of reactive lymph nodes (Szatmari et al. 2004). The ratio of CD1a^{-} to CD1a^{+} moDC varies among individuals and is negatively regulated by serum lipids and synthetic PPARγ ligands (Szatmari et al. 2006), which have been shown to ameliorate immune pathology in mice infected by highly pathogenic influenza virus (Aldridge et al. 2009). As the molecular background of the inflammatory nature of CD1a^{+} moDC was unknown, we examined whether these DC subsets differ in their ability to sense viral dsRNA by RLH, and if there is any difference in their pro-inflammatory and type I IFN responses.

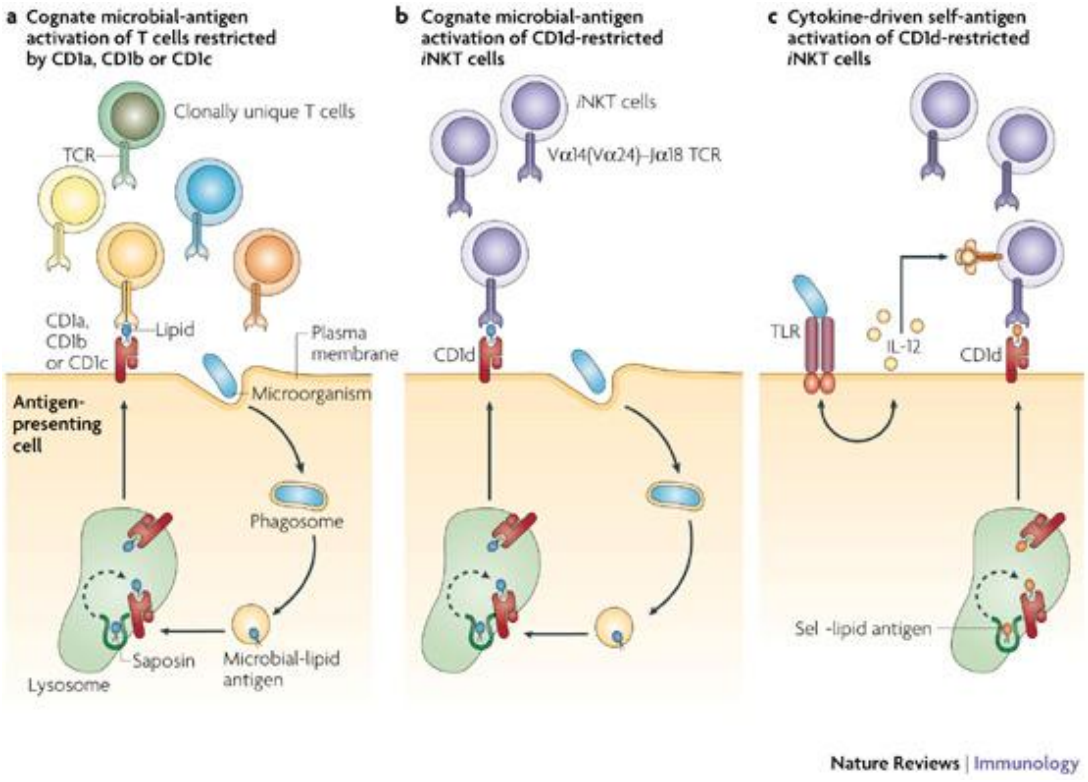

Figure 3. Antigen-presentation via CD1
Barral, D. C. and Brenner, M. B. (2007) Nat Rev Immunol 7:929-941.

2.2 Pattern recognition receptors and the regulation of inflammatory and interferon responses

2.2.1 PRRs: families and features

The immune system is an evolutionally conserved but advanced host defense mechanism against invading pathogens. Innate immune processes are triggered by microbial components that are essential for the given organism. Upon infection, these pathogen-associated molecular patterns (PAMPs) are recognized by specific PRRs that are germline encoded and are usually expressed constitutively in the host (Núñez 2011). The big picture, however, is far more complicated. Microbial moieties are also found in non-pathogenic microbes, and therefore the presence of different PAMPs cannot explain the discrimination between "pathogenic" and "non-pathogenic" life forms *per se*. Furthermore, certain PRRs also sense host-derived (*i.e.* "self") materials that become available during cellular/tissue injury. These endogenous PRR ligands have been termed "damage-associated molecular patterns" (DAMPs), and their impact on the immune homeostasis is yet to be clarified. A recent review focuses on the role of these endogenous molecules with the potential to elicit inflammation and cell death by activating innate PRRs (Rock et al. 2011). A growing body of evidence suggests an evolutionary linkage between innate immunity and cell death signaling. For example, several studies discuss the emerging role of mitochondria in the activation of innate signaling, and the connection between apoptotic cell death and innate immunity (Tal and Iwasaki 2011; West et al. 2011). According to the symbiotic theory, the mitochondrion is an organelle derived from Gram-negative bacteria. It is possible that in multicellular organisms the development of cellular machineries for cell death and innate defense against microbial pathogens is based on ancestral mechanisms associated with bacteria. This may shed light on the importance of and help us to understand the co-evolution of early bacteria and the immune system.

Thus far, five classes of PRRs have been identified. They cover:

1) Transmembrane TLRs, which are integrated to cell surface or endosomal membranes of various cell types;

2) Membrane CLRs characterized by the presence of a carbohydrate-binding domain;

3) Three additional families of intracellular sensors, which are located in the cytosol of various cell types and involve NLRs, RLHs and the recently described AIM2-like receptors (ALRs) all with nucleotide recognition capabilities (Barber 2011).

The type of microbes recognized by the immune system might depend on the cellular localization of PRRs. For instance, PRRs expressed in the cell surface could primarily sense extracellular bacteria, whereas cytoplasmic receptors could be involved in the detection of intracellular microbes such as viruses. However, it has also been shown that many extracellular bacteria can also be detected by intracellular sensors. There is evidence that extracellular *Staphylococcus aureus* and *Streptococcus pneumoniae* produce bacterial toxins that promote the transport of peptidoglycan fragments into the host cells, thereby helping the activation of NOD1 and NOD2, two members of the NLR family (Clarke and Weiser 2011). It is therefore tempting to speculate that extra- and intracellular PRRs serve another purpose: in accordance with their simultaneous activation patterns, the innate immune system can discriminate between pathogenic and non-pathogenic microorganisms. The review of Fontana and Vance (2011) supports this notion by presenting a two-signal activation model with the example of the obligate intracellular pathogen *Legionella pneumophila*. Indeed, several Gram-negative bacteria such as *L. pneumophila* and *Pseudomonas aeruginosa* use special secretion systems that allow them to translocate virulence factors into the cytosol of the host cell. These secretion systems form pores in the host cell membrane and can deliver flagellin into the cytoplasm, where it can trigger NLRC4 (another NLR family member) and lead to caspase-1 activation (Miao et al. 2006; Franchi et al. 2006). These observations confirm that cytoplasmic PRRs allow the innate immune system to discriminate between pathogenic and non-pathogenic signals through their compartmentalization and orchestrate the host response accordingly. Since our studies focus to the role of RLHs and TLRs in human DCs, the next section will cover only basic facts about NLRs, CLRs, and ALRs while provide with a more detailed description of TLRs and RLHs in *part 2.2.2*.

NOD-like receptors are built up by the combination of conserved structural and functional elements, including N-terminal caspase recruitment domain (CARD), baculovirus inhibitor repeat (BIR), pyrin domain (PYD), and central nucleotide-binding oligomerization (NOD) domain that mediates self-oligomerization during activation, and C-terminal leucine-rich repeats (LRRs) that define ligand specificity (Werts et al. 2011). Several NLRs participate in the formation and activation of inflammasomes,

which control the activation of caspase-1, a protease that carries out the cleavage of the pro-cytokines IL-1β and IL-18 and allows the secretion of the first-line pro-inflammatory cytokines (Rosenzweig et al. 2011) (Figure 4). ALRs (AIM2, DAI/ZBP1, IFI16, DHX9/36) belong to a family defined by the presence of a PYD and a DNA-binding HIN module involved in the cytosolic detection of self- and microbial molecules (Hansen et al. 2011). After activation by microbial or endogenous motifs, these innate sensors activate downstream signaling pathways, including NF-κB, interferon-regulatory factors 3 and 7 (IRF3/7) (type I IFN pathways), and/or inflammasomes, which results in the production of inflammatory cytokines, chemokines, and type I interferons important in the early antimicrobial response (Figure 4). C-type lectin receptors are cell surface sensors that can recognize several carbohydrates. Upon activation, CLRs trigger and tightly control the NF-κB pathway and interfere with the downstream signaling events of certain TLRs, thereby determining and fine tuning the secretion of specific cytokines that are important in the polarization of T cells (Geijtenbeek and Gringhuis 2009; Núñez 2011).

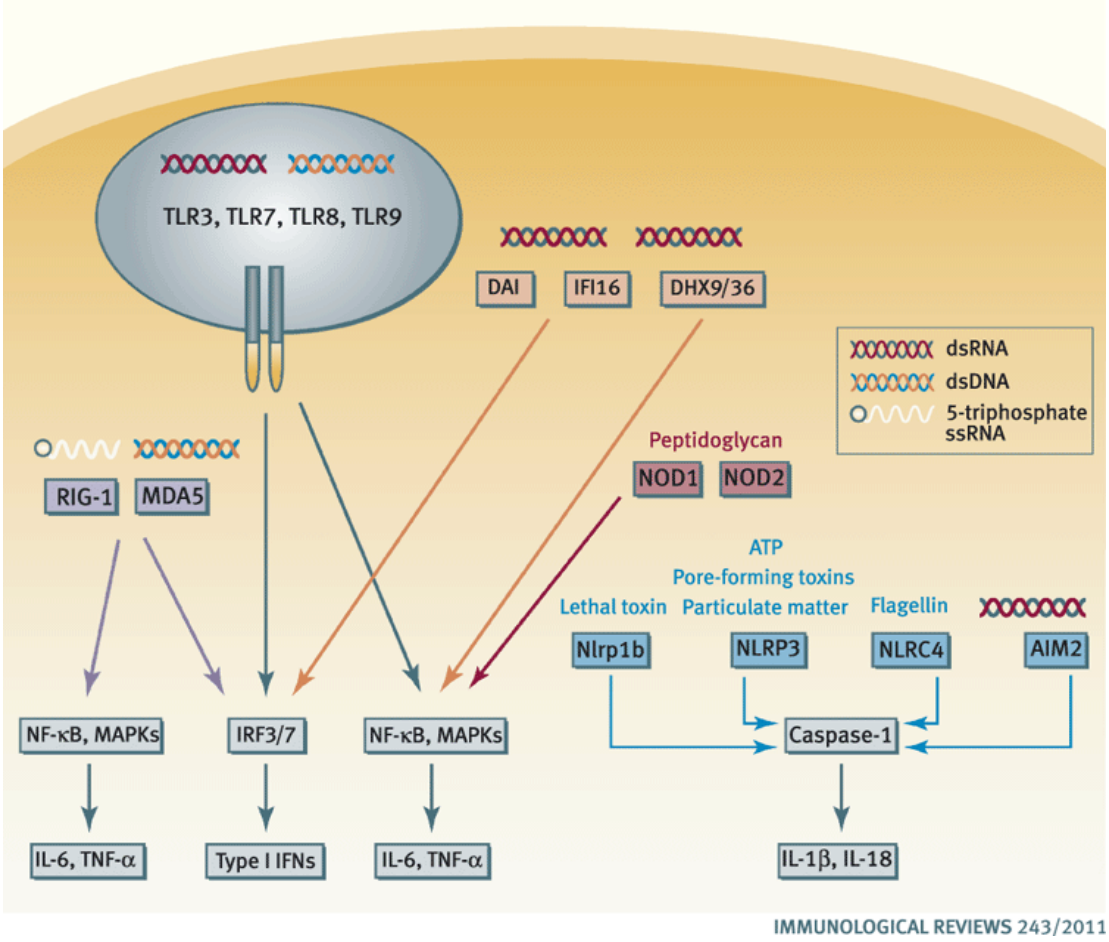

Figure 4. Signaling pathways of intracellular pattern recognition
Núñez, G. (2011) Immunol Rev ***243****:5-8.*

2.2.2 The function of and signaling through TLRs and RLHs

2.2.2.1 TLR signaling pathways and adaptors

As mentioned above, host defense mechanisms are able to sense pathogen invasion through different PRRs such as evolutionally conserved transmembrane TLRs, which represent the most ancient family and the earliest host defense mechanism found in insects, plants, and animals (Akira 2004; Uematsu and Akira 2008). An important event in the history of immunology was the description of the *Drosophila* Toll protein, which is essential for immune responses against *Aspergillus fumigates* (Lemaitre et al. 1996) (see part *2.2.1*). TLRs play a critical role in innate immune responses and as it was shown above, many PRRs and their activities are associated to opsonization, phagocytosis, complement cascades, proinflammatory signaling pathways, and apoptosis. The transmembrane protein Toll was identified as a vital component of dorsal-ventral embryonic development (Wu and Anderson 1997) and was also found to be involved in innate immune responses of the model organism *Drosophila melanogaster* (Lemaitre et al. 1996). The immune defense of the fruit fly is based on the production of different antimicrobial peptides induced by Toll signaling pathways (Tanji and Ip 2005). In the last decade milestones of Toll signaling were laid and several *Drosophila* adaptors and downstream signaling proteins involved in the induction of antimicrobial peptides were identified. Sun and colleagues described that after activation, the adaptors MyD88 and TUBE, together with the serine-threonine kinase PELLE, are recruited to the receptor (Sun et al. 2004). This process is followed by the activation of Cactus, a *Drosophila* IκB. Dif and Dorsal are species-specific transcription factors of the Rel protein family and are controlled by Cactus in the cytoplasm. Activation of the Toll pathway leads to the degradation of Cactus and the subsequent translocation of Dif and Dorsal into the nucleus, resulting in the production of antimicrobial peptides (Brennan and Anderson 2004; Hoffmann 2003; Hultmark 2003). These early studies provided important insights into the mechanism of pathogen recognition and host responses also in mammalian systems (Uematsu and Akira 2008).

The first described mammalian TLR (now termed TLR4) was identified through a database screening and shown to induce the expression of genes involved in inflammatory responses (Medzhitov et al. 1997). Further studies demonstrated that a mutation in the *tlr4* gene was responsible for hypo-responsiveness to LPS in C3H/HeJ mice (Poltorak et al. 1998); LPS was found to be the natural ligand for TLR4 (Hoshino

et al. 1999). To date, thirteen members of the TLR family have been identified, and ten of them are found in humans (Kawai and Akira 2011). TLRs are type I transmembrane glycoproteins characterized by a cytoplasmic signaling domain and different extracellular domains. The cytoplasmic "tail" shows high similarity to the interleukin-1 receptor family and is called the Toll/IL-1 receptor (TIR) domain. All TLRs have characteristic extracellular "leucine-rich repeats" (LRRs) (Akira 2004) that consists of 19-25 tandem LRR motifs (24-29 amino acids long each), containing the motif XLXXLXLXX. Each LRR is composed of a β–strand and an α–helix connected by loops. It is now known that the LRR domain of TLRs forms a horseshoe-like structure that is the actual ligand-binding part or "surface" of the receptor. However, three-dimensional representation of human TLR3 LRR motifs suggests that negatively charged dsRNA is more likely binds to the outside or convex surface. Recent crystallographic and high-resolution FRET analyses have revealed the ligand-receptor binding mechanisms of TLRs (Yoon et al. 2011; Kang and Lee 2011).

The TLR family is an important class of PRRs through which the innate immune system detects invasive microorganisms. TLRs are also important in the non-immediate phase of the immune response, such as the recruitment of phagocytes to infected tissue areas. Recent studies revealed that TLRs recognize several organisms, such as bacteria, fungi, protozoa, and viruses (Kawai and Akira 2011) (Table 1). Once TLRs have been activated, they initiate various signal transduction cascades that initiate and regulate the immune response through the transcription factors NF-κB and IRFs, mitogen-activated protein kinases (MAPKs), p38, ERK1/2, and c-Jun N-terminal kinase (JNK) (Figure 7). This process results in the expression of a common set of genes whose products, such as cytokines, chemokines, and co-stimulatory molecules, are essential in the orchestration of both innate and adaptive immunity.

Apart from MyD88, there are four other important adapter molecules that take part in downstream TLR signaling events:

1) The MyD88-adapter-like or TIR domain-containing adapter (TIRAP/Mal);
2) The TIR domain-protein TRIF/TICAM-1;
3) The TRIF-related adapter molecule (TRAM), also known as TICAM-2;
4) The protein that contains sterile α and HEAT-Armadillo motifs (SARM).

The existence of these different signaling adapters explains to some extent the separate cascades that are initiated and regulated by different TLRs. In fact, not all members of the family bind the same adapter(s) that specifically regulate a given pathway. A good

example is the regulation of IRF3 via the adapter TRIF, that can be induced only by TLR3 or TLR4 and initiate the TRIF-dependent activation of IRF3 and thereby the production of type I IFNs (Figures 6, 7). The exact molecular mechanisms of the initiation and regulation of various TLR signaling pathways have not been elucidated completely, but a vast amount of knowledge does exist and this part of the dissertation will offer a short review of the topic.

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TLRs	Stimulants	Origin
TLR1	Triacyl lipopeptides	Bacteria, mycobacteria
	Soluble factors, porin (PorB)	*Neisseria meningitides*
	OspA	*Borrelia burgdorferi*
TLR2	Lipoprotein/lipopeptides, peptidoglycan	Gram-positive bacteria
	Diacyl lipopeptides (Pam2/3CSK4)	Synthetic ligands
	Lipoarabinomannan	Mycobacteria
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria meningitides*
	Zymosan	Yeast
	Atypical LPS	*Leptospira interrogans, Porphyromonas gingivalis*
	Hsp70, Hyaluronan	Host
	Hemagglutinin	Measles virus
TLR3	dsRNA	Viruses
	Poly I:C	Synthetic ligands
TLR4	LPS	Gram-negative bacteria
	Flavolipin	*Flavobacterium meningosepticum*
	Taxol	Plant
	Fibronectin-extra domain A, Hyaluronan fragments	Synthetic ligands
	ER-112022, E5564, E5531	Synthetic ligands
	Fusion Protein	Respiratory synctial virus
	Envelope Protein	Mouse mammary tumor virus
	Hsp60	*Chlamydia pneumoniae*
	Hsp60, Hsp70	Host
	Hyaluronic acid, heparin sulfate, fibrinogen	Host
TLR5	Flagellin	Flagellated bacteria
TLR6	Diacyl lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
	FSL-1, MALP-2	Synthetic ligands
TLR7	ssRNA	Viruses
	Imidazoquinolines, bropirimine, guanosine analogs	Synthetic ligands
TLR8	ssRNA	Viruses
	Imidazoquinolines	Synthetic ligands
TLR9	Unmethylated CpG DNA	Bacteria, virus, yeast, insects
	Chromatin-IgG complexes	Host
TLR10	Unknown	Unknown
TLR11	Unknown	Uropathogenic bacteria

FSL-1: CpG-cytidine-phosphate guanosine; Hsp: Heat shock protein; LPS: Lipopolysaccharide; MALP-2: 2-kDa macrophage activating lipopeptide; Poly I:C: Polyinosinic:polycytidylic acid; TLR: Toll-like receptor. Modified from [18,73].

Source: Future Microbiol © 2008 Future Medicine Ltd

Table 1. Specificity of Toll-like receptors

MyD88 was the first adapter found to be essential for TLR signaling. MyD88 had already been identified as a protein generally expressed in myeloid tissues and could be induced when myeloid precursors were differentiated in the presence of IL-6 (Lord et al. 1990; Brikos and O'Neill 2008). Later, this protein was found to be crucial for the signaling processes of several TLRs and IL-1RI (Hultmark 1994; Yamagata et al. 1994; Medzhitov et al. 1998). In general, MyD88 is able to mediate the signaling of all TLRs except TLR3 and to some extent TLR4. The basic structure of MyD88consists of a death domain (DD) and a TIR domain, with an intermediate domain (ID) linking them. In 2006, a germ-line mutagenesis study offered further insight into the details of the interactions between MyD88 and TLRs (Jiang et al. 2006). MyD88-dependent signaling of TLRs is very similar to the downstream processes of IL-1RI in terms of the kinetics of activation and the induction of NF-κB and MAPKs. Thus the ligation of a TLR results in the association of MyD88 to IRAKs. Initially, IRAK-1 seemed to play an important role in IL-1RI signaling as the overexpression of IRAK-1 led to the activation of downstream signaling (Knop et al. 1998) and IRAK-1-deficient HEK293 cells did not respond to IL-1 (Li et al. 1999). Another member of the IRAK family, IRAK-4 (Li et al. 2002) was shown to phosphorylate itself as well as recombinant IRAK-1 *in vitro*. Interestingly, recombinant IRAK-1 could autophosphorylate, but failed to phosphorlyate IRAK-4 *in vitro*. IRAK-$4^{-/-}$ mice exhibited a much stronger phenotype than the IRAK-$1^{-/-}$ mice and had severely impaired IL-1- and TLR signaling (Suzuki et al. 2002). Further studies demonstrated that humans lacking IRAK-4 were extremely susceptible to pyogenic bacterial infections (Picard et al. 2003, 2011) proving the role of IRAK-4 in innate immunity. Two other members of the family, IRAK-2 and IRAK-M have been identified (Muzio et al. 1997; Wesche et al. 1999; Brikos and O'Neill 2008) and shown to be under the control of IRAK-1 by keeping them inactived by replacing a serine or an asparagine in the catalytic site with an aspartate residue (IRAK-1-D340). Altogether four murine isoforms of IRAK-2 were discribed, two inhibiting and two activating NF-κB (Rosati and Martin 2002). IRAK-M turned out to be a negative regulator of the TLR-MyD88-NF-κB pathway (Kobayashi et al. 2002) by hampering the dissociation of IRAK-1 from MyD88 and preventing the formation of the complex of IRAK-1 with TRAF6, the next element of the downstream pathway (Figures 6, 7).

The exact mechanism by which the IRAKs are recruited to the TLR/IL-1RI complexes via MyD88 has not yet been clarified. A recent study identified a novel protein, Unc5CL, which is involved in the IRAK-1/IRAK-4/TRAF6-dependent

activation of NF-κB, but the nature of the interaction is not known (Heinz et al. 2011). All of the complexes contain an N-terminal DD-motif, a domain which was thought to be important in the receptor-MyD88 interaction (Muzio et al. 1997). However, mutated IL-1RI that cannot interact with MyD88 still associates with IRAK-1. Furthermore, an alternative splice variant of MyD88 (MyD88s), which still possesses a functional DD region, inhibits IL-1R/TLR signaling (Burns et al. 2003). Recently, *in vitro* pull down assays were used to demonstrate that elements of MyD88 responsible for its interaction with IRAK DDs are found in the C-terminal region of the protein, proximal to the TIR domain (Lasker and Nair 2006; Brikos and O'Neill 2008).

After TLR activation, IRAK-1 becomes phosphorylated, dissociates from the receptor complex and binds to TRAF6, an essential cytosolic downstream element that links IL-1R/TLR complexes to the activation of NF-κB and MAPKs. During this process TRAF6 becomes polyubiquitinated by ubiquitin-conjugating enzyme E2 variant 1 (Uev1A) and ubiquitin-conjugating enzyme 13 (Ubc13). As a result, TRAF6 oligomerizes (Deng et al. 2000; Chen 2005) and associates with downstream proteins transforming growth factor (TGF)-β-activated kinase 1 (TAK1), and the TAK1-binding proteins, TAB1, TAB2, and TAB3 (Wang et al. 2001) (Figure 5). Once TAK1 has been ubiquitinated, it phosphorylates the inhibitor of NF-κB (IκB) kinases (IKK) complex, which also undergoes polyubiquitination by Uev1A and Ubc13. Subsequently, the activated IKKs phosphorylate IκB proteins present in the inhibitory complex with NF-κB dimers in the cytoplasm of resting cells. This event results in the release of NF-κB and its translocation to the nucleus. In humans, the predominant form of NF-κB is a heterodimer of p50 and p65 (also known as RelA) proteins. The p50 subunit is responsible for the interaction and assembly with IκBα, whereas p65 is the controller of the transactivation of gene expression (Brikos and O'Neill 2008). During the standard course of MyD88-dependent signaling, IκBα becomes phosphorlyated, polyubiquitinated, and degraded within 1-6 hours (Karin and Ben-Neriah 2000; Pasparakis 2011). Consequently, after the release of the p50/p65 heterodimer and phosphorylation of p65, NF-κB translocates to the nucleus and controls the transcription of genes responsible for inflammatory responses and chemotaxis.

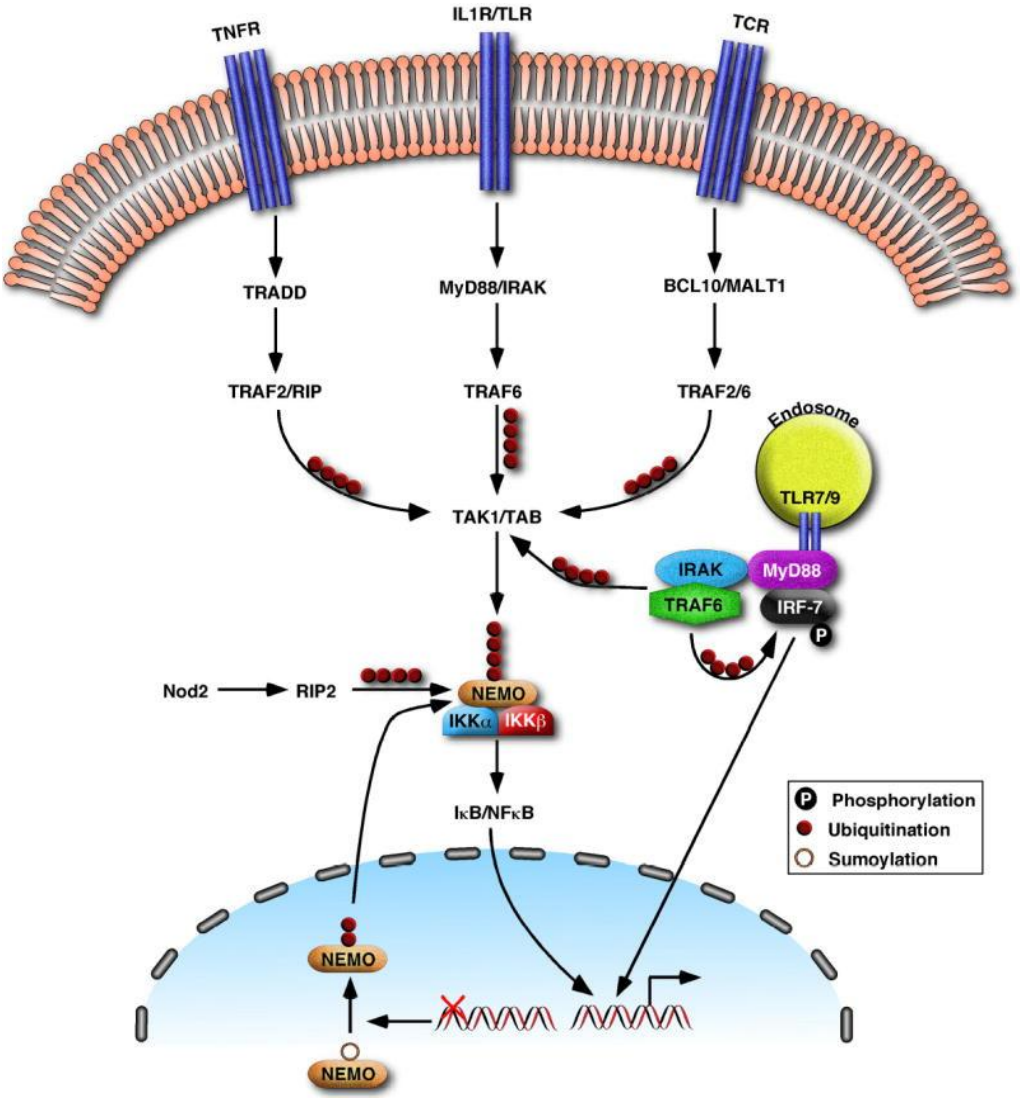

Figure 5. A central role for ubiquitin in multiple signaling pathways
*Chen, Z. J. (2005) Nat Cell Biol **7**:758-65.*

IKK consists of three subunits, the regulatory IKKγ, also termed as NF-κB essential modulator (NEMO), and two catalytic subunits, IKKα and IKKβ (Rottenberg et al. 2002; Pasparakis 2011). NEMO is a scaffold protein essential for the assembly of the IKK complex and connects the IKKs to IκB (Figure 6). TAK1 is not only vital for IKK activation, but plays a crucial role in MAPK signaling. TAK1 is linked to MAPK kinase 6 (MKK6) and other MAPKKs, such as MKK3 and MKK7. Two main pathways are triggered: phosphorylation of MKK6 results in the activation of JNK, and phosphorylation of MKK3 and MKK7 leads to the activation of p38. These two pathways converge into the activation of transcription factor activator protein-1 (AP-1), which is a main inducer of inflammatory-response and proliferation-factor genes (Brikos and O'Neill 2008; Liu et al. 2007).

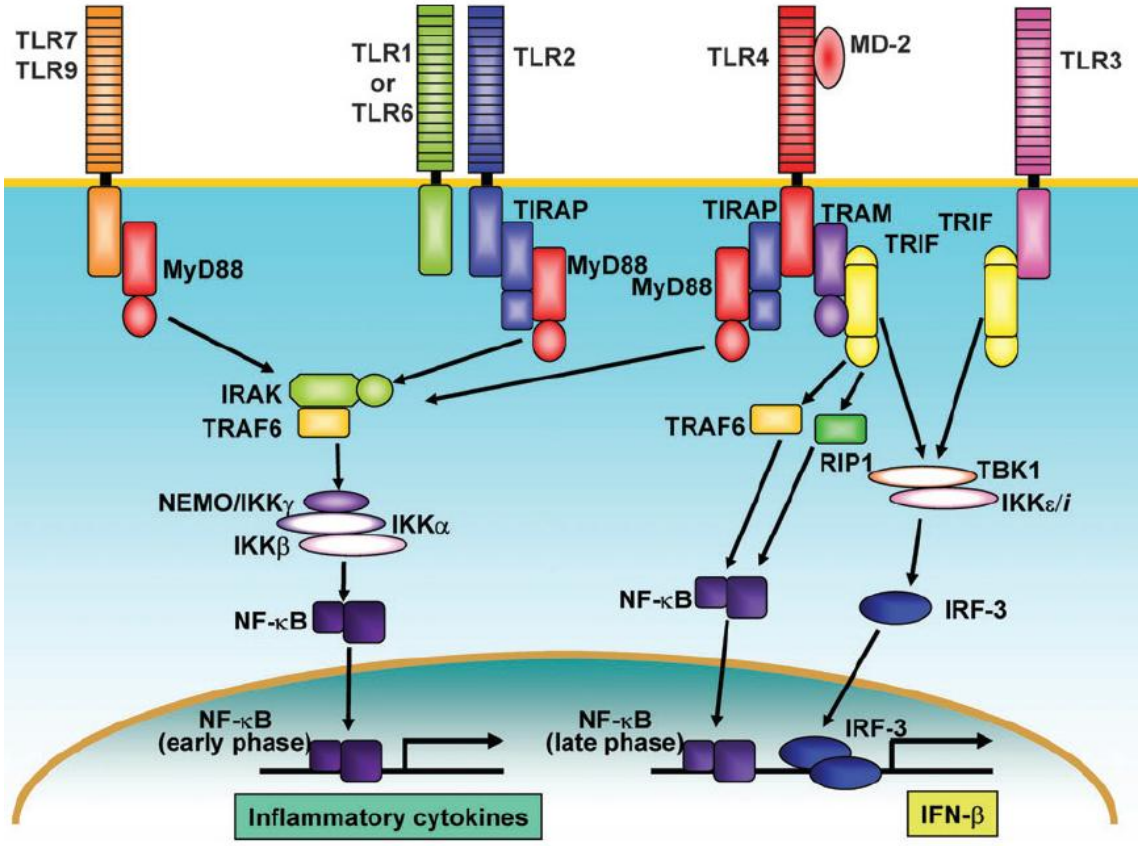

Figure 6. The main pathways of TLR signaling

Takeishi, Y. and Kubota, I. (2009) Front Biosci ***14****:2553-2558.*

2.2.2.2 RLH signaling pathways and adaptors

Besides NF-κB and MAPK triggering, the MyD88-dependent signaling pathway also leads to the activation of IRFs. This process is of extreme importance because these factors control the production and regulation of interferons, a cytokine family with strong antiviral and anticancer activity (González-Navajas et al. 2012; Honda and Taniguchi 2006). Our studies focused to three members of this group, IRF1, IRF3, and IRF7. These IRFs act as transcription factors, and after activation-induced phosphorylation they form homo- and heterodimers and translocate into the nucleus where they regulate type I interferon gene expression. Since only IRF1 and IRF7 are MyD88-dependent factors, they will be discussed here, while IRF3-mediated signaling regulating IFNβ production will be detailed later.

In myeloid DCs, MyD88 has been reported to associate with IRF1 (Brikos and O'Neill 2008; Negishi et al. 2006) and after phosphorlyation IRF1 dimers activate

several TLR-dependent genes and contributes to the induction of IFNα and β propduction. The expression of IRF1 is induced by IFNγ via the IFNγRI pathwayand thus affects TLR signaling indirectly. Although IFNγRI does not have TIR domain, it can recruit MyD88 and activate p38 (Sun and Ding 2006).

IRF7 was shown to participate in TLR7 and TLR9 signaling in pDCs but not in cDCs. MyD88, IRAK-1, IRAK-4, and TRAF6 were shown to interact directly with IRF7 (Hochrein et al. 2004; Honda et al. 2005; Uematsu et al. 2005) and upon activation phosphorylated and dimerized IRF7 translocates into the nucleus and binds to IFN-stimulated response element (ISRE) motifs, which leads to the production of type I IFNs (González-Navajas et al. 2012). IRAK-1 is a keyplayer in this process as IRAK-1deficient mice do not produce type I IFNs following TLR7/9 activation (Uematsu et al. 2005). The role of IRAK-1 is to phosphorylate IRF7 and this must be preceded by the stable association of MyD88 and the TIR domain of the TLR (Honda et al. 2005). Other adaptors also play significant roles in the MyD88-dependent pathway. One such adaptor, Mal/TIRAP was discovered based on sequence similarities to MyD88 (Fitzgerald et al. 2001; Horng et al. 2001). TIRAP was shown to have an important role in TLR4 signaling (Fitzgerald et al. 2001) (Figure 7), and this was confirmed by experiments with TIRAP-deficient mice (Horng et al. 2002). Mal/TIRAP$^{-/-}$ mice showed a phenotype and TLR4signaling features similar to MyD88$^{-/-}$ mice. In both mutant mice, the activation of NF-κB and MAPKs was delayed instead of being inhibited in response to TLR4 ligation. In addition, TLR2 signaling was affected as activation of NF-κB and p38 did not occur in TIRAP$^{-/-}$ mice. . These observations prove that Mal/TIRAP is part of the TLR2 and TLR4 MyD88-dependent pathways. The role of TIRAP in TLR2/TLR4 signaling was also demonstrated in humans as TIRAP-deficient individuals were found to be susceptible to pneumococcal disease, malaria, and tuberculosis (Khor et al. 2007). Recent studies revealed unique aspects of TIRAP-linked processes showing that in contrast to MyD88, TIRAP could associate directly with TRAF6. Furthermore, TIRAP protein could also interact with Bruton's tyrosine kinase (Btk) thatphosphorylates TIRAP and is a crucial player in TLR2 and TLR4 downstream signaling (Brikos and O'Neill 2008; Gray et al. 2006; Liljeroos et al. 2007). The discovery of Mal/TIRAP was the first evidence showing that different TLRs can use different adaptors.

In 2003 Fitzgerald and colleagues found that MyD88$^{-/-}$ mice presented signal transduction upon both TLR3 and TLR4 activation (Fitzgerald et al. 2003a,b). This

MyD88 independent pathway converged into the activation of IRF3 and IRF7 inducing type I interferon responses. The adaptor found to be essential for this pathway was TRIF (Yamamoto et al. 2002; Oshiumi et al. 2003) as in $TRIF^{-/-}$ mice pro-inflammatory cytokine production was unaffected upon TLR2, TLR5, TLR7/8, and TLR9 ligation, but was abolished when induced by TLR4.

However, the activation of IRF3 and consequent IFNβ production was impaired in both the TLR3 and TLR4 pathways (Yamamoto et al. 2003). Thus TRIF was the missing link essential for TLR3 and also required for TLR4 signaling. Experiments with TRIF/MyD88 double knock out mice revealed that TRIF was responsible for delayed NF-κB-mediated pro-inflammatory response in $MyD88^{-/-}$ and $TIRAP^{-/-}$ mice. TLR3/TLR4-TRIF signaling involves several previously identified elements; after receptor activation TRIF forms a complex with TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) (Fitzgerald et al. 2003a), the IKK homolog IKKε, and IRF3. These interactions result in the phosphorylation and activation of IRF3 by TBK1 and IKKε (Figure 6). IRF3 then binds ISRE and induces the transcription of IFNα/β (Sato et al. 2003). IRF7 is also regulated through this pathway by TBK1/IKKε (Uematsu et al. 2005). In TLR3 signaling, TRIF is also involved in the NF-κB-inflammatory response by binding to the TRAF6-TAK1-TAB2 complex that activates IKK (Jiang et al. 2004). This interaction is likely to occur through consensus TRAF6-binding motifs in the TRIF structure, though the nature of this phenomenon is yet to be explained. Another important downstream event is the interaction of TRIF with treceptor interacting protein (RIP) family members (Meylan et al. 2004). This involves the association of TRIF, RIP1, and RIP3. RIP1 activates NF-κB via TLR3, whereas RIP3 has a negative regulatory role and prevents the interaction between TRIF and RIP1. Although there are many similarities between the signaling cascades initiated by TLR3 and TLR4, there are important disparities as well. Unlike TLR3 TLR4 can indirectly activate NF-κBthrough TRIF that leads to the production of TNF-α (Figure 6). Secreted TNF-α then binds to its receptor (TNFR) and this signaling culminates in NF-κB activation (Covert et al. 2005). Through this mechanism, endotoxin can cause a prolonged activation that is important for host defense. Another difference between TLR3 and TLR4 is the way how IRF3 is activated (and therefore the regulation of ISRE-dependent response). Activation of TLR3 leads to the homodimerization and ISREbinding of IRF3; in the case of TLR4 activation however, IRF3 forms a heterodimers with p65 (called “enhanceosome”) which then binds ISRE

(Wietek et al. 2003). Apart from the cascades mentioned above, TRIF is involved in other important processes, such as the induction of apoptosis (together with RIP1, FADD and caspase-8) and the modulation of MHC-II expression in DCs (Brikos and O'Neill 2008; Kamon et al. 2006).

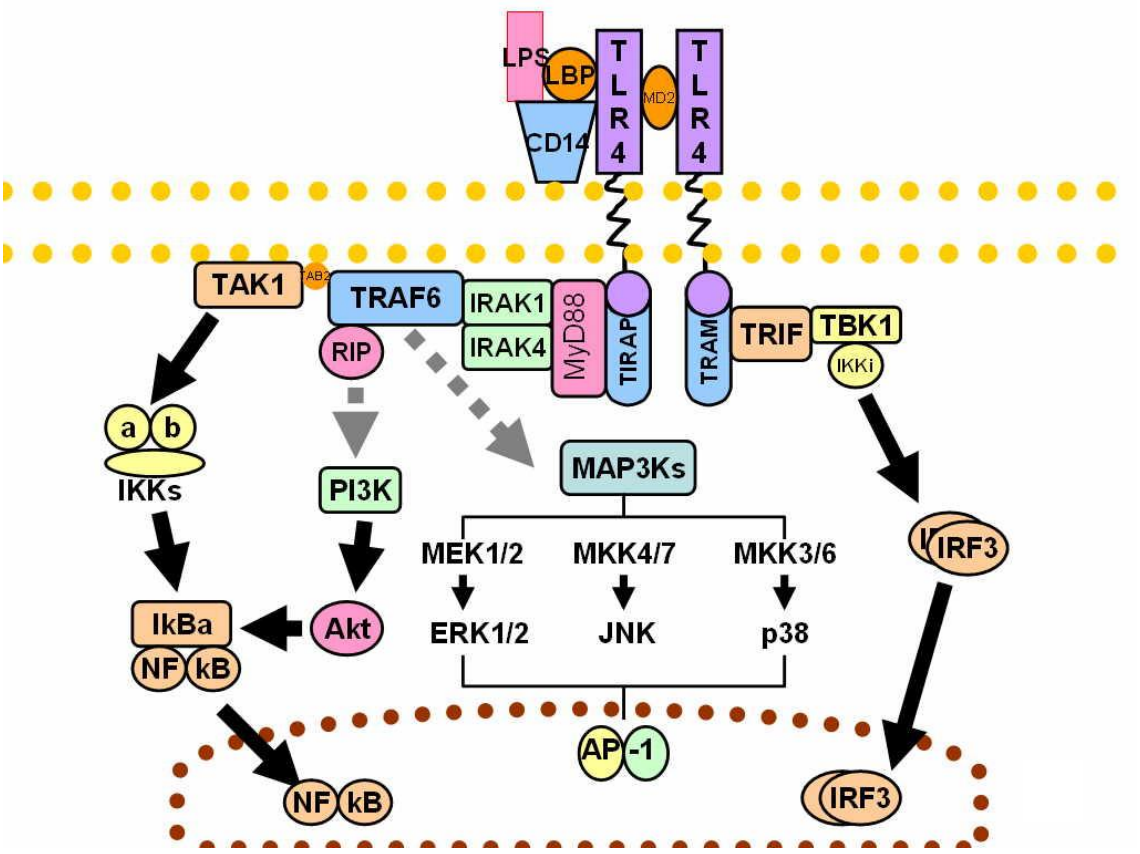

Figure 7. Comparative topology of the MyD88-dependent- and independent TLR pathways
Source: Wikipedia (http://en.wikipedia.org/wiki/Toll-like_receptor)

It is clear that TRIF interacts directly with TLR3; however, TLR4 requires another protein to make contact with TRIF. This bridging adapter is called TRAMand seems to be exclusively used by TLR4 (Fitzgerald et al. 2003b; McGettrick et al. 2006; Rowe et al. 2006) (Figure 7). Like TIRAP, TRAM is also associated with the plasma membrane and its activation is dependent on the phosphorylation of a serine residue (Ser16) by protein kinase Cε. As mutation of this serine renders TRAM completely inactive this modification is needed for the proper signaling of TRAM but its exact role is not yet known (Kenny and O'Neill 2008).

Intracellular occurence of viral nucleic acids or extracellular pathogens such as bacteria or fungi represent a danger signals for the innate immune system that mobilizes

a broad spectrum of response mechanisms that eliminate the invading pathogen. DCs and macrophages acting as professional APCs display the widest spectrum of TLRs. However, the two major DC lineages, cDCs and pDCs express a unique combination of TLRs ensuring the detection of a broad range of pathogenic or damaged self structures (Figure 8). As other body cells express at least one or a few TLRs, these receptors may have evolved to act as regulators of physiological functions during a response to hazardous signals (Yamamoto et al. 2004). As mentioned above, TLR-mediated signaling results in the activation of NF-κB, but TLR4 ligands may also induce IRF3. TLR3 activation is linked to both IRF3 and IRF7 however, the baseline expression level of IRF3 in DCs and in most cells is far higher than that of IRF7, and thus the primary target of TLR3-mediated activation is IRF3 (Benko et al. 2008; Mamane et al. 1999; Hansen et al. 2011), whereas the ligation of TLR7, TLR8, and TLR9 triggers IRF7 activation (Figure 4). Signaling through TLRs in DCs leads to pro-inflammatory and IFN responses and results in the recruitment of other inflammatory cell types such as granulocytes and natural killer (NK) cells. Thus, the coordinated activation and interaction of different cell types involved in the mobilization of innate cells are able to create a local microenvironment that allows the regulated activation of adaptive immunity (Iwasaki and Medzhitov 2004). As potent inducers of innate responses, natural and synthetic TLR ligands act as adjuvants of the initiation of robust antigen specific immune responses and support the formation of long term immunologic memory.

Other PRR families also play a significant role in innate host defense; these include conserved intracellular sensors such as NACHT-LRRs (Martinon and Tschopp 2005) or NLRs (Petrilli et al. 2007a,b), RLHs, and intracellular DNA sensors (Creagh and O'Neill 2006; Ishii and Akira 2006) that recognize PAMPs. As all these molecular sensors are expressed differently in DC subtypes, the combination of distinct stimuli processed by DCs will determine the magnitude and quality of the induced immune response (Figure 8).

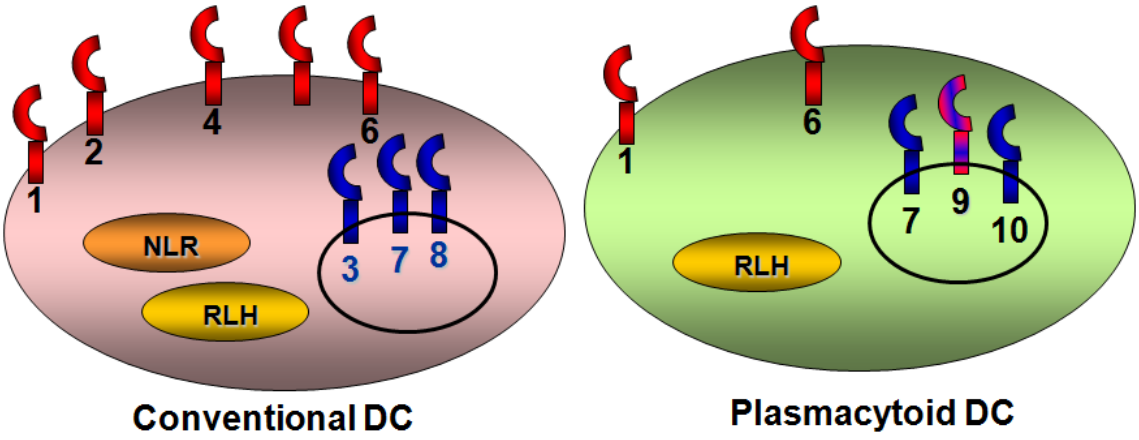

Figure 8. Expression and specificity of Toll-like receptors (TLRs) and RIG-I-like helicases (RLHs) in conventional and plasmacytoid dendritic cells (DCs) *Conventional and plasmacytoid DCs express different sets of TLRs localized to the cell or intracellular membranes. NLRs and RLHs are expressed in DCs, but the expression pattern and functional activity of the individual members of these intracellular sensors have not yet been studied systematically in various DC subtypes. Activation of the two major lineages of DCs by TLRs and presumably by NLRs and RLHs results in the secretion of different combinations of inflammatory cytokines. Benko, S. et al. (2008) Biol Chem* ***389****:469-85.*

RLHs include RIG-I, melanoma-differentiation associated gene-5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), all of which possess a DExD/H box helicase domain (Yoneyama et al. 2004, 2005). RIG-I was first described in promyelocytic leukemia cells (Sun 1997; DiSepio et al. 1998), whereas MDA5 was found in a melanoma cell line (Kang et al. 2002). RIG-I and MDA5 are structurally homologous proteins with RNA helicase activity, being able to unwind double stranded RNA in an ATP-dependent manner (Takahasi et al. 2008). However, this event does not lead to downstream signaling *per se*. The two CARD domains of RIG-I and MDA5 are located at the N-terminal end of the proteins (termed CARD1 and CARD2). RIG-I- and MDA5-mediated signaling is coordinated by another CARD domaincontaining protein, the CARD adaptor inducing interferon-β (CARDIF)alternatively referred to as IFNB-promoterstimulator-1 (IPS1), mitochondrial antiviral signaling protein (MAVS), or virus-induced signaling adaptor (VISA) (Figure 9). Direct interaction of CARD domains in RLH and CARDIF/IPS1 leads to downstream transmission of the activation signal (Kawai et al. 2005; Seth et al. 2005; Xu et al. 2005). This adaptor is localized to the mitochondrial membrane and thus links innate immunity to an organelle that evolutionarily originated from aerobic bacteria (McWhirter et al. 2005; West et al. 2011). As mitochondria play a pivotal role in regulating apoptosis and silencing of IPS1

was reported to induce apoptosis, this pathway may also be important in protecting virus-infected cells from programmed cell death (Seth et al. 2005). Although RIG-I and MDA5 are IFN-inducible helicases, IFN treatment alone does not induce the transcription of IFN genes. This phenomenon led to the establishment of the RIG-I autorepression model, which proposes that a certain region of RIG-I acts as a repressor domain that carries out auto-regulation. This repressor domain is also found in LGP2, but not in MDA5 (Saito et al. 2007).

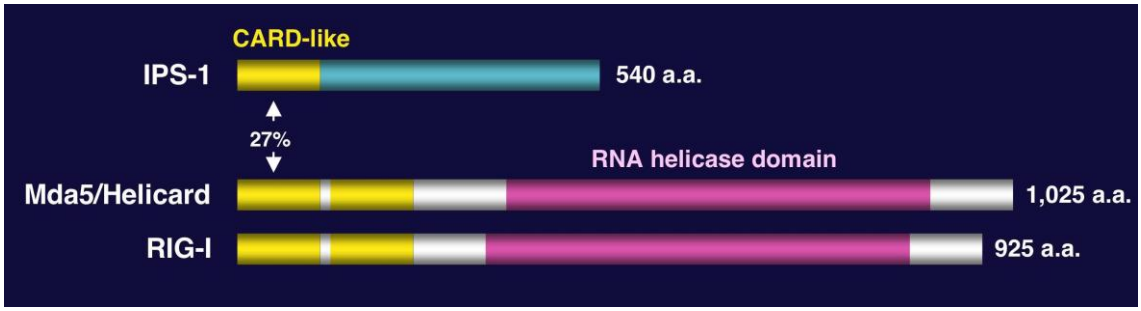

Figure 9. Structure of the CARD-proteins RIG-I, MDA5 and IPS-1
Source: http://www.biken.osaka-u.ac.jp/e/topics.php?year=2005

RLHs are essential for the production of type I IFNs and pro-inflammatory cytokines in response to viral infections. Double knock-out experiments revealed that cells lacking RIG-I/MDA5 produce marginal levels of type I IFNs upon viral induction (Kato et al. 2006). However, RLHs exhibit different specificity and "select" among different virus taxa. Studies demonstrated that RIG-I is highly specific for RNA viruses including influenza A (*Orthomyxoviridae*), Newcastle disease virus (NDV) (*Paramyxoviridae*), Sendai virus (*Paramyxoviridae*), vesicular stomatitis virus (VSV) (*Rhabdoviridae*) and Japanese encephalitis virus (JEV) (*Flaviviridae*) (Kato et al. 2005, 2006), whereas MDA5-deficient cells fail to produce interferon in response to picornaviruses, including encephalomyocarditis virus (EMCV), Mengo virus, Theiler's virus, murine norovirus-1 (*Calciviridae*), and murine hepatitis virus (*Coronaviridae*) (Gitlin et al. 2006; McCartney et al. 2008; Roth-Cross et al. 2008). Furthermore, some virus species are recognized both by RIG-I and MDA5, such as hepatitis C (HCV), Dengue and West Nile virus (*Flaviviridae*) (Loo et al. 2008; Fredericksen and Gale 2006). The third member of the RLH family, LGP2, lacks CARD and is consequently incapable of activating IFN- and pro-inflammatory cytokine responses during viral

stimulus, so it was considered to be a negative regulator of RIG-I/MDA5 (Yoneyama et al. 2005; Komuro et al. 2008). A recent study by Satoh and colleagues showed that $LGP^{-/-}$ mice produce decreased amounts of IFN upon infection of VSV or EMCV (Satoh et al. 2010) suggesting that LGP2 may cooperate with RIG-I and MDA5 in order to sense viral nucleic acids in the cytoplasm. The *Janus-faced* activity of LGP2 through inhibiting or facilitating RLH activity is still a subject of controversy.

Besides viruses as natural ligands for RLHs, several other molecules are able to induce RIG-I and MDA5 activation under experimental conditions. Poly-riboinosinic:poly-ribocytidylic acid (poly I:C or pI:C) is a synthetic dsRNA that acts as a potent synthetic inducer of type I IFNs that selectively activates TLR3 and RLHs (Alexopoulou et al. 2001; Kato et al. 2008). Although its background it not fully understood, MDA5 is essential for the recognition of pI:C (Kato et al. 2006, 2011; Gitlin et al. 2006). This specificity is due to the length of the nucleic acid chain rather than its base composition (Kato et al. 2008), as commercial or "long" pI:C is not detected by RIG-I. However, partial digestion of pI:C by the specific endonuclease RNaseIII results in the generation of trimmed "short" pI:C of about 300 bp in length, which is able to activate RIG-I but not MDA5. The mechanisms behind this size discrimination is not yet known, but Kowalinski and colleagues (Kowalinski et al. 2011) recently reported differences in the length of the CARD2-helicase linker regions of RIG-I and MDA5. This difference together with electrostatic properties of the binding interface may contribute to size discrimination and highlight the diverse biological functions of helicases (O'Neill and Bowie 2011).

Subsequent to the discovery of RLH induction by pI:C another RNA species, 5'-triphosphate containing RNA (5'pppRNA) was found to be a highly selective ligand for RIG-I (Hornung et al. 2006; Pichlmair et al. 2006). 5'pppRNA is a general genome constituent or the product of *in vitro* transcripts of most RNA viruses. Endogenous 5'ppp moieties are removed by adding a 7-methyl-guanosine cap in host cells, therefore these self-patterns are refractory to detection by RIG-I. In addition, the recognition of 5'pppRNA is strictly dependent on the presence of a short double-stranded section of the molecule, since short dsRNAs can weakly trigger RIG-I while a single 5'ppp strand by itself cannot. It was also demonstrated that the transfection of AT-rich dsDNA (synthetic polydAdT:polydAdT) can lead to the production of type I IFNs. This phenomenon was shown to be RIG-I-dependent and was shown to be mediated by a host DNA-dependent RNA polymerase III that transcribed polydAdT:polydAdT to

5’pppRNA (Ablasser et al. 2009; Chiu et al. 2009). This latter mechanism may explain IFN induction by some DNA viruses and intracellular bacteria. A recent study reported that besides RIG-I, PKR can recognize the 5’ppp signature however, this does not lead to IFN response and hence the biological significance of this simultaneous activation is still obscure (Nallagatla et al. 2007).

Upon activation by dsRNA, RIG-I undergoes a conformational change that exposes its CARD domain. This is followed by an oligomerization step that involves the interaction of the CARDs of RIG-I and IPS1. MDA5 also signals through IPS1 in the same manner. IPS1 activates the IκB kinase (IKK)-related kinases, IKKi and TBK1 via TRAF3 (Fitzgerald et al. 2003a; Häcker et al. 2011). These kinases subsequently phosphorylate IRF3 and IRF7, leading to the production of type I IFNs. In addition, IPS1 activates NF-κB through the FADD and caspase-8/10 pathway thereby regulating the expression of proinflammatory cytokine and chemokine genes (Takahashi et al. 2008; Kato et al. 2011) (Figure 10). Recent studies reported that an endoplasmic reticulum-localized protein, stimulator of IFN genes (STING) also interferes with RIG-I (but not with MDA5) signaling. STING is also expressed on the outer membrane of mitochondria where it interacts with IPS1 and IRF3 and directly amplifies the signaling of RIG-I (Ishikawa and Barber 2008; Zhong et al. 2008).

As we have seen in the case of TLR pathways, the ubiquitin-dependent regulation is essential for the appropriate control of the innate immune system (Figure 5). Ubiquitination of RLHs is reported to be important for both positive and negative regulation. RIG-I is exclusively regulated by tripartite motif 25 (TRIM25) at Lys63; this

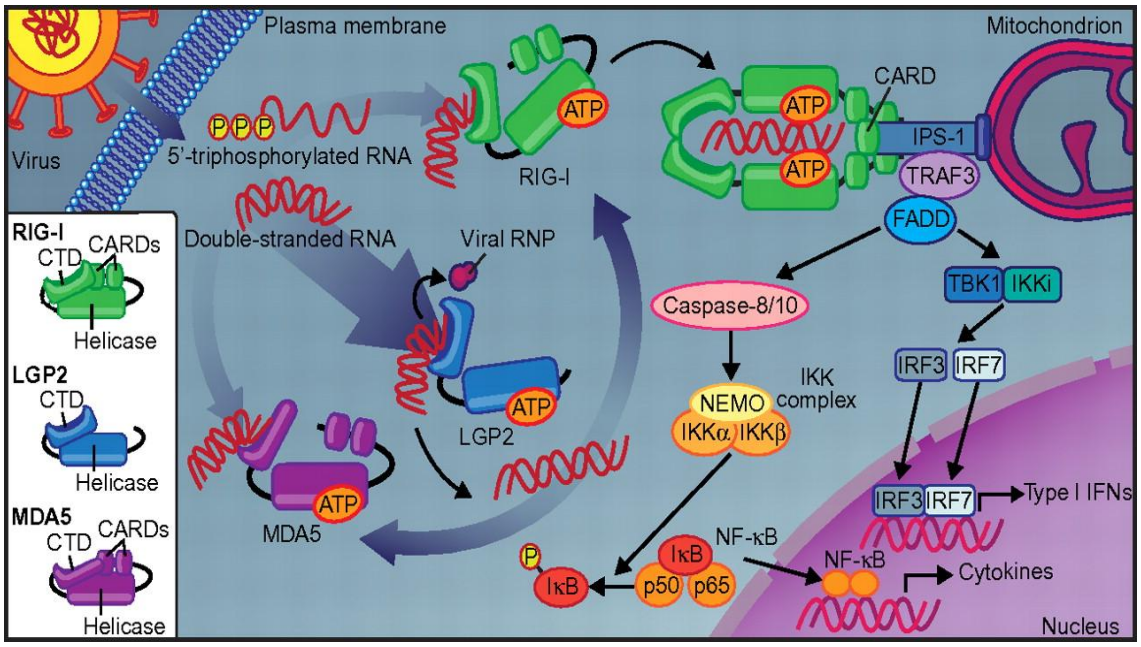

Figure 10. Signaling pathways of the RLH family
Moresco, E. M. and Beutler, B. (2010) Proc Natl Acad Sci U S A ***107****:1261-1262.*

ubiquitination step was shown to be crucial for the efficient activation of RIG-I signaling (Gack et al. 2007). Another E3 ubiquitin ligase, RNF125, was shown to promote RIG-I signaling via Lys63 (Oshiumi et al. 2009). The other important regulatory site of RIG-I is Lys48, where RNF125 can polyubiquitinate the helicase and targeting it for proteasomal degradation. This mechanism is thought to be important for the prevention of aberrant RIG-I activation. RNF125 also plays a pivotal role in the suppression of MDA5- and IPS1-coupled signal transduction by conjugating ubiquitin to these proteins (Arimoto et al. 2007). RIG-I is ISGylated by the ubiquitin-like protein ISG15, which also controls the activity of RNF125 and UbcH8, an E2 ubiquitin-conjugating enzyme that suppresses ubiquitination of RIG-I by RNF125, and this steady-state effect can be relieved by ISG15 (Arimoto et al. 2008; Kato et al. 2011).

In the last decade, cumulative studies revealed the details of virus sensing and signaling machinery of RLHs. It is now clear that RIG-I and MDA5 are of critical importance in innate antiviral host defense. They exhibit different specificity and are able to discriminate self and non-self RNAs by strict detection of viral signatures. However, discoveries of many signaling regulators as well as other PRRs coupled to interacting signaling processes make the picture more complicated. Indeed, many aspects of RLH pathwayregulation need to be clarified, especially because RLHs were shown to be potential disease modifiers in humans (Wang et al. 2007; Smyth et al.

2008; Nejentsev et al. 2009). These studies showed that RLHs are involved in the regulation of systemic immune homeostasis to a much greater extent than initially expected. Recently, Dann and colleagues reported that RLHs have a protective role in brain inflammation, making RLH-specific oligonucleotide ligands potential therapeutics for multiple sclerosis (Dann et al. 2011). The efficacy of future RLH-based therapies is highly dependent on understanding the details of TLR and RLH signaling and their interaction. The last part of the chapter will focus on this topic.

2.2.3 Cross-talk of RLH and PRR induced signaling

Collaboration of PRRs and RLH and the consequent secretion of type I interferons and pro-inflammatory cytokines can be highly efficient against pathogens. After viral infections, innate defense mechanisms are activated promptly and allow the development of adaptive immunity. DCs play an essential role in the orchestration of humoral and cellular immunity and the induction and maintenance of long-term immunological memory (Banchereau and Steinman 1998; Benko et al. 2008). Modified nucleotides of extra- or intracellular origin generated in infected or damaged cells are sensed through highly specific PRRs (Table 2). Conventional DCs exhibit high expression of TLR3 and are able to mediate dsRNA-induced signaling. However, the interplay of TLR3 and RLH mediated signaling is unknown but may have an impact on vaccine design.

Interaction of microbes with the innate immune system involves the induction of multiple PRR pathways triggered simultaneously by various PAMPs of the whole pathogen. The cross-talk of TLRs and NLRs is presumably crucial to maintain the balance of immune effectors and enhance the innate immune response in general. For example in professional APCs the activation of TLRs leads to the activation of the NF-κB signaling pathway, which in turn results in the expression of pro-IL-1β. However, the biologically active cytokine is secreted only after the conversion of pro-IL-1β to its fully mature form. This process is mediated by caspase-1, the enzyme component of NOD/Nalp-inflammasomes and is activated upon sensing pathogenic motifs or other danger signals (see also *part 2.2.1*). The cross-talk of TLRs and NLRs occurs in DCs at various molecular levels (Kufer and Sansonetti 2007), which may involve the up- or downregulation of TLR and/or NLR proteins or the members of their signaling pathways. It has already been reported that TLR agonist treatments can upregulate NOD1 and NOD2 gene expression (Rosenstiel et al. 2003; Takahashi et al. 2006).

Furthermore, MDP was shown to increases the expression of the critical adaptor MyD88 (Takada et al. 2002). NOD2 expression can also be induced in epithelial cells by flagellin, a TLR5 agonist (Begue et al. 2006). Another level of cross-talk is represented by the direct protein-protein interaction of different PRRs and the elements of downstream signaling pathways. Besides its binding to NOD1 or NOD2, RIP2 for instance is able to interact with caspase-1 of the inflammasome via CARD-CARD interaction (Sarkar et al. 2006). RIP2 was also shown to bind TRAF6, thereby taking part in the regulation of the NF-κB pathway (Meylan and Tschopp 2005) (Figure 6). Thus, RIP2 seems to balance the formation of inflammasomes and NOD-signalosomes at a molecular level. The cross-talk of TLR2 and NOD2 signaling pathways is another good example. It was reported that the ligation of TLR2 by peptidoglycan (PGN) or NOD2 by muramyl dipeptide (MDP) induces the secretion of IL-12 through NF-κB. However, this PGN-induced IL-12 production is inhibited by the concomitant induction of NOD2 signaling with MDP (Watanabe et al. 2004, 2006) suggesting that activation of the NOD2 pathway may interfere with the TLR2-mediated response. If so, this may explain the susceptibility of NOD2-deficient humans to Crohn's disease, presumably caused by the loss of NOD2-mediated control of TLR2 signaling. The distinct timing of signaling kinetics may also contribute to the dual effects of NOD2 (Yang et al. 2007; Benko et al. 2008).

Infection by RNA viruses is detected by RLHs and TLR7/8 and results in the production of type I IFNs. The interplay between TLRs and RLHs has an important role during viral infections. Kumagai and colleagues found that in NDV infected mice the major source of type I IFN was not pDCs (Kumagai et al. 2007) even though they were capable to produce vast amounts of IFNs in the absence of alveolar macrophages (AM) suggesting that pDCs play an important role when the first line of AM-mediated defense is disrupted. Since many viruses evolved RLH evasion mechanisms, pDCs may function as a backup for antiviral immunity when RLH signaling is shut down (Kawai and Akira 2011). Detection of HCV is carried out by both TLR3 and RIG-I (see also *part 2.2.2*) but the virus evades type I IFN responses by expressing a viral protein called NS3-NS4A. This HCV protease cleaves TRIF and IPS1 and renders hepatocytes incapable of producing IFNβ (Lemon 2010). On the other hand the presence of HCV induces a robust type I IFN response by pDCs, which infiltrate the liver during infection.

RECEPTOR	RECOGNIZED NUCLEOTIDES	EXPRESSION ON APC	RECOGNIZED PATHOGENS	RECOGNIZED HOST STRUCTURES
TLR3	**dsRNA**	**cDC**	**dsRNA, positive- and negative-sense ssRNA viruses (herpes simplex virus, influenza virus, respiratory syncytial virus)**	**Necrotic cells, virus infected dying cells**
TLR7	**ssRNA**	**pDC**	**Negative-sense ssRNA viruses (vesicular stomatitis virus (VSV) and influenza virus)**	**Dead or dying cells, immune complexes of small nuclear ribonucleoproteins (snRNP) and IgG**
TLR8		**cDC**	**Influenza virus**	**Dead or dying cells**
TLR9	**dsDNA, hemozoin**	**pDC**	***Mycobacterium tuberculosis, Neisseria meningitidis, Candida albicans,* herpes simplex virus, *Trypanosoma cruzi***	**Dead or dying cells, immune complexes of host DNA and IgG**
RLH (RIG-I-like helicases)	**dsRNA, 5'pppRNA**	**cDC**	**Negative- and positive sense ssRNA viruses (VSV and Sendai virus), Influenza virus**	**not known**

Table 2. Expression of nucleotide recognizing TLRs and RLHs in dendritic cells. *Different DC subtypes express both common and unique pattern recognition receptors that respond to a wide variety of pathogens, as well as recognize certain host structures. (by the author)*

HCV RNA is delivered to pDCs by a direct cell-to-cell contact between infected hepatocytes and pDCs. This process then leads to type I IFN production via TLR7. Moreover, stimulation of TLR7 or TLR9 by selective ligands can also upregulate the cytoplasmic expression of RIG-I protein in pDCs in a type I IFN-independent manner showing the importance of collaborative signaling between these two PRR families (Szabo et al. *unpublished results*).

While these sensors were shown to be crucial for innate and adaptive host defense, their inappropriate activation has been associated with autoimmunity and inflammatory diseases. Hence, a more complete appreciation of these PRRs and their complex signaling processes will provide important insights into new therapeutic modalities that can either enhance immune responses or inhibit functions to diminish the deleterious effects of uncontrolled inflammation.

An interesting approach to the topic has been carried out by using contemporary systems biology, bioinformatics, and biophysics as tools of understanding. This approach proposed that instead of single cell analyses one should move towards a more holistic understanding of signaling systems. The meta-network of biological entities is

considered to possess both microscopic and macroscopic dynamics as observed in physical sciences. In case of fluid dynamics for instance- one can examine the motion of each individual particle being highly unpredictable, while at macroscopic level the velocity of the flow follows the fundamental law of fluid mechanics (*i.e.* the law of conservation of mass, energy and momentum). The origin of averaging effects from stochastic responses of a single cell when collected to form a population should also be taken into account (Zhao et al. 2012). It is very likely that the emergence of an average cell deterministic response (*e.g.* following a TLR or RLH stimulus) from single cell stochastic responses complement each other (Gutiérrez et al. 2010; Selvarajoo 2011). Thus the stochastic fluctuations in the IFN response of a single DC or a single signaling pathway are necessary to induce probabilistic differentiation from identical cells or interacting pathways of the same PRR family. This might allow multicellular organisms or complex, interacting PRR signaling networks to switch cell fates or states to yield diversity, fine-tuning and reach the proper response that cannot be achieved by a purely deterministic system. Recent studies of multi-component, non-linear modeling of different TLR pathways verified the success of this approach (Selvarajoo et al. 2008; Selvarajoo 2011) by identifying cross-talk mechanisms between the MyD88- and TRAM-dependent pathways and proposed the concept of signaling flux redistribution (SFR). This concept is based on the law of conservation where the removal of MyD88 leads to increased activation of the entire alternative TRAM-pathway. Thus total signaling flux information from receptor through final downstream gene activation in the network is conserved. The group experimentally validated the SFR theory using $MyD88^{-/-}$ and $TRAF6^{-/-}$ KO mice (Selvarajoo et al. 2008) and their data generated interesting interpretations, which may open up new avenues towards the deeper understanding of cellular signaling processes.

2.2.4 Two edges of a sword: signaling through NF-κB and IRF3 pathways

In DCs, signaling events downstream from TLRs and RLRs branch into two directions: the MyD88-mediated inflammatory and chemotactic NF-κB, and the TRIF-controlled type I IFN regulating IRF3/7 pathways. In our work we investigated the functional signal distribution of these downstream pathways leading to the production of inflammatory cytokines, chemokines, and IFNβ, after the simultaneous, specific ligation of TLR3 and RLRs by polyI:C. We performed TLR3 and RLR specific siRNA

knockdown experiments in human moDCs prior to polyI:C activation to reveal the dominant downstream signaling events after specific receptor triggering.

2.3 Dendritic cells as targets of vaccination

Novel vaccination strategies that target DCs are emerging and involve delivering antigens to the appropriate DC subset along with stimulatory signals to result in prompt activation of innate immunity (Pashine et al. 2005; Frick et al. 2010). DCs continuously monitor their environment with the help of their internalizing receptors that mediate the uptake of soluble and particulate material (Figdor et al. 2002; Gogolak et al. 2003). Immature DCs are ideal targets of vaccine design due to their high phagocytic capacity (Benko et al. 2008). They possess unique mechanisms to promote antigen processing and presentation (Trombetta and Mellman 2005; Dudziak et al. 2007). Particulate antigens, such as intact microbes or their products, apoptotic cells, artificial beads, or other particulate formulates used for vaccination (Table 3) can be delivered to distinct intracellular compartments of DCs based on their expression pattern of PRRs (Blander and Medzhitov 2006). Microbes or artificial beads carrying both antigenic structures and TLR ligands induce phagosome maturation and the presentation of relevant peptide segments via MHC class II proteins on the cell surface. However, in the absence of coupled TLR signals peptides from phagocytosed structures (*e.g.* non-infected apoptotic cells) even if internalized together with microbes are not immunogenic because of incomplete antigen processing and phagosome maturation (Blander and Medzhitov 2004). The means how DCs get activated has an impact on their cytokine profile and physical interactions with other cells, and thus the mode of DC activation influences the functional activity of other immune cells. For example, proper DC modulation allows DCs to regulate the effector functions of T cells, the magnitude and characteristics of antibody responses and long-term immunologic memory.

Adjuvants – as general immune potentiating agents – can significantly increase immunogenicity, sustain immune responses, optimize the qualitative features of humoral and cellular immunity, induce local mucosal and systemic responses and decrease the required dose of antigen (Benko et al. 2008; Singh and O'Hagan 2003). Aluminum-based adjuvants (aluminum phosphate or hydroxide, termed as "Alum") have been used for more than 60 years as a delivery system for human and veterinary vaccines (Hem and White 1995) (Table 3). In general, Alum is safe and can provide

long-term protective immunity, but was found to be incapable of inducing Th1 immune responses required for protection against many intracellular pathogens. It is well known that DCs can internalize alum-adsorbed antigens more efficiently than soluble ones (Morefield et al. 2005) thereby obtaining enhanced antigen presenting potential and exhibiting augmented expression of costimulatory molecules and secretion of IL-1β and IL-18 cytokines (Sokolovska et al. 2007). These cytokines in combination with IL-4 and IL-13 predominantly support Th2 polarization, whereas the combination of IL-1β, IL-6, and IL-23 stimulates the differentiation of human Th17 cells that exert immunity against extracellular bacteria and fungi (Chen and O'Shea 2007). As the expression of IL-1β mRNA is constitutive in DCs and unaffected by alum, Matsue and colleagues suggested that alum induces the caspase-1-mediated cleavage of IL-1β and IL-18 (Matsue et al. 1992). In a murine model system, Gr-1^{hi}CCR2^{+} monocytes were identified as the precursors of DCs recruited to epithelial surfaces after skin or mucosal administration of antigen together with Alum. These cells could mediate the cross-priming of $CD8^{+}$ T-lymphocytes (Le Borgne et al. 2006) and facilitate the activation and differentiation of naïve B-lymphocytes into antibody secreting cells (Jordan et al. 2004).

The traditional complete Freund's adjuvant (CFA) (Table 3) is a preferred water-to-oil system that increases the efficacy of immunization. CFA contains heat killed mycobacteria and is a strong inducer of cell mediated immune responses as it can strengthen humoral immunity against various antigens. The minimal essential component of this ajuvant is MDP, a degradation product of the PGN cell wall component of Gram-negative and Gram-positive bacteria. Muropeptides are released during the standard life cycle of bacteria or as a result of the host response or antibiotic treatment. MDP alone was found to elicit a weaker response than TLR-induced activationhowever, MDP can synergize with TLR agonists to induce a much stronger response. Novel delivery and adjuvant systems, such as liposomes, vesicles, micelles, cationic peptides or microparticles, and biomaterial drug vehicles offer new avenues for targeting DCs to improve the efficacy of vaccines (Benko et al. 2008) (Table 3).

Artificial systems like polymer particles and liposomes can protect the antigen from degradation, and after internalization by DCs they enable its intracellular release facilitating antigen presentation by both MHC class I and class II molecules. Large particles are usually phagocytosed, while microparticles and other small biomaterials are taken up by macropinocytosis or receptor-mediated uptake. In the last two decades

vaccination studies revealed that particle size is a crucial factor for lymphatic uptake- and synthetic adjuvants coupled to various nano- or microparticles were shown to help robust DC activation (Pashine et al. 2005). Since thymic, splenic, tissue resident and approximately half of LN-resident DCs are in immature state (Wilson and Villadangos 2004), both tissue and lymphoid organ resident DCs are ideal targets for synthetic adjuvant-assisted vaccination. However, the most potent DC subset for vaccine targeting has not yet been found. The traditional routes of vaccine administration are intramuscular and subcutaneous injections. Epidermal $CD1a^+$ LCs are the major APCs of the skin that are replenished from resident precursors (Romani et al. 2003), whereas circulating blood derived pre-LCs are recruited to the epidermis only in the dramatic loss of LCs after severe trauma (*e.g.* high dose UV-irradiation) (Merad et al. 2002). $CD1a^-$ DCs of the skin resemble tissue resident DCs, while moDCs give rise to both $CD1a^-$ and $CD1a^+$ subsets. All of these cells are migratory DC subtypes, which deliver their antigenic cargo to draining LNs following appropriate stimuli. The activation of DCs by PRR ligands can be used for fine tuning DC-mediated functions.

A possible route of nucleic acid-based PRR vaccination is subcutaneous administration of synthetic dsRNAs. It has recently been reported that the synthetic dsRNA analog pI:C stabilized with poly-L-lysine (called polyICLC) that targets both TLR3 and MDA5 is a potent strategi to achieve the same efficacy as live viral vaccines (Caskey et al. 2011). An important regulatory pathway here is associated with DC-dependent activation of regulatory T-cells (T_{regs}) that is controlled by TLR-induced IL-6 secretion (Schnare et al. 2001; Frick et al. 2010). Therefore natural inhibitors or regulators of the PRR signaling pathways are also potential targets of vaccine design.

Splice variants of TLR2, TLR3, and TLR4 for instance may act as dominant-negative isoforms that effectively interfere with TLR-mediated signaling (Mbow and Sarisky 2005). The LPS-binding protein (LBP) or soluble CD14 can also facilitate TLR4 signaling (Miyake 2004) and the flagellin-triggered TLR5-specific response can be enhanced by a soluble TLR5 ortholog of fish acting as a natural adjuvant (Tsujita et al. 2006). As different PRRs signal through distinct adaptors and mediate well defined signaling pathways, overexpression of these adaptors may promote (Sasai et al. 2006), whereas inhibition of receptor-adaptor interactions by synthetic agents can block PRR-associated functions (Ii et al. 2006). Specific ligands of PRRs, liposomes, and microparticles can activate DCs directly, whereas mineral salts and various antigen delivery systems exert indirect effects on DCs through the stimulation and recruitment

TYPE	MODE OF ACTION	DC TYPE
Mineral		
Aluminum salts; Hydroxide/phosphate; Alum	antigen delivery, uptake, presentation, co-stimulation, IL-1b, IL-18, Th2/Ab response	**cDC**
Emulsion		
Freund's adjuvant (FA) - water-to-oil emulsion; complete (CFA); incomplete (IFA); micro-fluidized detergent stabilized emulsions;	delayed antigen release, „depo-effect", antigen delivery to APC, CFA contains heat killed mycobacteria, MDP-NOD2/Nalp3 ligand	**cDC**
Particulate		
Immunostimulatory complexes; saponin, cholesterol, phospholipid, (30–80 nm), amphipathic protein antigen on surface; Liposomes (w or w/o saponin) (100–1000 nm), antigen internal; Biodegradable macrospheres (w or w/o saponin), Polylactide polyglycolide PLG (>1000 nm), antigen internal; **IC31®**	Targeting to APC and LN, co-stimulation, reduced antigen dose, CTL-response, IL-1b, IL-6; Protect antigen from degradation/delayed antigen release; Enhanced, prolonged immune responses; Intracellular release of antigen; **delivers ligands to endosomal TLRs**	**cDC**
Cationic microparticles		
	Targeting to APC, concentrate, display and co-localize antigen with immunostimulators;	**cDC**
Microbial products I.		
Nucleic acids: intermediers of virus infection, ingested through infected or dying cells; Peptidoglycan cell wall component of Gram-/+ bacteria, muramyl-dipeptide (MDP); Endotoxin: bacterial lipopolysaccharide (LPS); Exotoxin: cholera/pertussis toxin, *E. coli* heat labile toxin;	Natural PRR ligands; co-stimulation, mucosal immunity, etc.	**cDC/pDC**
Microbial products II.		
TLR ligands/imidazoquinolines (R848, R837); NLR ligands/MDP analogs; Oligonucleotides/CpG ODN/ISS1018;	Synthetic PRR ligands; Inflammatory/IFN stimulants;	**cDC/pDC**

Table 3. Type, mode of action and DC target of various adjuvants grouped on the basis of their source, composition and physicochemical properties (mineral, microbial, emulsion, particulate) *(by the author)*

of other blood (McLachlan et al. 2008) or stromal cells (De Gregorio et al. 2009). It is also well established that the combination of multiple stimulatory signals is able to boost adjuvant effects (Trinchieri and Sher 2007; Wack et al. 2008) and induces the accumulation of inflammatory cells at the injection site (Kool et al. 2008). However, the molecular mechanisms mediating these complex effects are still poorly characterized.

IC31® is a two-component adjuvant consisting of the artificial antimicrobial cationic peptide KLK as a vehicle (Fritz et al. 2004) and the TLR9stimulatory oligodeoxynucleotide ODN1a (Kritsch et al. 2005; Schellack et al. 2006). Several *in vivo* studies in the murine system revealed the Th1 and/or Th17 polarizing effect of IC31® when used as an adjuvant in combination with mycobacterial antigens (Agger et al. 2006; Kamath et al. 2008a,b), and the efficacy of IC31® in anti-mycobacterial vaccination of healthy volunteers was also shown (van Dissel et al. 2010, 2011). Besides its beneficial immunological effects, the unique functional characteristics of IC31® and its components have also been described. KLK was shown to facilitate the uptake and delivery of ODN1a into TLR9-positive intracellular vesicular compartments of human moDC (Aichinger et al. 2008, 2011) and based on these properties IC31® was implicated to have a profound effect on immune responses triggered by TLR9 agonists (Lingnau et al. 2007). TLR9-mediated stimulation has been intimately linked to type I interferon production and previous studies in a mouse model revealed the capability of IC31® to induce peptide-specific cytotoxic T cell (CTL) activation in a Stat1 phosphorylation dependent manner, indicating the role of interferons as adjuvants. However, the exact mechanisms involved in IC31®'s actions have not been identified (Pilz et al. 2009; Prchal et al. 2009). We hypothesized that type I interferon production contributed to the adjuvant effects of IC31® and sought to analyze the effects of IC31® in human DCs. In the human immune system, TLR9 expression and function has been associated with pDCs owing to the production of high amounts of type I interferons upon viral infections or ligation by specific TLR9 ligands (Kaisho 2010; Kawai and Akira 2011), whereas the role of TLR9 in cDCs is still controversial (Benko et al. 2008).

2.3.1 Characterization of the effects of IC31® on DC differentiation, activation, and subset distribution

The major goal of our study was to identify the mechanism of IC31® adjuvant action in human leukocytes at the molecular and functional levels by comparing the

distribution and accumulation of the adjuvant in human PBMCs, monitoring its effects on DC differentiation, subset distribution, activation of key transcription factors and signaling cascades, and cytokine secretion. This was approached by characterizing the phenotypic and functional properties of the $CD1a^+$ and $CD1a^-$ moDC subsets generated in the absence or presence of IC31® or its components both at resting and activated states.

2.4 Aim of the studies

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

3. MATERIALS AND METHODS

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

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

Phenotyping of resting and activated DCs was performed by flow cytometry using anti-CD83 and anti-CD1a antibodies (Abs) and isotype-matched control Ab (BD Pharmingen, San Diego, CA). Fluorescence intensities were

measured by FACS Calibur cytometer (BD Biosciences, Franklin Lakes, NJ), data were analyzed by the FlowJo software (Tree Star, Ashland, OR). Fluorescent dye conjugated KLK(-FITC) and ODN1a(-Cy5) uptake was performed in cell culture medium at 37°C and 0°C as control. The cell population of interest was gated according the forward and side light scatter properties.

Activation of dendritic cells

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

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

RNA isolation, cDNA synthesis and QPCR

Real-time quantitative polymerase chain reaction (QPCR) was performed as described previously (Gogolak et al. 2007). Briefly, total RNA was isolated by TRIzol reagent (Invitrogen; Carlsbad, CA). 1.5-2 μg of total RNA were reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)15

primers (Promega, Madison, WI). Gene-specific TaqMan assays (Applied Biosystems) were used to perform QPCR in a final volume of 25 µl in triplicates using AmpliTaq DNA polymerase and ABI Prism 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA). Amplification of 36B4 was used as normalizing control. Cycle threshold values (Ct) were determined using the SDS 2.1 software. Constant threshold values were set for each gene throughout the study.

siRNA experiments

Gene-specific siRNA knockdown was performed by SilencerSelect siRNA (Applied Biosystems) transfection at day 3 of *in vitro* DC differentiation using GenePulser Xcell instrument (Bio-Rad, Hercules, CA). Silencing of helicase genes was performed by RIG-I and MDA5 siRNA mix, Silencer Negative Control non-targeting siRNA (Applied Biosystems) was used as a negative control. The efficacy of siRNA treatments was tested before and after DC activation on days 5 and 6, respectively by Q-PCR and Western blotting.

Western blotting

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

Scientific, Rockford, IL). After the membranes had been probed for the target protein, they were stripped and re-probed for β-actin.

Cytokine measurements

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

IFNγ ELISPOT assay

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

Immunohistochemistry (IHC) and immunofluorescence (IF) staining

Immunostaining was performed on human tissues obtained from formalin-fixed and/or paraffin-embedded surgical specimens. RIG-I and MDA5 Ab were used at a dilution of 1:50 (Cell Signaling and Lifespan, respectively). As a reference antibody, affinity purified rabbit Ab to S100 protein 1:1000; Novocastra) was used. Biotin-free

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

Statistics

Data are presented as mean ± standard deviation (SD). p values were calculated using Student's *t* test. $p < 0.05$ was considered as statistically significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ and ****$p < 0.001$.

4. RESULTS

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

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

Several groups reported that TLR4 and IRAK-1 proteins are degraded in response to long-term LPS triggering in DCs and in macrophages (Albrecht et al. 2008; Li et al. 2000; Nomura et al. 2000) whereas the inhibitory protein IRAK-M can be upregulated upon chronic DC activation (Hedl et al. 2007). In this study, we compared TLR4 expression in moDCs developing with or without 5 ng/mL LPS for 2 days using flow cytometry or Western blot and found no sign of decreased TLR4 expression in the presence of LPS (data not shown). Thereafter we studied IRAK-1 and IRAK-M protein levels in moDCs developing in the presence or absence of LPS using western blot and we detected the downregulation of IRAK1 by day 2 in the presence of LPS (Figure 11A). IRAK-M levels slightly decreased as well, indicating that an upregulation of IRAK-M might not stand as the mechanism underlying moDC endotoxin tolerance. In order to determine whether decreased IRAK-1 levels could play an important role in DC inactivation we transfected developing moDCs with IRAK1-specific siRNA. As shown in Figure 11B, decreased IRAK-1 expression resulted in low IL-12 production when moDCs were activated on day 2 by LPS or the TLR7/8 ligand CL075. These results indicate that the activation-induced IRAK1 downregulation might play an important role in the functional exhaustion of moDCs as this event alone can lead to decreased cytokine production by activated DCs.

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

Previous studies have indicated a developmental blockade in moDC differentiation in response to persistent TLR activation (Bartz et al. 2006; Palucka et al. 1999; Rotta et al. 2003) or an impaired TLR signaling as the underlying mechanism for LPS-induced tolerance (Biswas and Lopez-Collazo 2009; Liew et al. 2005; Escoll et al. 2003; Taganov et al. 2006; Albrecht et al. 2008; Chang et al. 2009). To better

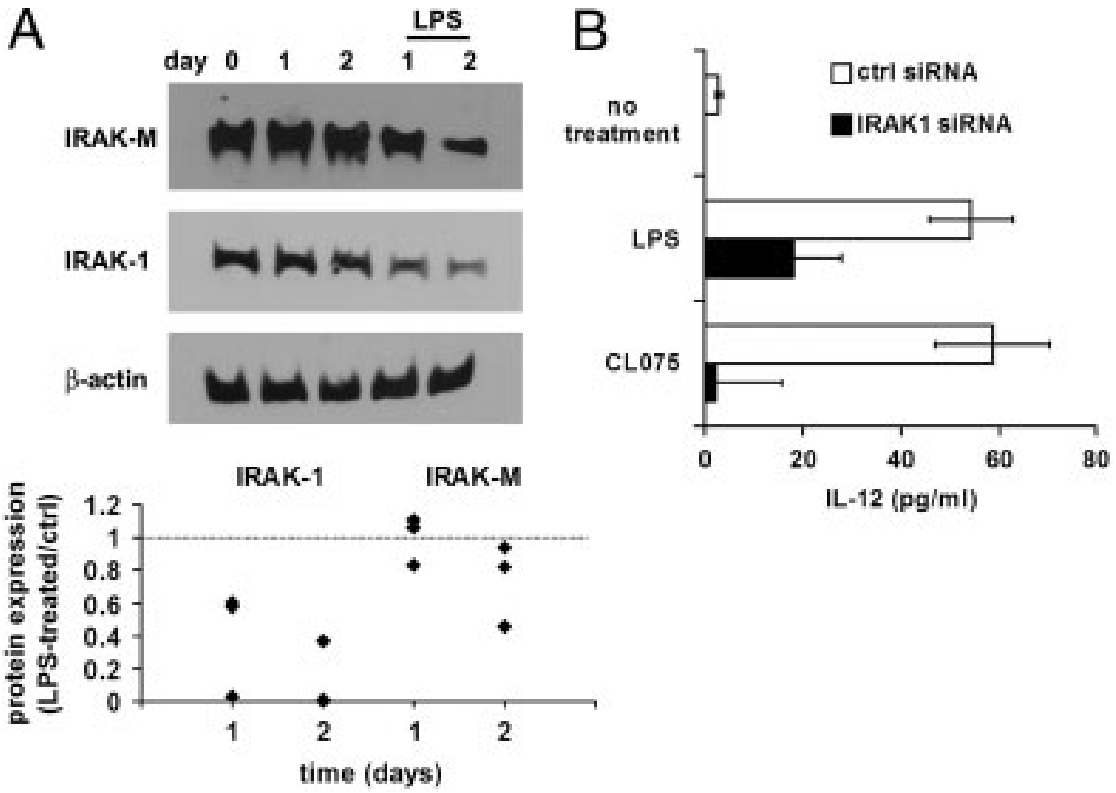

Figure 11. Downregulation of IRAK-1 in MoDCs developing in the presence of LPS. ***(A)*** *IRAK-M and IRAK-1 expression was studied in monocytes or in moDCs developing in the presence of 5ng/mL LPS for 1 or 2 days using western blot. The effect of LPS on IRAK-1 and IRAK-M protein levels is shown in the lower panel calculated from the band intensities measured in three independent experiments.* ***(B)*** *Monocytes were transfected with IRAK-1-specific or control siRNA molecules and then moDC cultures were established and maintained for 2 days. The cells were activated for 24 h using LPS (100 ng/mL) or CL075 (1 mg/mL) and IL-12 concentration was measured in the supernatants. Data are shown as mean±SD calculated from three replicate measurements. A representative result of three independent experiments is shown.*

understand how early activation may influence moDC functionality we studied the survival, differentiation and signaling abilities of moDCs developing in the presence of LPS.

As shown in Figure 12A, TLR ligands, TNF cytokine or CD40L had variable effects on moDC differentiation by day 2 and none of the stimuli led to a substantial increase in apoptosis. Ligation of TLR2 by zymosan, or HKSA and CL075, led to the retention of high CD14 expression on a subset of cells and blocked CD1a expression. Other signals, however, did not have a major impact on the expression of moDC differentiation markers despite their ability to decrease the sensitivity to further activation (data not shown). Monocyte activation may thus prevent DC differentiation

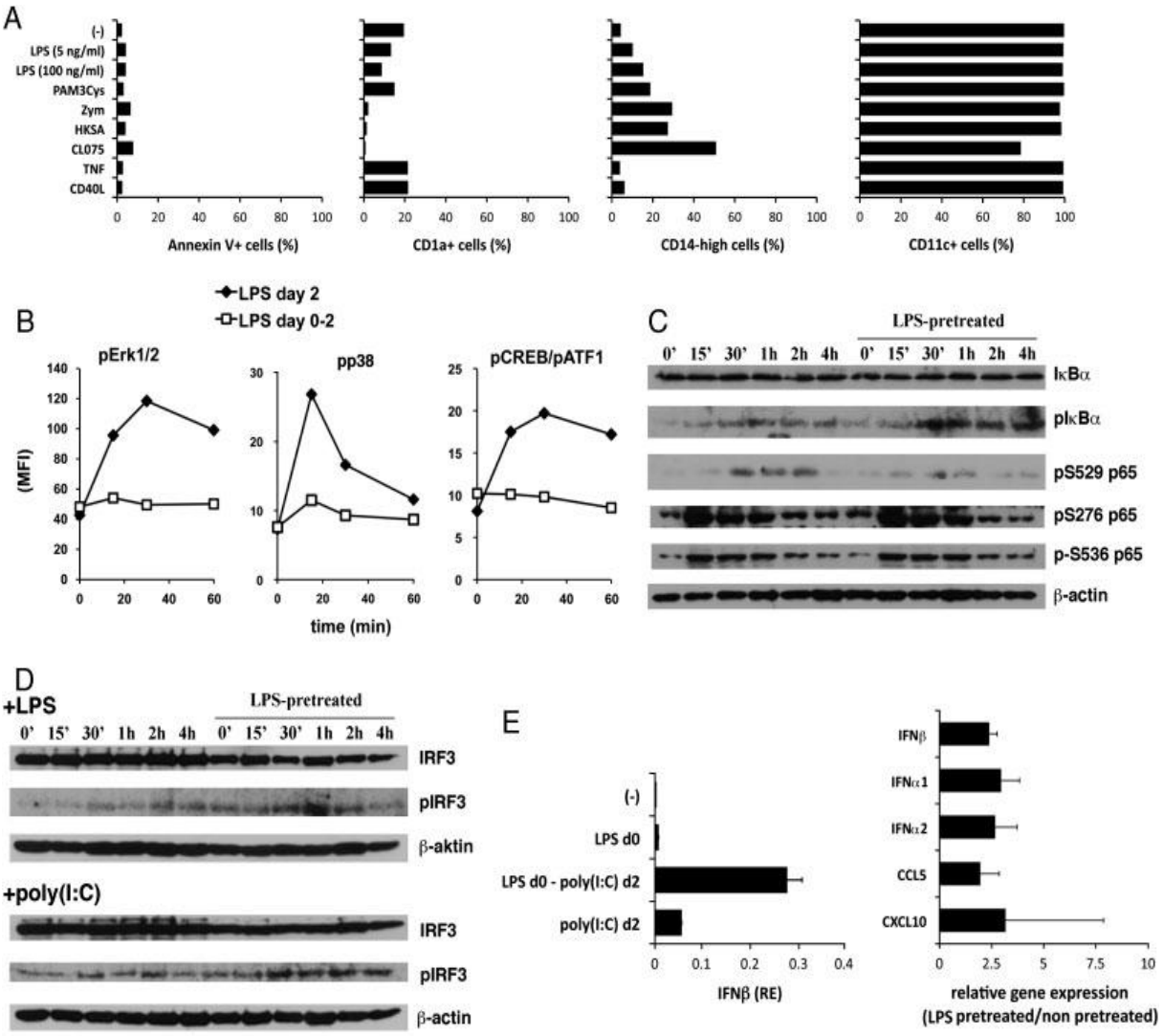

Figure 12. Early activation of moDCs modulates differentiation and TLR signaling. ***(A)*** *Survival and the expression of the differentiation markers CD1a, CD14 and CD11c were measured in moDCs cultured in the presence of various activation signals for 2 days.* ***(B)*** *moDCs were cultured in the presence (open squares) or absence (black diamonds) of 5 ng/mL LPS for 2 days. Thereafter the cells were activated with 100 n/mL LPS and the phosphorylation of Erk1/2, p38 MAP kinases and the transcription factors CREB/ATF1 were studied by flow cytometry.* ***(C)*** *NF-κB signaling was studied in LPS-activated moDCs that were cultured for 2 days in the presence or absence of 5 ng/mL LPS. IκBα levels, phosphorylation of the IκBα and p65 (on serine 529, 276 and 536) were analyzed by western blot.* ***(D)*** *IRF3 phosphorylation in response to LPS (top) or poly(I:C) (bottom) was studied by western blot in moDCs cultured for 2 days in the presence or absence of 5ng/mL LPS.* ***(E)*** *IFNβ gene expression in response to poly(I:C) activation was studied by real-time PCR in day-2 moDCs pre-cultured or not with 5 ng/mL LPS (left). Mean±SD values were calculated from three replicate measurements. In similar assays we compared the induction of the IFNα1, IFNα2, CCL5 and CXCL10 genes in response to poly(I:C) between LPS pretreated and non pretreated MoDCs (right). Mean±SD values were calculated from four independent experiments. In all other panels representative results of at least three independent experiments are shown.*

in the case of some particular TLR ligands; however, such effect does not fully overlap with the tolerizing ability of the different stimuli.

In order to identify which TLR-induced signaling pathways are impaired in moDCs that received an early LPS stimulation we studied MAPK, NF-κB and IRF-3 activations in these cells. Activation of MAPKs is attributed to signals transmitted by the MyD88-dependent arm of the TLR pathways that might be particularly affected by the downmodulation of IRAK-1. Accordingly, LPS-induced phosphorylation of the Erk1/2 and p38 kinases as well as phosphorylation of the CREB/ATF-1 transcription factors, often occurring via p38 activation, were abrogated by LPS pre-treatment of developing moDCs (Figure 12B). On the contrary, DCs differentiating in the absence of LPS responded readily with Erk1/2, p38 and CREB/ATF-1 phosphorylation to LPS stimulation.

The primary step of NF-κB activation is the phosphorylation-dependent degradation of the IκB components, a prerequisite for NF-kB nuclear translocation (Hayden and Ghosh 2011; see also part *2.2.2*). Interestingly, LPS-induced IκBα phosphorylation occurred similarly in LPS pre-treated and control moDCs and we did not detect a different level of total IκBα protein in these samples either (Fig. 12C). These results indicate that NF-κB might be activated by TLR-dependent signals in LPS-tolerized moDCs. Further activity of NF-κB is tuned by enzymatic modifications that include phosphorylation at multiple residues. The NF-κB subunit p65 is phosphorylated at S276 in order to gain strong transcriptional activity, whereas its functions are further modulated by phosphorylations at other sites of the protein (Viatour et al. 2005). We found similar S276 and S536 phosphorylation patterns in response to LPS in both LPS pre-treated and control moDCs (Figure 12C). S529 phosphorylation was, on the other hand, inhibited in LPS-pretreated DCs, indicating a partial impairment of NF-κB regulation following persistent LPS signals. However, the functional significance of S529 phosphorylation is not known.

The partial activation of NF-κB in spite of decreased MyD88-dependent signal transduction might indicate functional MyD88-independent, TRIF-dependent signal routes. Indeed, we found strong IRF-3 phosphorylation in response to TLR3 or TLR4 ligation by pI:C and LPS respectively, in both LPS pretreated and control moDCs (Figure 12D). IRF-3 phosphorylation was rather elevated in LPS pretreated cells (3.8- and 2.1-fold higher than in non-LPS pretreated cells when using 2h LPS or pI:C stimulation respectively, as calculated from the densitometry analysis of three

independent experiments) and increased IRF-3 activation was accompanied by higher IFNβ expression in LPS pretreated moDCs as compared to control cells (Figure 12E). Similar to the observed effect on IFNβ, pI:C induced higher expression of other genes sensitive for TRIF-dependent regulation (IFNα1, IFNα2 and CCL5) when the cells received LPS pretreatment, whereas we did not observed a similar consistent effect on CXCL10 expression. Overall, our results indicated the downregulation of MyD88-dependent TLR signals in response to LPS pretreatment of developing moDCs. The TRIF-dependent TLR pathways, on the other hand might remain functional following persistent LPS stimulation.

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

We compared gene expression levels of chemokines in moDCs cultured with or without LPS for 2 days and observed a significant increase in the expression of CCL5, CCL18, CCL19, CCL23, CCL24, CCL26, CXCL1, CXCL2 and CXCL5 in the presence of LPS that suggests an increased ability of the LPS-treated moDCs to attract both resting and activated T cells, as well as granulocytes (Figure 13A). In addition to such possible contribution to the cellular influx associated with tissue inflammation, LPS-treated moDCs might increase their motility by cleaving extracellular matrix constituents as suggested by the elevated MMP7, MMP9 and MMP12 mRNA levels in these cells.

In order to understand whether moDCs that received activation signals at early stages of their development could obtain migratory potential towards lymphoid tissues and contribute to naïve T-cell activation, we studied their chemokine receptor pattern during the first day of culture in the presence of a wide range of activation stimuli. As moDCs responded readily with strong cytokine production when receiving activation signals (day 1) and became later functionally exhausted and incapable to respond to further stimulation (day 2), early modulation of chemokine receptor expression might be a prerequisite for the migration of functional, non exhausted moDCs to lymphoid tissues. We studied CCR5 and CCR7 expression on moDCs that received activation signals during the first day of their culture and compared these cells to moDCs that received the same activation signals at a more differentiated stage, at day 5 of the culture. Interestingly CCR5, a chemokine receptor that primes migration to inflamed

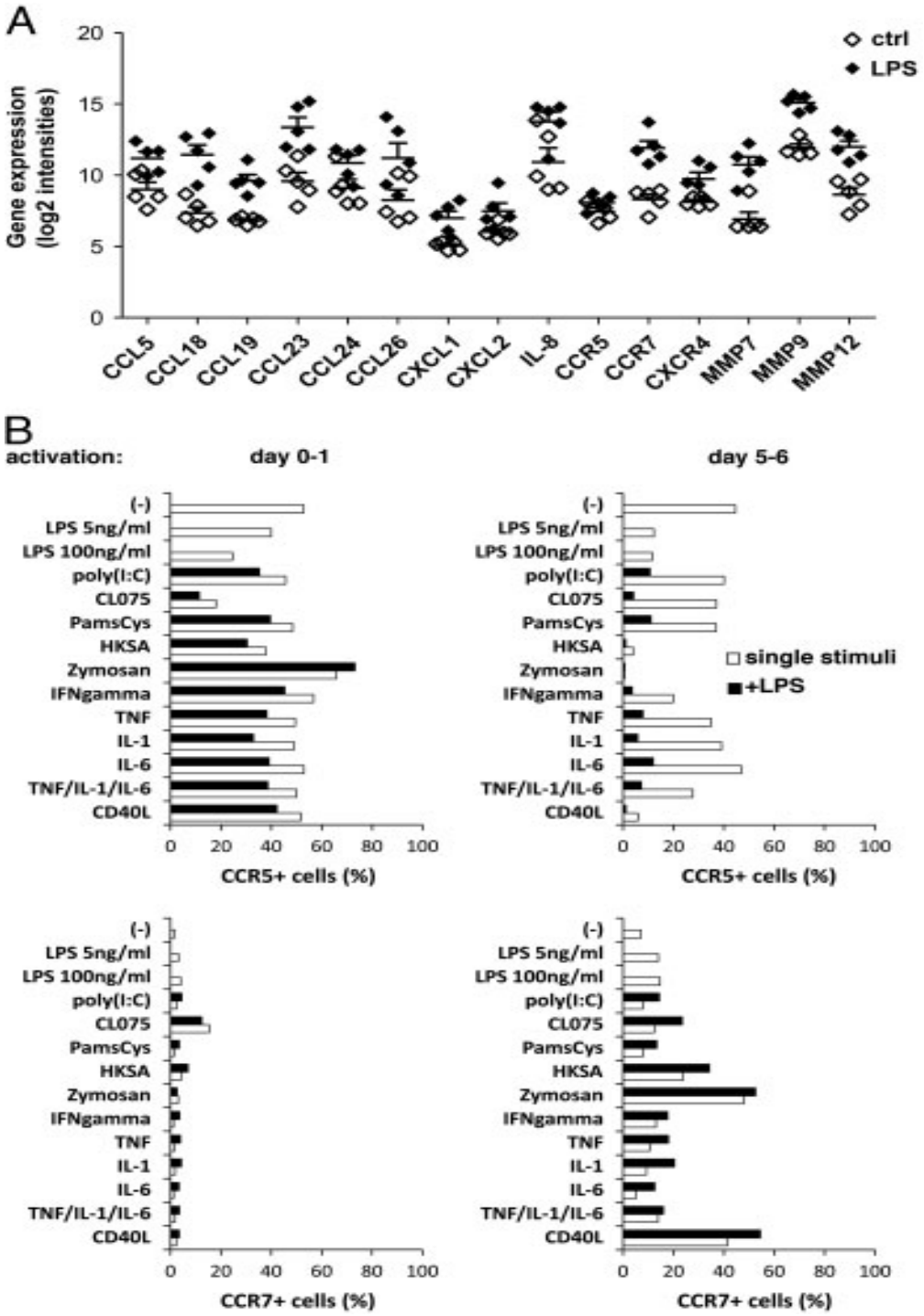

Figure 13. Early activation of developing moDCs induces chemokine production but no change in CCR7 and CCR5 cell surface expression. (A) *Microarray data show gene expression of chemokines, chemokine receptors and matrix metalloproteinases in developing moDCs extracted from the complete list of differentially expressed genes. Monocytes were isolated from five different blood donors and were cultured in the presence (black diamonds) or absence (open diamonds) of LPS for 2 days.* ***(B)*** *Expression of CCR5 (top) and CCR7 (bottom) was analyzed in moDCs using flow cytometry at day 1 (left) or day 6 (right) following the activation of cells using a range of TLR ligands, cytokines or CD40L alone (white bars), or in combination with LPS (white and black bars). Representative results of three independent experiments are shown.*

peripheral tissues, was not downregulated and CCR7 was not induced when moDCs received activation signals early in their development. On the contrary, moDCs that developed for 5 days without activation downregulated CCR5 in response to LPS, HKSA, zymosan or CD40L and several of the tested activation signals induced the expression of CCR7 on these cells (Figure 13B).

These results showed that the inability of moDCs to modulate their chemokine receptors early during their differentiation might limit egress from peripheral tissues and predispose these cells to short-term inflammatory functions in the periphery. Longer differentiation free of activation signals might be required for the acquisition of a migratory phenotype in response to late activation; however such differentiation pattern may not occur in inflamed tissues.

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

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

The cytosolic RLR sensors RIG-I and MDA5 share dsRNA specificity with membrane TLR3 but their role in human DC biology is poorly understood. We first mapped the expression of RIG-I and MDA5 in resting moDC and found that they express both sensors albeit at lower levels than monocytes (Figure 14A), but inducible by ATRA, LPS and pI:C (Figure 14C-E). The results obtained with donors 1, 2 and 3, characterized by increasing ratios of CD1a$^+$ cells suggested that mRNA expression of the helicases may correlate to the presence of CD1a$^+$ cells, previously identified as PPARγ-negative DC with the capability to produce high amounts of inflammatory cytokines and chemokines (Gogolak et al. 2007).

ATRA, LPS and pI:C are potent inducers of RIG-I and MDA5 expression in various cell types (Wang et al. 2007) but their activity to increase RLR expression in human moDC has not been analyzed. Moreover pI:C is a ligand of both RLRs (Kato et al. 2006) and TLR3, although endosomal TLR3 has been shown to detect endocytosed pI:C (Matsumoto and Seya 2008), whereas RIG-I and MDA5 are able to sense pI:C penetrating through the cell membrane by an unknown mechanism (Wang et al. 2010). To compare the effects of these activators on moDC, we first measured RIG-I and MDA5 mRNA expression after activation by graded doses of ATRA, pI:C or LPS (Figures 14C-E). ATRA did not result in moDC activation monitored by CD83 and

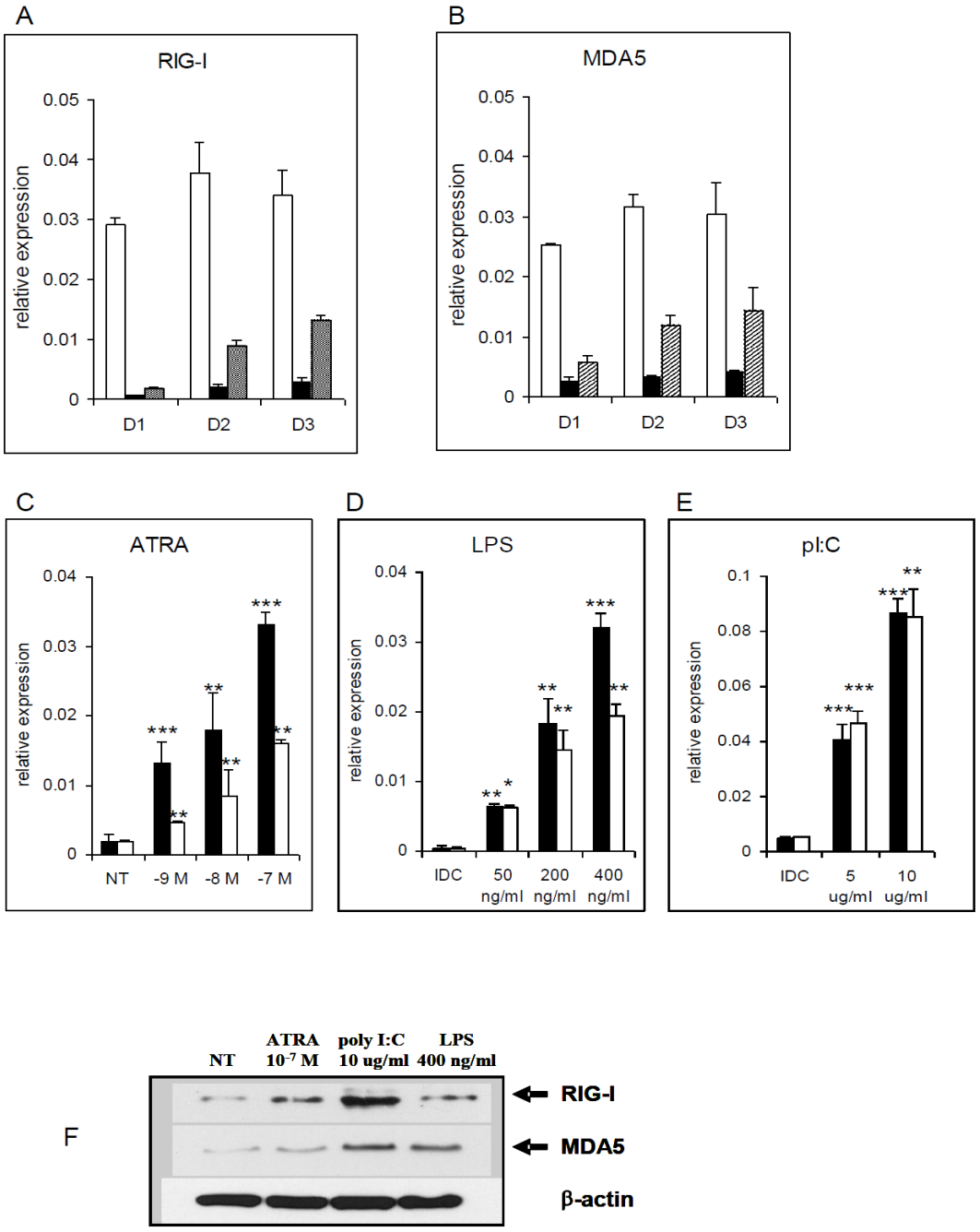

Figure 14. Relative expression of RIG-I and MDA5 upon moDC differentiation and activation. (A-F) *moDC were differentiated in the presence of GM-CSF and IL-4 as described previously (Gogolak et al 2007). Relative levels of mRNA were measured in triplicates by QPCR and mean±SD values were calculated. The ratio of CD1a^{+} DC in donors 1, 2 and 3 was 8, 37 and 60 %, respectively. Comparison of the relative expressions of RIG-I* ***(A)*** *and MDA5* ***(B)*** *in monocytes (empty bars), resting DC (black bars) and DC activated by 400 ng/ml LPS (dashed bars). On day 5 of culture DC were activated by 10^{-9} – 10^{-7} M ATRA* ***(C)****, 50 – 400 ng/ml LPS* ***(D)*** *or 5 – 10 µg/ml pI:C (E), and the relative expression of MDA5 (**C-E**: black bars) and RIG-I (**C-E**: white bars) were measured.* ***(F)*** *Protein expression was detected by Western blotting of cell lysates prepared from non-treated (NT) or ATRA, pI:C or LPS-activated DC. Representative experiments out of 3-5 are shown.*

CD86 expression, but LPS and pI:C stimulation resulted in >80% activated cells (data not shown). Despite the inactivity of ATRA to induce moDC activation, it increased RIG-I and MDA5 expression (Figure 14C) similar to the response induced by the TLR4 ligand LPS (Figure 14D) or the TLR3 ligand pI:C (Figure 14E). in a dose dependent manner detected both at mRNA (Figures 14C-E) and protein (Figure 14F) levels, and pI:C turned out to be the most potent enhancer of RLH expression. These results altogether demonstrate that besides membrane TLR3, resting moDC also express cytosolic helicases that can be induced by various stimuli.

4.2.2 Subset dependent expression and activity of RLH-related genes in moDC

PRR are distributed in a cell type specific manner and upon stimulation by specific ligands they may provoke collaborative or inhibitory signals (Kumagai and Akira 2010; see also part *2.2.3*). To assess the expression of RIG-I and MDA5 and the components of the related signaling cascade in moDC subsets we sorted the cells to $CD1a^+$ and $CD1a^-$ fractions and subjected them to mRNA and protein analysis. Both resting moDC subsets were shown to express RIG-I and MDA5, the downstream transcription factor IRF3 and the effector cytokine IFNβ1 (Figure 15). Interestingly, $CD1a^+$ cells exhibited significantly higher baseline expression of RIG-I, MDA5, IRF3 and IFNβ1 genes than $CD1a^-$ cells (Figures 15A – C). Furthermore, activation by increasing doses of pI:C resulted in the coordinated upregulation of RLH, IRF3 and IFNβ1 mRNA preferentially in $CD1a^+$ cells (Figure 15D). Activated $CD1a^+$ moDC also displayed higher levels of RIG-I and MDA5 proteins as compared to the $CD1a^-$ subset (Figure 15E). Our results showed that ATRA and LPS stimulation also resulted in upregulation of RIG-I/MDA5 expression preferentially in $CD1a^+$ cells as compared to its $CD1a^-$ counterpart (Figure 15E) however, unlike pI:C these stimuli did not show clear subset-specific differences in the enhancement of IRF3 and IFNβ1 gene expression levels (data not shown). Although ATRA upregulated the expression of RIG-I and MDA5 genes, it failed to trigger IRF3 phosphorylation and to induce IFNβ signaling (data not shown) indicating that it does not contribute to cytokine secretion.

These results demonstrate that the baseline and activation-induced expressions of RIG-I, MDA5, IRF3 and IFNβ1 genes show a significant difference in the $CD1a^+$ and $CD1a^-$ moDC subsets.

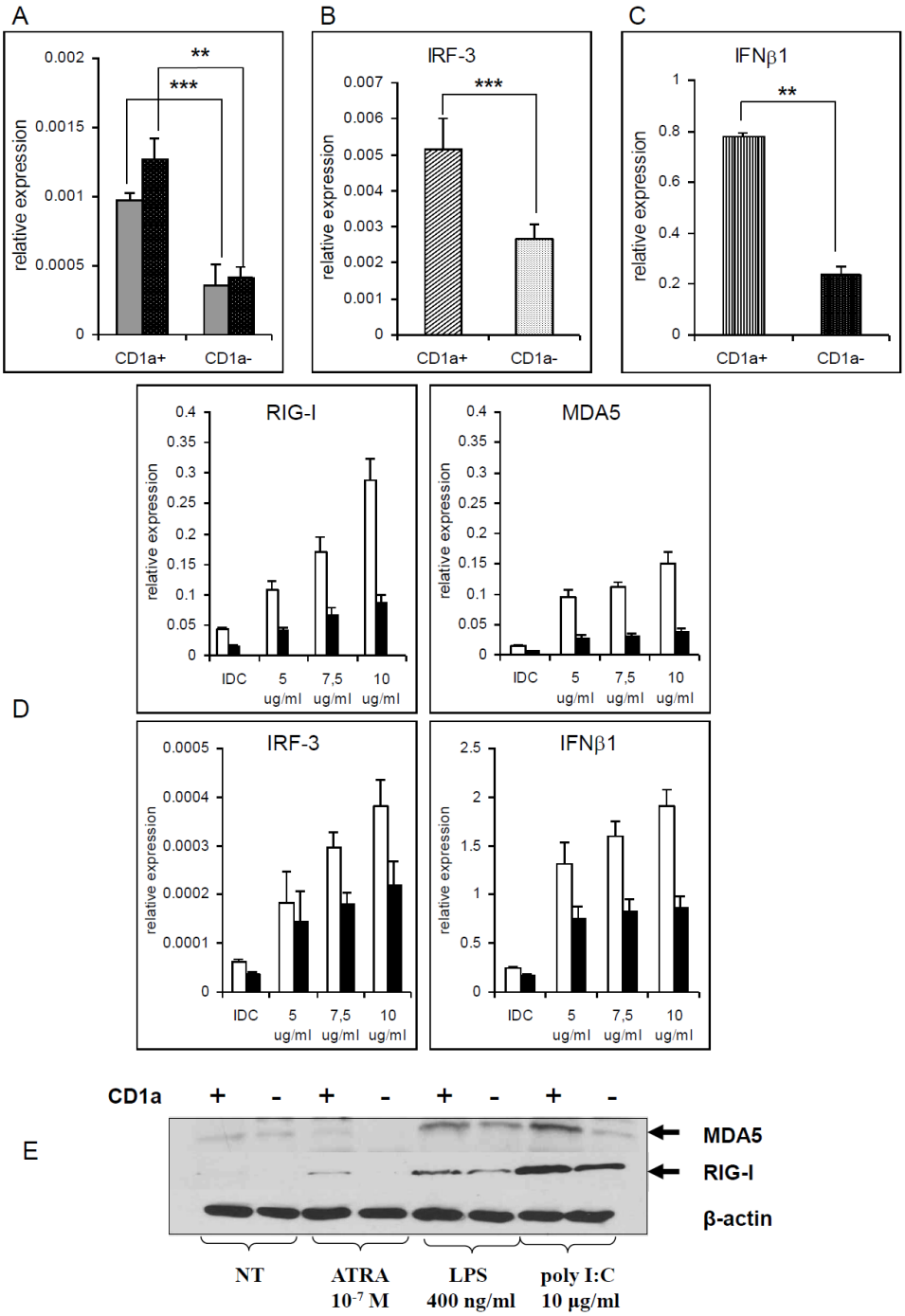

Figure 15. Expression of RLH, IRF3 and IFNβ1 genes in separated moDC subsets. *The expression of RIG-I, MDA5, IRF3 and IFNβ1 genes was measured at mRNA level in CD1a$^+$ and CD1a$^-$ cells isolated by flow cytometry before activation.* ***(A)*** *Comparison of relative mRNA expressions of RIG-I (grey bars) and MDA5 (black bars) in resting, immature DC.* ***(B, C)*** *Baseline expressions of IRF3 and IFNβ1 genes in CD1a$^+$ and CD1a$^-$ DC are shown as mean±SD of three independent experiments.* ***(D)*** *Expression profiles of RIG-I, MDA5, IRF3 and IFNβ1 in activated CD1a$^+$ (white bars) and CD1a$^-$ (black bars) cells. Activation of sorted cells was induced on day 5 of culture by 5, 7.5 and 10µg/ml of pI:C. Mean±SD values of three independent donors are presented.* ***(E)*** *Expression of RIG-I and MDA5 proteins tested by immunoblot in resting and ATRA, LPS or pI:C activated CD1a$^+$ and CD1a$^-$ DC. A representative experiment out of three is presented.*

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

To determine the contribution of NF-κB and the IRF3 – IFNβ signal transduction pathways in CD1a$^+$ and CD1a$^-$ moDC we compared the levels of phosphorylated IκBα and IRF3 (Figure 16A). As a result of pI:C stimulation we detected rapid activation of both pathways and found obvious differences in IκBα and IRF3 phosphorylation levels detected in the two DC subsets. Concordant to the differential expression of RIG-I and MDA5 receptors, CD1a$^+$ and CD1a$^-$ moDC not only differed in the expression levels of the key molecules (Figure 15), but also in the functional activity of the downstream signaling machinery (Figure 16A).

To assess the impact of differential helicase-mediated signaling on DC functional activity we measured the concentration of inflammatory cytokines and chemokines secreted by activated CD1a$^+$ and CD1a$^-$ moDC. The tested cytokines involved TNFα, IL-6, IL-12p70 and the CXCL10 chemokine as signatures of the NF-κB pathway, and IFNβ controlled by IRF3. Figure 16B shows the concentration of the secreted soluble factors in a typical experiment out of 9. Comparison of the relative cytokine levels in all donors revealed statistically significant differences for IL-6 (p=0.009), TNFα (p=0.036), IL-12p70 (p=0.002), CXCL10 (p=0.007) and IFNβ (p=0.006). These results indicate that due to their distinct RLH expression and signaling activity the CD1a$^+$ and CD1a$^-$ moDC subsets participate in the inflammatory and interferon responses to different extent.

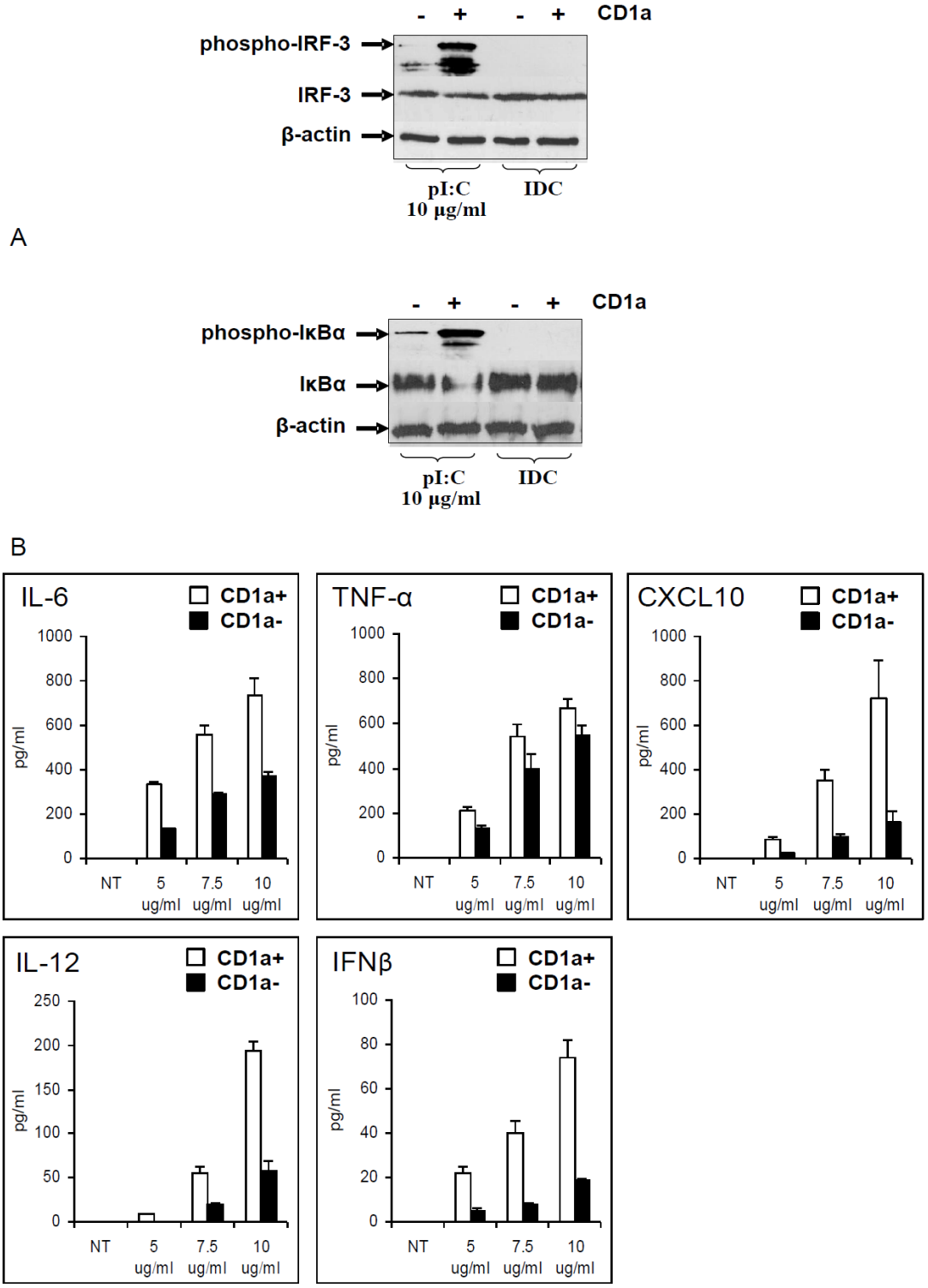

Figure 16 Activation of the NF-κB and IRF3 – IFNβ pathways in CD1a$^+$ and CD1a$^-$ DCs. *CD1a$^+$ and CD1a$^-$ DC fractions were activated by 10µg/ml pI:C. Cell culture supernatants were collected 24 hours after activation for cytokine measurements.* ***(A)*** *Phosphorylation of IκBα and IRF3 analysed by Western blotting before and after activation by pI:C. Phospho-IκBα levels were measured 30, phospho-IRF3 was detected 60 minutes after activation.* ***(B)*** *Concentration of inflammatory cytokines secreted by CD1a$^+$ (empty bars) and CD1a$^-$ (black bars) cells measured by ELISA. Data of a representative donor out of 9 are shown.*

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

The interferon and inflammatory cytokine responses of DC is mediated by the collaboration of multiple converging signaling pathways that involve the NF-κB, IRF3 and AP-1 transcription factors (part *2.2.2*; Joffre et al. 2009; Kawai and Akira 2011). We showed previously that upon pI:C activation, the production of inflammatory cytokines and type I IFNβ is primarily attributed to CD1a$^+$ moDC (Figures 15 and 16). As the dsRNA analogue pI:C is a shared ligand of both RLHs and TLR3, to dissect the roles of RLH- and TLR3-mediated signaling cascades we used the siRNA technology and silenced the RLH or TLR3 genes in CD1a$^+$ cells. The efficacy of gene silencing was checked by QPCR and Western blot (data not shown). Silencing of TLR3 gene resulted in dramatic down regulation of the secretion of pro-inflammatory cytokines IL-6, TNFα and CXCL10 known to be under the control of NF-κB (Figure 17A). Interestingly, the production of these cytokines was not affected significantly, when the RIG-I/MDA5 genes were downregulated, whereas the production of IFNβ decreased significantly (Figure 17B). These results show that upon pI:C stimulation the production of IFNβ is controlled preferentially by RLH expression and function, whereas TLR3 acts as a regulator of pro-inflammatory cytokine secretion in human CD1a$^+$ moDC.

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

The role of type I IFNs in priming adaptive T-cell responses is well established in both mice and human (Pace et al. 2010). Type I IFNs are able to enhance T-cell activity via APCs, which exert a direct effect on the autocrine and/or paracrine secretion of these cytokines (Huber and Farrar 2011) and influenza virus is known to activate RIG-Ispecifically (Rehwinkel et al. 2010; Siren et al. 2006). We hypothesized that CD1a$^+$ DC demonstrated to produce large amounts of IFNβ should be more efficient to prime T-cells in an *in vitro* human virus-infection model than CD1a$^-$ cells. To determine the functional relevance of DC subtype-specific regulation of IFNβ secretion we sought to analyse the involvement of CD1a$^+$ and CD1a$^-$ moDC in priming influenza virus-specific T lymphocyte responses. In these experiments moDC were infected by live, or incubated with inactivated seasonal H1N1 influenza virus, and then co-cultured with naive CD4$^+$ or CD8$^+$ autologous T lymphocytes. Infection of DC by live influenza virus provoked robust CD8$^+$ and lower but reproducible levels of CD4$^+$ virus-specific T cell

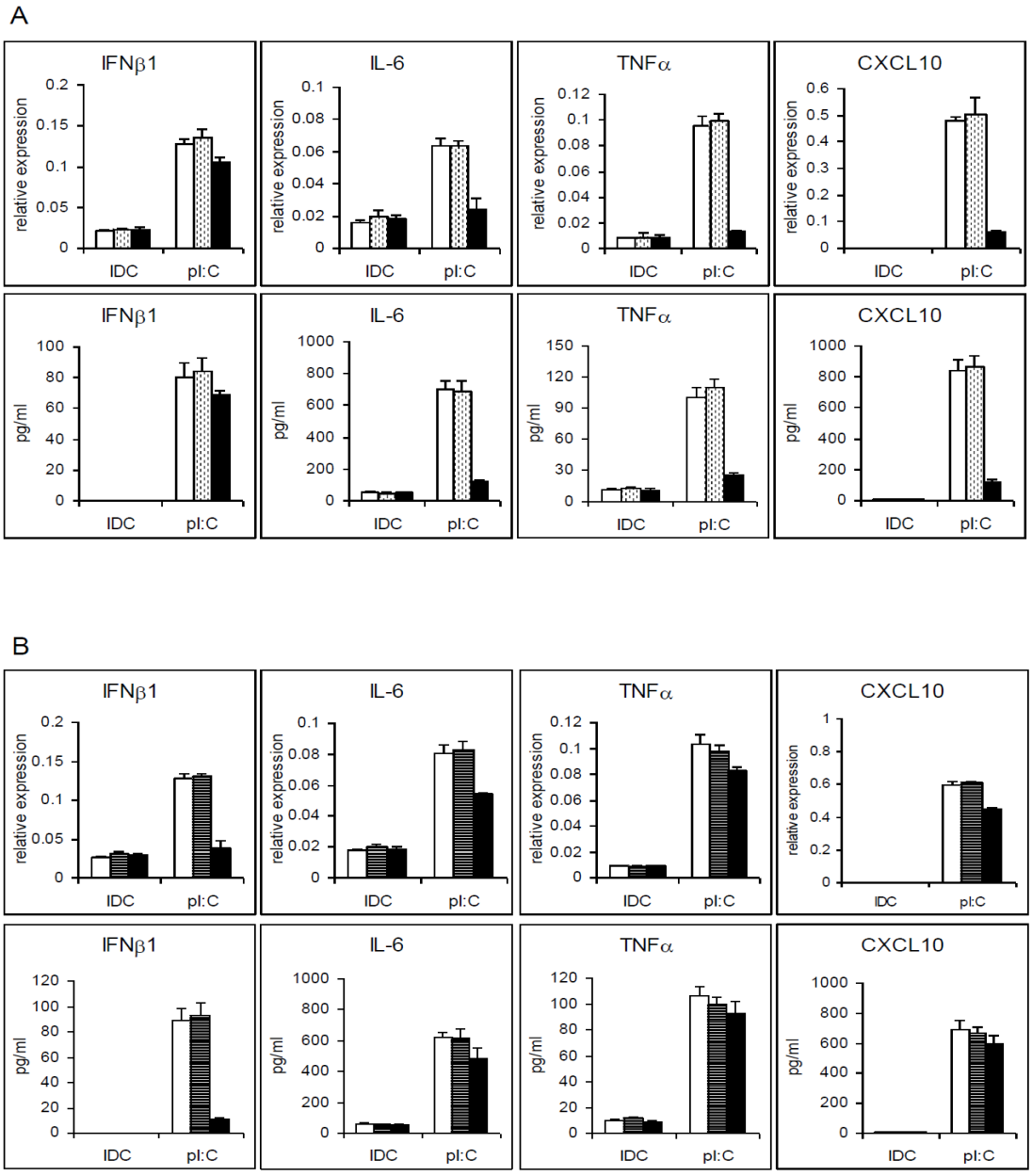

Figure 17. Functional dissection of the RLH- and TLR3-mediated signaling pathways in CD1a$^+$ moDC. *moDC were harvested on day 3 and transfected by siRNA specific for RIG-I/MDA5 (BiMix), TLR3, control siRNA or left untreated. Differentiated DC were collected on day 5 and a fraction of cells was stimulated by 10μg/ml pI:C. Cell lysates of resting and activated moDC were subjected to mRNA quantitation, supernatants of the cultures were used for cytokine measurements.* ***(A)*** *The effect of TLR3 siRNA treatment on mRNA expression and secreted cytokine levels before and after activation by 10μg/ml pI:C. Empty and dotted bars represent non-treated and control siRNA treated samples, black bars show the effect of TLR3-specific siRNA-treatment.* ***(B)*** *The effect of RIG-I/MDA5 silencing on gene expression and secretion of cytokines in immature (IDC) and pI:C (10μg/ml) activated moDC. Empty and stripped bars represent non-treated and control siRNA treated samples, whereas black bars show the effect of RLH-specific siRNA-treatment. Mean+SD values of triplicate measurements in a representative donor out of 3 are shown.*

responses, while the number of IFNγ producing $CD8^+$ T cells induced by inactivated virus remained low (Figure 18A).

When the autologous naïve $CD8^+$ T cells were activated by sorted $CD1a^+$ or $CD1a^-$ DC infected by live virus, the number of IFNγ producing T cells was significantly higher in the co-cultures containing $CD1a^+$ cells (Figure 18B, white bars) as compared to cultures with $CD1a^-$ cells (Figure 18B, black bars) underlying the preferential contribution of the $CD1a^+$ DC subset in virus-specific CTL stimulation. Silencing RIG-I/MDA5 expression in moDC by specific siRNA resulted in 68 $\pm$ 5% (n=3) reduction in the number of IFNγ secreting T lymphocytes in both subsets demonstrating the RIG-I/MDA5 dependence of the $CD8^+$ T cell response (Figure 18C).

These results confirm the functional role of RIG-I/MDA5 and the related signaling pathway in regulating influenza virus-specific $CD8^+$ T cell activation triggered preferentially by $CD1a^+$ moDC. Our studies also indicate that $CD1a^+$ cells but not $CD1a^-$ DC acquire a differentiation state that is sensitized to the rapid activation of the RLH cascade ensured by high basal expression levels of the sensors and components of the downstream signaling pathway.

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

Monocyte-derived DC has been classified as migratory and inflammatory cells that could be indentified in both peripheral tissues and lymph nodes (Jeras et al. 2005). To analyze the tissue distribution of RIG-I/MDA5 expressing DC we performed immune histochemistry (IHC) and immune fluorescence (IF) staining of tonsil and resting or reactive lymph node tissues. Immunoperoxidase (IP) staining revealed the appearance of MDA5 (Figure 19B) and RIG-I (Figure 19C) expressing cells in perifollicular regions of non-reactive lymph nodes. Double staining with S100, a typical DC marker demonstrated that a subset of DCs was positive for RIG-I (Figure 19D).

MDA5 and RIG-I positive DCs with typical morphology have also been detected in the interfollicular areas of reactive lymph node (Figures 19E – H). These results show that $CD1a^+$ DCs expressing RIG-I and/or MDA5 are detectable under physiological and pathological conditions and can be identified in lymphoid tissues.

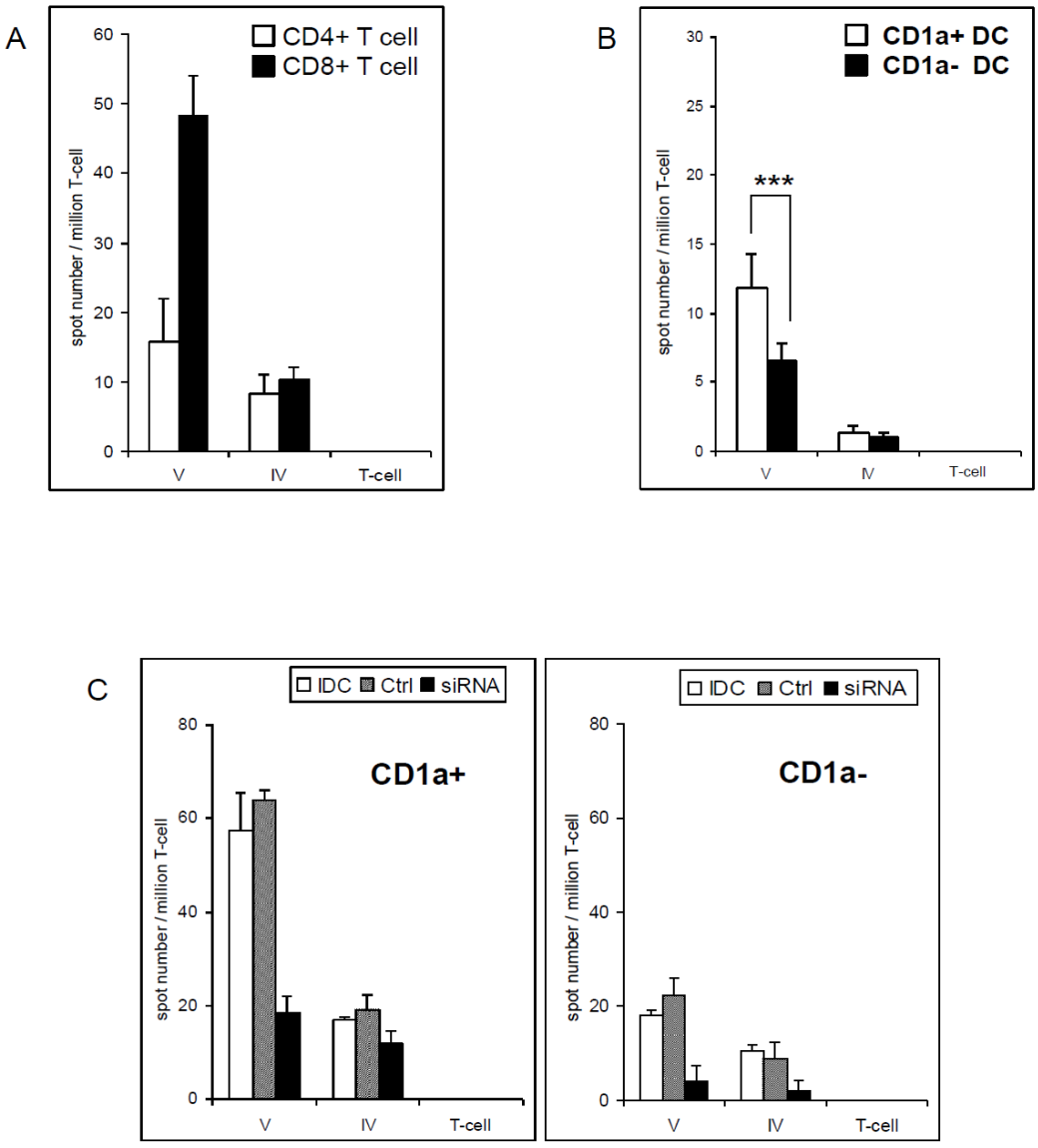

Figure 18. Role of RIG-I in triggering influenza virus specific T lymphocyte responses. *Dendritic cells were activated by influenza virus for 24h, and then co-cultured with naive autologous CD4$^+$ or CD8$^+$ T lymphocytes for 5 days. The number of primed, IFNγ secreting T-cells was assessed by ELISPOT assay.* ***(A)*** *Induction of IFNγ production by naive CD4$^+$ (empty bars) and CD8+ (black bars) T cells induced by CD1a$^+$ moDC infected by live virus (V) or loaded by inactivated virus (IV).* ***(B)*** *T cell activation provoked by CD1a+ (empty bars) and CD1a- (black bars) moDC previously stimulated by live (V) or inactivated influenza virus (IV).* ***(C)*** *The effect of RIG-I/MDA5 silencing on the T cell response induced by CD1a$^+$ (left panel) and CD1a$^-$ (right panel) moDC subsets. Bars represent non-treated "immature" DCs (IDC – empty bars), negative control siRNA treated DCs (Ctrl – dashed bars), and RLH siRNA transfected DCs (siRNA – black bars). DC were transfected by RLH-specific siRNA on day 3, separated by flow cytometry on day 5, activated by virus for 24h, and then co-cultured with naive autologous CD8$^+$ T lymphocytes and tested as described in Figure 18B. Figures 18A and 18B represent Mean+SD values of triplicate measurements of three independent donors. In Figure 18C, data of a typical donor out of 5 are shown.*

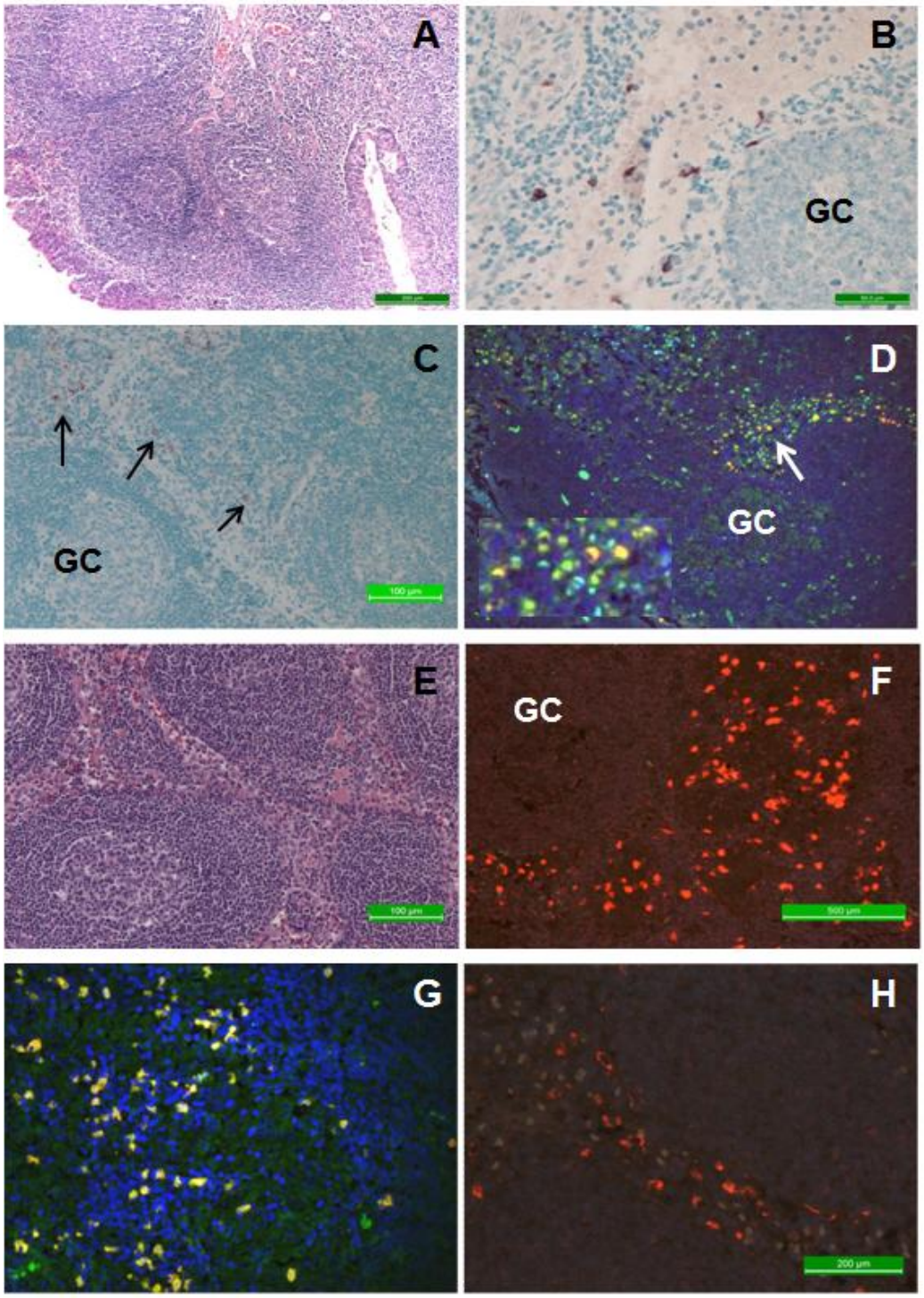

Figure 19. Immunohistological analysis of RLR expression in human tissues
(A-D) *Normal tonsil. A: HE, 10x; B: IP staining for MDA5 positive cells in the perifollicular sinus system (40x); C: RIG-I positive cells in the perifollicular region (20x). D: Double IF for RIG-I (red) and S100 (green) photographed with overlay technique. The composite color, yellow fluorescence indicates DCs that express both S100 and RIG-I (20x).* ***(E-H)*** *Reactive lymph node. E: HE, 20x; F: Perifollicular cells with cytoplamic projections that express MDA5 (20x). G: Double IF for MDA5 (red) and S100 (green) shows DC expressing both proteins (yellow fluorescence; 40x). H: Interfollicular RIG-I positive cells (red fluorescence) in part with DC morphology (40x). D and F-H: IF with DAPI nuclear counterstaining (blue).*

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

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

Cell type-specificity and cellular compartmentalization of PRRs has an important impact on the functional activity of various adjuvants (Palm et al. 2009). Thus, we first tried to identify the cell types of human PBMC preferentially involved in the accumulation of IC31® by using fluorescence-labeled KLK and ODN1a components of the adjuvant. We hypothesized that IC31® targets phagocytes and professional APCs. When we compared fluorescence intensities of different subsets of human PBMC, we found that monocytes and MHC class II-positive cells, which involve mostly B-lymphocytes, were indeed stained very strongly by KLK-FITC as compared to MHC class II-negative cells (Figure 20A, left column; Table 4). The highest intensities were measured in monocytes as compared to other MHC class II-positive cells (Figure 20A, left upper and middle panels; Table 4). The left column also indicates temperature-independen staining of the cells with KLK-FITC in contrast to ODN1a-Cy5, which exhibits a temperature-dependent accumulation in all cell fractions (Figure 20A, middle column; Table 4). As a component of IC31®, staining of ODN1a was shifted to higher fluorescence intensities and its temperature dependency was lost (Figure 20A right column; Table 4) indicating that KLK renders the cells capable to interact with the adjuvant in a temperature-independent manner. Since IC31® was most efficiently accumulated in monocytes, which can give rise to moDCs *in vivo*, we performed further experiments with moDCs testing the effects of IC31® on cellular functions.

We have previously shown that based on the expression of CD1a moDCs involve two distinct subsets with different functional abilities (Gogolak et al. 2007). Thus we compared the fluorescence intensities in $CD1a^+$ and $CD1a^-$ moDCs both at 0^oC and 37^oC (Figure 20B) and found only marginal differences suggesting the contribution of both cell types in interacting with IC31® and its components in a temperature-independent manner.

		ΔMFI values					
Treatment		**KLK**		**ODN1a**		**IC31®**	
Fluorescence		**KLK-FITC**		**ODN1a-Cy5**			
Temperature		**37 °C**	**0 °C**	**37 °C**	**0 °C**	**37 °C**	**0 °C**
Cell types	monocytes	**2066.55**	**1615.52**	**91.71**	**9.75**	**9907.81**	**9907.75**
	HLA-D^+ lymphocytes	**80.99**	**44.49**	**12.29**	**1.29**	**983.19**	**564.09**
	HLA-D^- lymphocytes	**4.6**	**12.07**	**10.2**	**0.36**	**50**	**124.51**

Table 4. **Distribution of IC31® and its components in peripheral blood mononuclear cells**

4.3.2 Effect of IC31® on moDC differentiation and activation

Our previous studies revealed that $CD1a^-$ and $CD1a^+$ DCs are generated by consecutive differentiation steps and their ratio varies among individuals (Gogolak et al. 2007). To check the long and short term effects of IC31® we set up various *in vitro* experimental systems. In the first system (*protocol A*) moDCs were differentiated in the presence of IC31® or its components added to monocytes on day 0 and to differentiating moDCs on day 2. In the second system (*protocol B*) the procedure was extended by an additional treatment on day 5 together with the activation of moDCs by LPS+IFNγ or by various TLR ligands to mimic inflammatory conditions. In the third system (*protocol C*), IC31® and its components were added on day 5 only, combined with the activation stimuli.

When monocytes were differentiated according to protocol A, IC31® dramatically inhibited the generation of $CD1a^+$ cells and this effect could be attributed to KLK, as ODN1a had no effect (Figure 21A). IC31® also inhibited the LPS+IFNγ-induced activation of DCs as measured by CD83 expression after 24 h, while ODN1a had no effect (Figure 21B) indicating that IC31®interferes with moDC differentiation and activation, and KLK had an essential role in these effects.

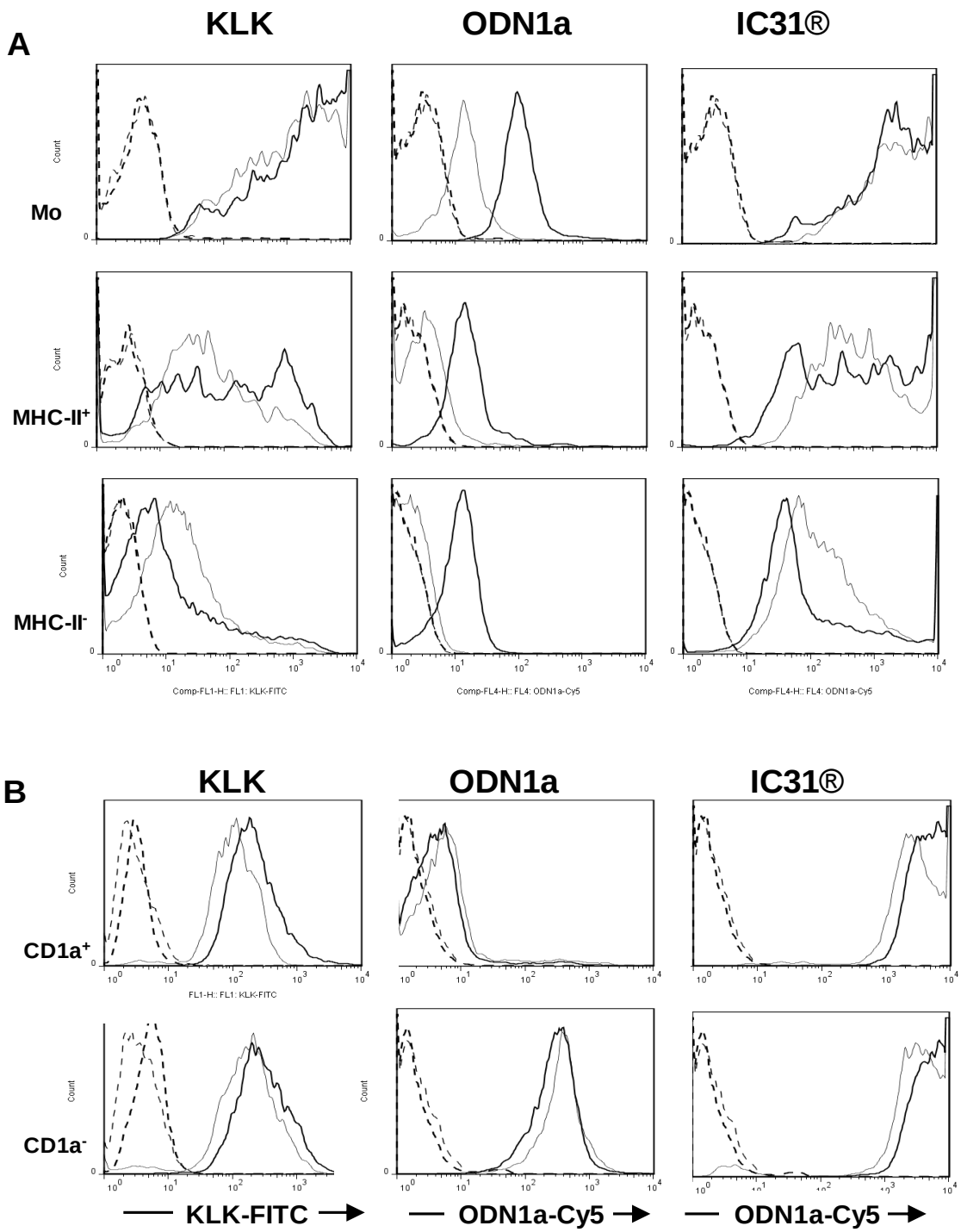

Figure 20. Distribution of fluorescent ODN1a, KLK and IC31® in human peripheral blood mononuclear cells and in monocyte-derived dendritic cell subsets

(A) *Human peripheral blood mononuclear cells (PBMC) were incubated with Cy5-conjugated ODN1a, FITC-conjugated KLK and IC31® containing both fluorescent components for 1 hr either on ice (thin line) or at 37 ºC (thick* line*). Fluorescence intensities were measured by flow cytometry in monocytes (upper panel) identified by their light scatter properties, MHC class II+ (middle panel) and MHC class II- (lower panel) lymphocyte populations discriminated by PE-conjugated HLA-D-specific antibodies. Unstained controls are shown as thick (37 ºC) or thin (0 ºC) dashed lines. Cells stained by KLK-FITC (left column) or ODN1a-Cy5 (middle and right columns) are shown. A typical experiment out of three is documented.*

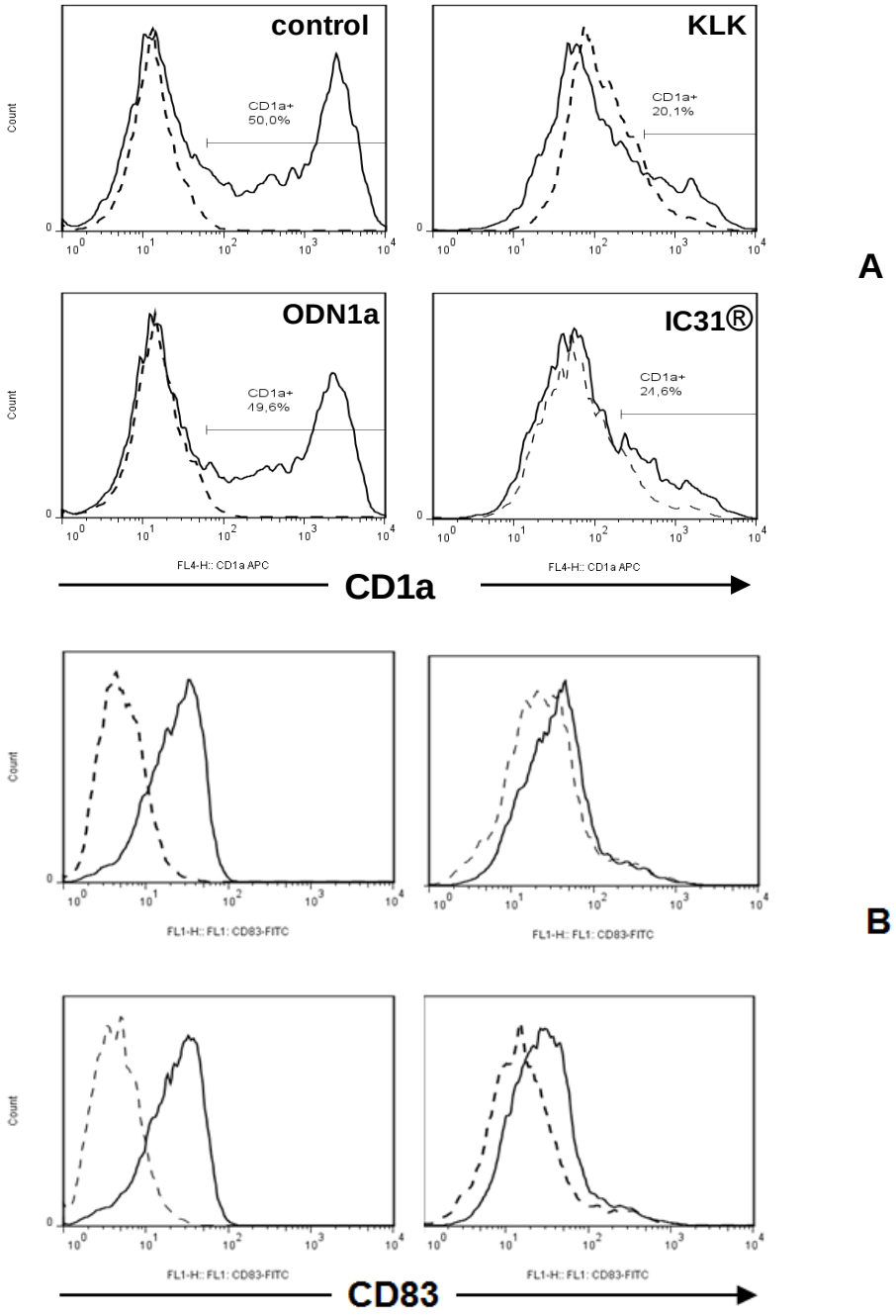

Figure 21. The effect of KLK, ODN1a and IC31® on the differentiation and activation of monocyte-derived dendritic cell subsets

(A) *Human monocyte-derived DCs were differentiated for 5 days in the absence (control) or presence of KLK, ODN1a or IC31®, added on day 0 and 2 to the cultures (Protocol A) together with the differentiating cytokines IL-4 and GM-CSF. The distribution of $CD1a^-$ and $CD1a^+$ cells was monitored by flow cytometry; samples stained by CD1a-specific antibody are shown as compared to isotype-matched control antibody (dashed line).* ***(B)*** *Human monocyte-derived DCs were differentiated as described in Figure 21A and on day 5 they were activated by 100ng/ml LPS + 10ng/ml IFNγ in combination with KLK, ODN1a and IC31® for 24 hrs (Protocol B). Activation of DCs was measured on day 6 by the cell surface expression of CD83 detected by specific antibody by flow cytometry. A typical experiment out of three independent experiments performed with DCs generated from different healthy donors.*

4.3.3 IC31® modulates the cytokine profile of human moDCs

Consistent with its inhibitory effect on moDC differentiation, we found that IC31® in combination with LPS+IFNγ according to protocol B inhibited the secretion of TNF-α and IL-6, but not IL-1β. We also observed a statistically significant increase of IFNβ secretion (Figure 22A) induced by IC31® and its KLK component, whereas ODN1a had no effect. To clarify the molecular background of these modulatory effects we sought to investigate the activation of NF-κB- and IRF3-mediated signaling pathways, respectively (Figure 22B). Our results revealed that IC31® and KLK abolished IκB-α phosphorylation, while slightly increased and extended the phosphorylated state of IRF3. As the NF-κB and IRF3-mediated signaling cascades mutually influence each other's functional activities, this effect could be attributed either to the inhibition of IκB-α activity or to modulating the balance of IκB-α and IRF3-mediated pathways (signaling flux redistribution) (Selvarajoo et al. 2008). These results suggest that IC31® is not able to activate the NF-κB pathway but rather supports the type I interferon response and this modulatory effect is attributed to KLK, the peptide component of IC31®.

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

Considering the moderate effect of the adjuvant on interferon secretion, we tested the effects of IC31® and its components present in the course of the entire *in vitro* moDC differentiation process. In this system IC31® or its components were added on day 0, 2 and 5 and the cells were activated by LPS+IFNγ on day 5. The expression profiles of IRF and type I IFN family genes in DCs showed that repeated administration of IC31® during DC differentiation increased the expression of all IRF and type I IFN genes as compared to a single adjuvant treatment at day 5 (Figure 23). Furthermore, IC31® increased IRF3 gene expression both after a single (day 5) or repeated (day 0, 2 and 5) administration to similar levels. These findings suggest that if circulating monocytes as precursors of moDCs encounter IC31®, they stimulate type I interferon production during moDCs development, but it has a less prominent effect on already differentiated moDCs. These unique effects seem to be restricted to IC31®, as they could not be induced by the single components of the adjuvant.

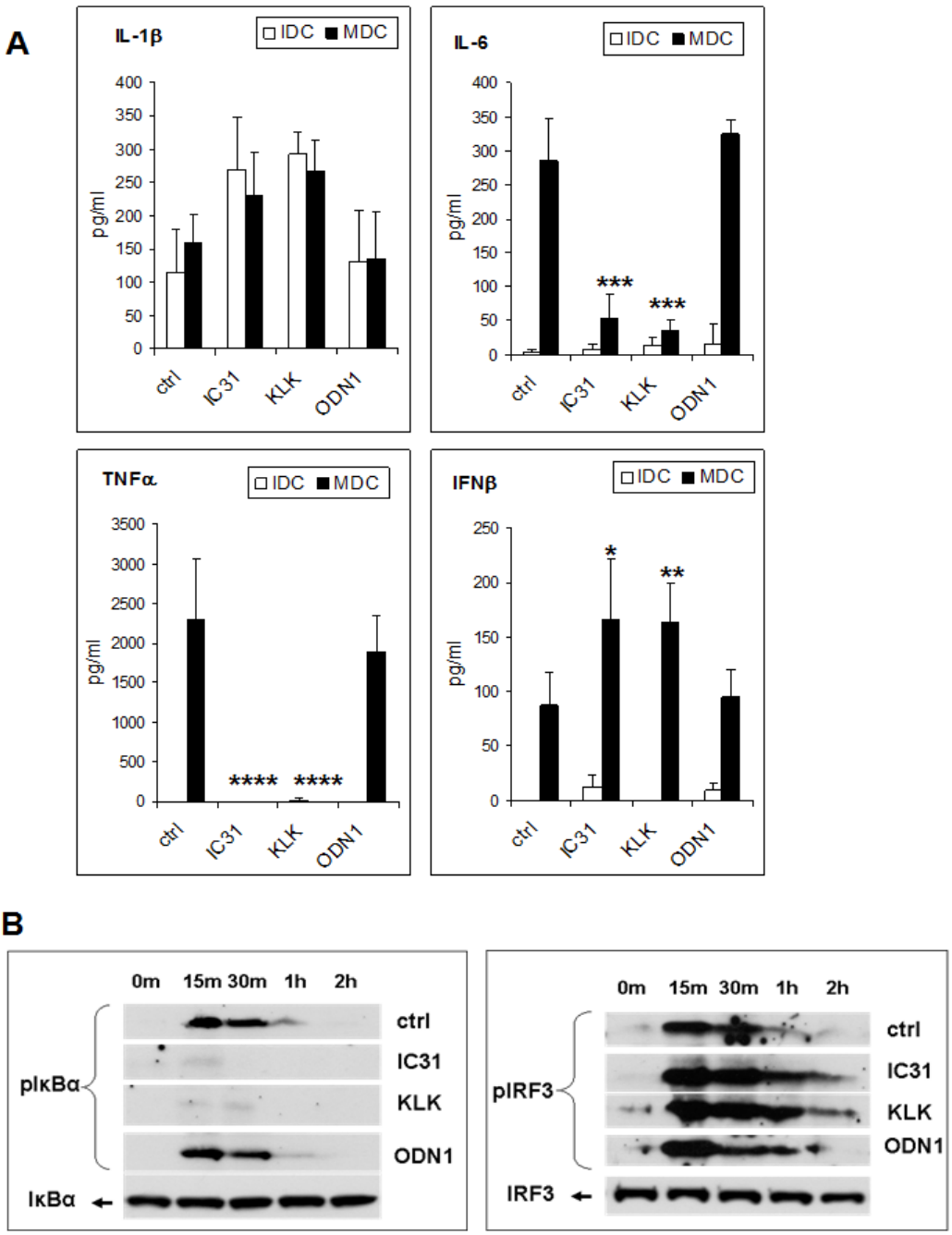

Figure 22. The effect of KLK, ODN1 and IC31® on the cytokine production of monocyte-derived dendritic cells.

Human monocyte-derived DCs were differentiated as in Protocol B and activated by 100ng/ml LPS + 10ng/ml IFNγ for 24 hrs (non-activated immature dendritic cells: IDC; activated mature dendritic cells: MDC). ***(A)*** *Supernatants of activated DC cultures were collected on day 6 and the concentration of IL-1β, IL-6, TNF-α and IFNβ was measured by ELISA. Mean ± SD values of 3 independent experiments performed with monocyte-derived DCs obtained from different donors are presented. Statistically significant changes (n=3) were calculated as described in the Materials and Methods.* ***(B)*** *Activation of the NF-κB and IRF3 pathways was detected in cell lysates of DC generated as described in Figure 22A, by phosphorylation of IκBα and IRF3 using Western blotting. Data of a representative DC donor out of three is shown.*

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

Type I IFN responses can be mediated by various PRRs localized either to the cytosol (RLRs) or to intracellular vesicular compartments (TLRs). To test the involvement of RLRs in IC31®-modulated production of IFNβ induced by polyI:C we silenced the RIG-I and MDA5 genes by specific siRNA (Figure 24A). Down-regulation of RLRs had no effect on polyI:C-induced IC31®-enhanced expression of IFNβ measured at both gene (Figure 24B) and protein levels (Figure 24D). Conversely, silencing of TLR3 (Figure 24A) resulted in dramatic abrogation of IFNβ mRNA and cytokine levels (Figure 24C, E) underlying the type I interferon-enhancing activity of IC31® acting through the vesicular TLR3-mediated signaling pathway (Figure 24B, D). This observation led us to hypothesize that IC31® primarily interacts with PRRs localized to the endo/lysosomal membrane of cDCs.

cDCs express TLR3, TLR7, TLR8 and TLR9 and previous studies demonstrated that in human moDCs IC31® co-localizes with TLR9-positive compartments (Aichinger et al. 2008). To check the possible booster effect of IC31® on IFNβ production we used CL075 and CpG2216 for triggering TLR7/8 and TLR9, respectively. Similar to TLR3-targeted stimulation, activation of TLR7/8 resulted in significantly increased IFNβ production in the presence of IC31® treated according to protocol B (Figure 24F). Interestingly, CpG2216 stimulation of DCs did not induce IFNβ production per se however, repeated pre-treatments by IC31® or ODN1a (protocol B) resulted in moderate secretion of IFNβ (Figure 24G). Thus IC31® was able to render TLR9 functional in moDCs and this effect could be attributed to the ODN1a component. As TLR9 gene expression was not affected by the adjuvant (data not shown) we propose that targeting IC31® or ODN1a to endosomal vesicles (Aichinger et al. 2008) ensures sufficient concentration of the CpG2216 ligand to trigger TLR9 in human moDCs.

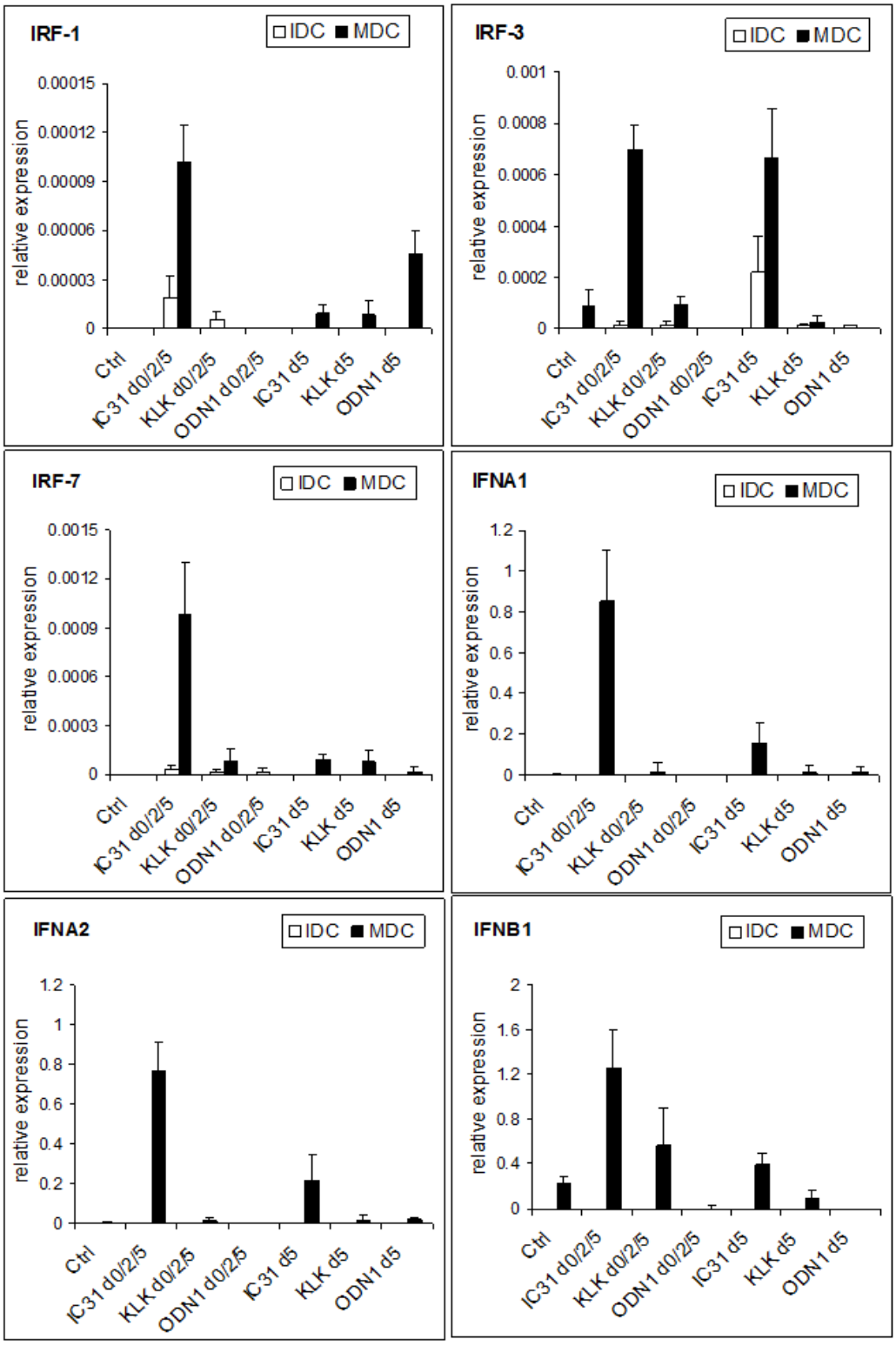

Figure 23. The effect of KLK, ODN1a and IC31® on the expression of IRF family transcription factors and type I interferons

Human monocyte-derived dendritic cells were differentiated for 5 days in the absence (control) or presence of KLK, ODN1a or IC31®, added on day 0, 2 and 5 (Protocol B) to the cultures or on day 5 only (Protocol C). Relative expression of IRF1, IRF3 and IRF7 as well as IFNA1, IFNA2, IFNB1 genes was measured in resting (empty bars) and 100ng/ml LPS + 10ng/ml IFNγ-activated (black bars) DCs by Q-PCR. Mean ± SD values of triplicate measurements were calculated from data of 3 independent donors.

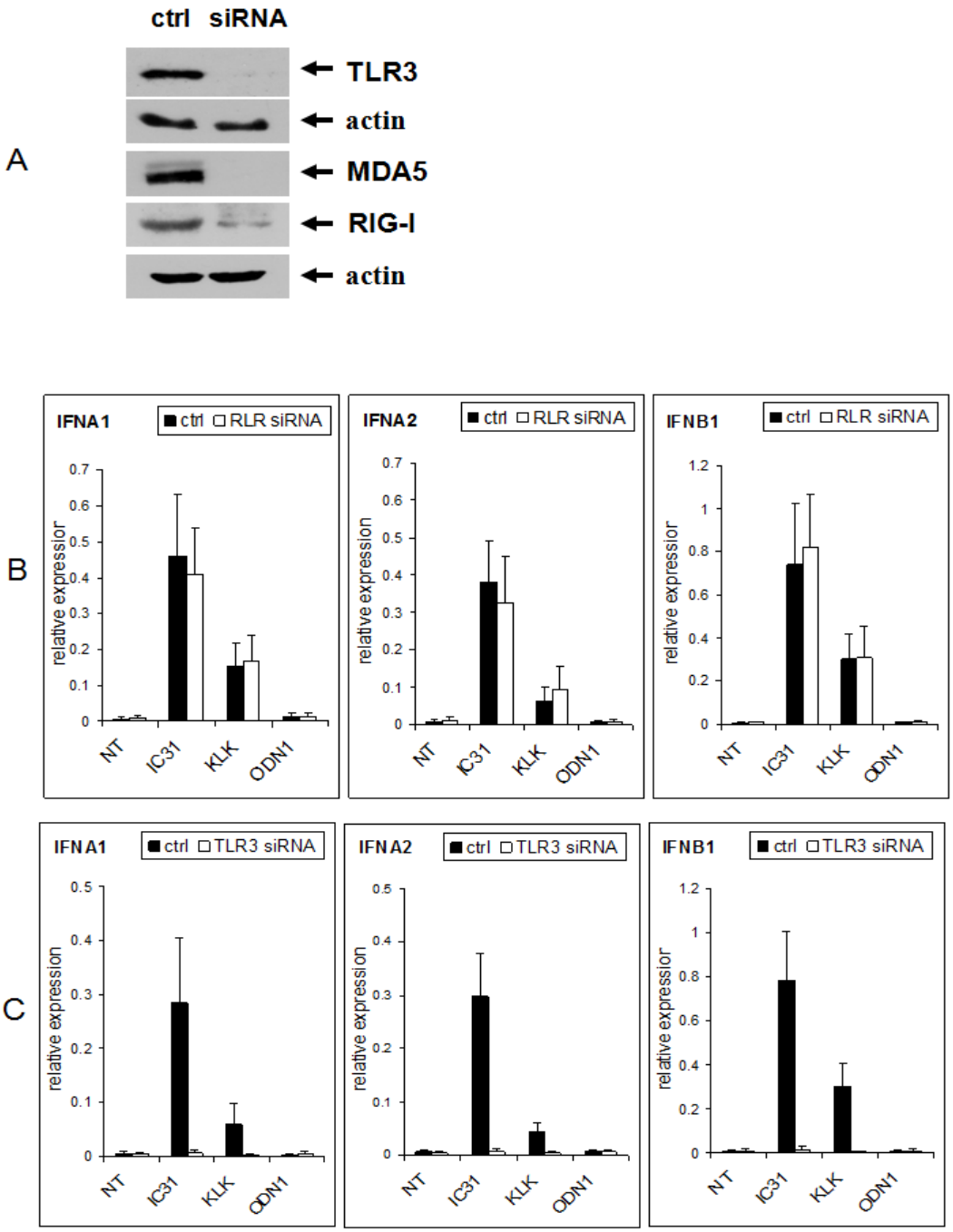

Figure 24. *(see figure legend below!)*

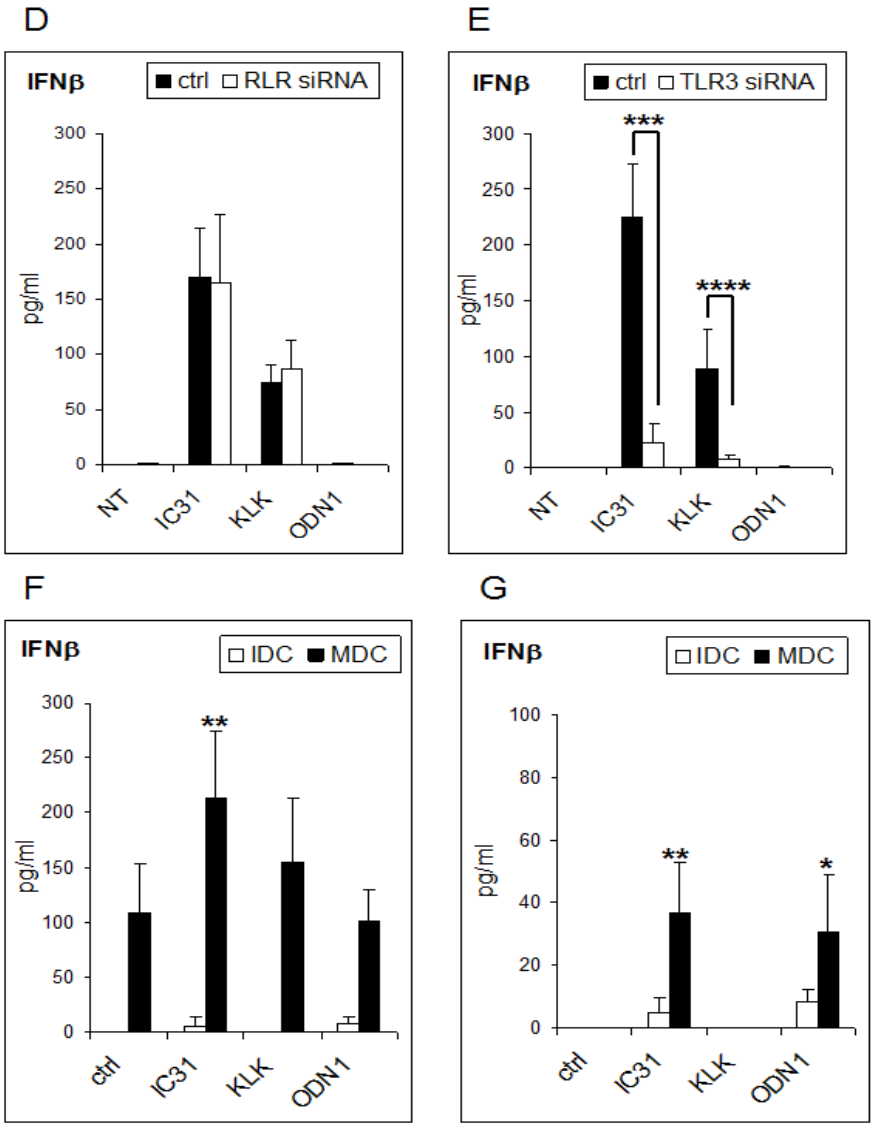

Figure 24. Identification of the receptors and signaling pathways involved in the enhancement of the type I interferon response by IC31®

Human moDCs were differentiated for 5 days in the absence (control) or presence of KLK, ODN1a or IC31® (Protocol B). In Figures 5B, 5C, 5D and 5E, moDCs were activated on day 5 by 20 µg/ml poly-I:C for 24 hrs. In Figure 5F DCs were subsequently activated by 1 µg/ml CL075 and in Figure 5G by 5 µg/ml CpG2216.

***(A)** Validation of siRNA activity specific for TLR3 and RLR (RIG-I/MDA5) by Western blotting.**(B)** Comparison of gene expression of type I interferons in control and RLR siRNA-treated DCs.*

***(C)** Comparison of gene expression of type I interferons in control and TLR3 siRNA-treated DCs.*

***(D)** Comparison of the levels of secreted IFNβ in control and RLR siRNA-treated DCs.*

***(E)** Comparison of the levels of secreted IFNβ in control and TLR3 siRNA-treated DCs.*

***(F)** Effect of TLR7/8 activation by CL075 on IC31®-, KLK- or ODN1a-treated DCs monitored by IFNβ ELISA.*

***(G)** Effect of TLR9 activation by CpG2216 on IC31®-, KLK- or ODN1a-treated DCs monitored by IFNβ ELISA. In Figures 5B – 5G mean + SD values of duplicates performed with DCs of two independent donors are shown.*

5. DISCUSSION

Persistent macrophage and DC activation by TLR ligands leads to powerful inhibitory mechanisms blocking further activation by the same or heterologous stimuli (Biswas and Lopez-Collazo 2009). Several inhibitory factors induced in response to TLR stimulation have been identified but it is still unclear, how these factors contribute to tolerance for further activation. Some pathways have been connected, like miR146a and IL-10 might both contribute to decreased IRAK1 expression (Chang et al. 2009; Bartz et al. 2006), but the present view supports several coexisting inhibitory pathways operating in activated DCs and macrophages. Whether these pathways are redundant, additive or synergistic or act at different conditions or time frames is yet to be understood. Since DCs developing from monocyte precursors in inflamed tissues might be particularly affected by the constant presence of microbial compounds and inflammatory mediators, we decided to study which inhibitory pathways are activated in moDCs in the presence of early and persistent TLR4 stimulation. Although not tested here, it is possible that certain inhibitory factors could modulate the expression of particular genes of DCs thereby inducing a qualitative tuning of cellular functions.

We found, that IRAK-1 downregulation occurring in moDCs receiving early activation signals through TLR4 during differentiation might by itself be sufficient to inhibit further activation through TLR, as demonstrated by the strong inhibitory effect of siRNA induced IRAK-1 downregulation on IL-12 secretion. We showed IRAK-1 downregulation and decreased MyD88-dependent signaling activity in response to early LPS activation in moDC development in the absence of any detectable change in the survival rate. Some activation stimuli, including zymosan, HKSA or CL075 inhibited the upregulation of CD1a and the downregulation of CD14 on a subset of the developing moDCs by day 2. Other factors like PAM3Cys, TNF or CD40L had on the other hand no effect on phenotypic moDC differentiation although these molecules were able to induce a functional moDC exhaustion. These findings suggest that downmodulation of TLR signal intensity during early moDC activation might induce tolerance to further activation irrespective of the differentiation stage of the cells. IRAK-M was recently identified as an important suppressive factor involved in endotoxin tolerance of murine bone marrow-derived DCs (Cole et al. 2012).

Most studies on macrophage or DC inactivation by persistent TLR stimulation have been limited to *in vitro* conditions. Endotoxin tolerance of monocytes has been

described in septic patients (Escoll et al. 2003; Manjuck et al. 2000) however, a broader significance of macrophage and DC exhaustion in response to persistent activation signals is still unknown. moDCs might be affected by the inhibitory signals originated from constant activation when differentiating in inflamed tissues. A recent study showed a very rapid DC differentiation of peripheral blood monocytes followed by their lymph node homing in mice that received LPS injections (Cheong et al. 2010). Circulating monocytes might thus differentiate into migratory DCs within a time frame short enough to preserve their full functionality. Such rapid differentiation was not observed when ligands for other TLRs were injected, suggesting that migratory DC differentiation from blood monocytes might be a mechanism specifically triggered by Gram-negative bacteria. Other data however showed that intradermal injection of Gram-negative bacteria or LPS to mice blocked the differentiation of migratory DCs from monocyte precursors (Rotta et al. 2003). These data suggest that migratory DC differentiation in the peripheral tissues might be impaired if the activation signals reach the monocyte precursors before their commitment to the DC differentiation pathway. Our data support this hypothesis by showing that activation of early moDC precursors leads to inflammatory cytokine and chemokine production but the cells, at early stage of DC differentiation, have a limited ability to modulate their chemokine receptor expression required for lymph node homing. The cytokine producing ability of the developing inflammatory moDCs can be terminated by the functional exhaustion before the cells differentiate to mature DCs capable of reprogramming their chemokine receptor profile. Early activation of developing moDCs may thus set the threshold of DC migration to LNs, thereby limiting the continuous transfer of inflammatory signals to T lymphocytes.

Identification of DC subsets with specific functions has recently emerged as a new challenge of DC biology. DCdeveloping under inflammatory conditions support protection against pathogens, while DC subsets differentiating under steady state play a role in the control and resolution of inflammation and tissue destruction (Heath and Carbone 2009; Merad and Manz 2009). This functional divergence is determined by the destination site of DC precursors and the subtype-specific and compartmentalized expression of PRRs. In contrast to the restricted expression of nucleotide recognizing TLR (Barton and Kagan 2009), RLH specialized for the recognition of shorter or longer dsRNA are present in multiple cell types (Takeuchi and Akira 2008).

In our study we compared the expression and functional activity of RLH family members in two previously characterized human moDC subsets distinguished by the expression and activity of PPARγ that controls the expression of type I CD1 molecules (Szatmari et al. 2004). The $CD1a^+$ and $CD1a^-$ moDC subtypes were shown to differ in the uptake of apoptotic cells (Majai et al. 2010) and their potential to provoke inflammatory T lymphocyte responses (Gogolak et al. 2007) and has been shown to ameliorate immune pathology in influenza virus infection (Aldridge et al. 2009). To assess the functional importance of $CD1a^+$ and $CD1a^-$ moDC subsets in the RLH-mediated inflammatory cytokine and type I IFN responses, and their role in priming influenza virus-specific T cell responses we performed *in vitro* and *ex vivo* experiments. Our results show that the baseline expression of RIG-I/MDA5, IRF3 and IFNβ, key elements of the downstream signaling cascade, are significantly higher in $CD1a^+$ cells than in their $CD1a^-$ counterparts. Activation of these DC subsets by specific ligands, *i.e.* pI:C or influenza virus revealed that the coupled signaling machinery is more active in the $CD1a^+$ moDC subset than in $CD1a^-$ cells. These results altogether show that in human moDC the RLH cascade acts in a subtype specific manner. Furthermore, the results of our siRNA experiments revealed that in the inflammatory $CD1a^+$ moDC subset, the TLR3 – NF-κB and RLH – IFNβ pathways control the production of different sets of cytokines and due to their subset-specific activity work independently. The putative *in vivo* role of the $CD1a^+$ RLH expressing DC subtype is suggested by the appearance of these cells in tonsils and reactive lymph nodes.

It has recently been shown that the cross-talk of human pDC and moDC subtypes is essential for optimal antiviral immune responses (Kramer et al. 2010). TLR3 and RLH have also been shown to have different yet complementary functions upon pI:C-induced virus-specific CTL responses in mice, where TLR3 was shown to be critical for priming, whereas MDA5 for supporting $CD8^+$ memory responses. Moreover, simultaneous pI:C triggering of TLR3 and RLH in cDCs combined with RLH activation in NK cells is required for potent IFNγ responses and elimination of viruses (Perrot et al. 2010). A global quantitative proteomic approach has recently been utilized to demonstrate the DC subset-specific expression of TLR, NLR and RLH and components of the related signaling cascades in murine viral infections (Luber et al. 2010). The authors identified splenic $CD4^+$ and double negative DCs specialized to viral recogition by RIG-I/MDA5 instead of the $CD8\alpha^+$ DC type with cross-presenting potential. Supporting the interpretation of these authors, we also suggest that due to the high basal

levels of the RLH sensors and the major components of the coupled signaling pathways, human $CD1a^+$ DC exhibit a sensitized state for priming virus-specific immune responses, whereas $CD1a^-$ cells remain incompetent for these functions.

The human homologue of mouse $CD8\alpha^+$ DC with efficient T cell priming activity has recently been identified and characterized by the expression of the C-type lectin DNGR-1/CLEC-9A, the chemokine receptor XCR1, high expression of TLR3 and efficient cross-presentation (Bachem et al. 2010; Poulin et al. 2010). This DC type, also known as BDCA3 DC, is considered as an independent lineage based on the expression of IRF8 and BATF3 transcription factors. However, this minor cell population does not express RIG-I or MDA5 (Hildner et al. 2008). Hence, we conclude that the $CD1a^+$ migratory moDC subset with the unique potential to activate IFNβ secretion and anti-viral cellular immune responses is a unique human DC subset specified by high CD1a and low PPARγ expression. As the regulation of CD1a and CD1c gene and protein expression, controlled by PPARγ is tightly linked (Majai et al. 2010) this interpretation is supported by recent data showing that blood $CD1c^+$ DC display the capacity of secreting of high amounts of IL-12 (Jongbloed et al. 2010).

Human CD1 proteins are closely linked to the presentation of mycobacterial lipid, glycolipid, and lipopeptide antigens for T lymphocytes (Felio et al. 2009; Vincent et al. 2003). Interestingly, the largest proportion of transcripts changed in *M. tuberculosis* infection was recently found among neutrophil-dependent IFN-inducible genes presenting a typical signature that correlates with disease severity (Berry et al 2010). Thus the expression of CD1a in human DCs marks a subset specialized for the recognition of and protection against intracellular pathogens through the elevated baseline expression of cytosolic RLH sensors and components of the coupled signaling machinery, which support type I interferon responses and polarization to inflammatory T lymphocyte differentiation.

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

The distinct functional attributes of $CD1a^+$ and $CD1a^-$ moDC subsets may result in reasonable functional complementarity based on their inflammatory and phagocytic activities. At the early stage of infection bone marrow derived $CCR2^+$ monocytes and monocyte-derived TNF/iNOS producing DCs rapidly act as potent local APCs for $CD8^+$ T cells, but upon infection by highly pathogenic influenza viruses, their recruitment correlates with immune pathology (Aldridge et al. 2009). In a mouse model of influenza virus infection the major source of IL-12 was associated to a monocyte-derived DC subset that enters inflamed lymph nodes directly from the blood guided by CCR2 (Nakano et al. 2009). In our model we suggest that dead infected epithelial cells can efficiently be internalized by resident, highly phagocytic but less inflammatory PPARγ-positive $CD1a^-$ DC that may use TLRs for the recognition of nucleotides in engulfed apoptotic cells and confer immunomodulatory effects (Majai et al. 2010). In contrast, sensing dsRNA and triggering IFNβ responses through RIG-I and MDA5 is mediated by IL-12p70 producing inflammatory $CD1a^+$ moDCs that are able to provoke CTL activation against viruses and other intracellular pathogens. Cheong *et al* have recently shown that repeated inoculation of mice with LPS results in the migration of circulating monocytes to lymph nodes and their rapid differentiation to DC-$SIGN^+$ moDC. Since these cells act as effective APCs with strong cross-presenting capacity they are considered as "authentic" DCs (Cheong et al. 2010). As human moDCs are widely used in various clinical settings and considered as gold standards of DC biology, we propose that identification of human moDC subsets with specialized functions may have an important impact on designing vaccines against viruses and tumor cells.

Provoking potent cellular immune responses against intracellular pathogens, which often cause persistent infections, requires the priming and/or boosting of inflammatory T-lymphocytes by properly activated DCs (reviewed by Palucka et al. 2010). Analyzing the response of human monocytes and differentiating moDC to IC31® we found that i) IC31® was efficiently accumulated in human blood-circulating monocytes and moDCs; ii) in the presence of IC31® the generation of inflammatory $CD1a^+$ DCs was inhibited, and failed to induce phenotypic changes of DC activation while decreased the secretion of TNF-α and IL-6 cytokines with a concomitant increase of IFNβ secretion; iii) long term presence of IC31® prevented IκB-α phosphorylation but extended the phosphorylated state of IRF3 indicating their independent activation; iv) the IC31® adjuvant exhibited a booster effect on ligand-induced vesicular TLR-mediated induction of IFNβ secretion.

The adjuvant effect of IC31® has already been characterized (Agger et al. 2006; Kamath et al. 2008a,b; van Dissel et al. 2010, 2011) and its intracellular localization (Aichinger et al. 2011) and role in TLR9-dependent moDC activation has also been shown (Pilz et al. 2009). However, complete understanding of its mode of action and its effects on PRR-coupled signaling cascades in human moDCs has not been analyzed sofar. To assess the long and short term *in vitro* effects of IC31 on moDC differentiation we set up different treatment protocols. The experimental data revealed that IC31® and its KLK component inhibited the transition of $CD1a^-$ cells to $CD1a^+$ moDCs and interfered with moDC activation. As a functional consequence these cells were unable to exhibit their typical phenotypic and functional changes upon moDC activation. This semi-mature differentiation and activation state of moDCs was associated with inactive IκB-α with the unusual capability to secrete high amounts of IFNβ. Thus IC31® could be identified as a moDC-modulatory adjuvant that has a profound effect on the balance of NF-κB and IRF3-mediated signaling pathways and thus fails to induce pro-inflammatory cytokine secretion. Further analysis of the type I interferon and NF-κB-mediated signaling pathways demonstrated that both IC31® and KLK increased type I interferon responses mediated by vesicular TLRs. This modulatory effect could be attributed in part to KLK, which facilitates the transport and accumulation of TLR ligands to the endosomal compartment to stimulate resident TLRs. Although these TLRs may interfere with each other's functional activities (Berghofer et al. 2007; Butchi et al. 2010), our results point to the synergistic action of IC31® and TLR-induced interferon signaling and secretion. Besides increasing the magnitude of adaptive immune responses, adjuvants also play an instructive role in driving immune responses to the most appropriate directions to confer protection against pathogens (Pulendran et al. 2010). TLR activation inducing lysosome maturation is a crucial requirement of antigen presentation by DCs, efficient $CD4^+$ T cell activation and Th1 polarization (Blander and Medzhitov 2006; Joffre et al. 2009). As DCs produce increased levels of IFNβ when IC31® is co-administered with endosomal TLR-ligands we propose that augmented type I IFN responses known to facilitate cross-presentation may potentiate both $CD4^+$ and $CD8^+$ T cell responses. We also propose that IC31® is an effective adjuvant of microbial proteins acting through the endosomal TLR system of moDCs and thus offers a potent tool for future vaccination strategies against intracellular pathogens and cancer.

6. SUMMARY

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

We found, that:

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

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

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

ÖSSZEFOGLALÁS

A veleszületett immunitás a behatoló kórokozókkal és a szervezetben képződő káros anyagokkal szembeni védelem filogenetikailag legősibb formája. Működését az **idegen és saját**, valamint az **ártalmatlan és veszélyes** molekulák *felismerése*, a védelmi reakciót kiváltó *jeltovábbítás* és a *végrehajtó sejtek összehangolt mozgósítása* biztosítja. A veleszületett immunitás fontos résztvevői az ezeket a feladatokat ellátni képes dendritikus sejtek (DS), melyek folyamatos "őrszemként" működve biztosítják a többsejtű szervezetek immunológiai felügyeletét és a megfelelően szabályozott válaszfolyamatok elindítását. A hivatásos antigen bemutató sejtek (APS), köztük a monociták, makrofágok és DS-ek kiemelt szerepet játszanak a védelmi mechanizmusok szabályozásában és koordinálásában. Munkánk során a DS-ek által kifejezett Toll-szerű és a RIG-szerű mintázatfelismerő receptorok (TLR és RLR) szerepét tanulmányoztuk emberi DS populációk gyulladásos és I. típusú interferon válaszának szabályozásában. Az két komponensű IC31® adjuváns szerepét emberi fehérvérsejtekben és monocita eredetű DS-ekben tanulmányoztuk.

Kimutattuk, hogy

- a TLR4-közvetítette, LPS által kiváltott IRAK1 kifejeződés csökkenése önmagában elegendő a moDS-ek funkcióinak tartós gátlásához;
- a sejtaktiváció által kiváltott negatív visszacsatolási mechanizmusokat elemezve kimutattuk, hogy csak a fejlődő és még nem véglegesen differenciált moDS-ek esetében a korai TLR4 receptoron keresztüli aktiválás a gyulladáskeltő citokinek átmeneti és korlátozott megjelenését váltja ki, ami a végrehajtó funkciók szabályozott csökkenéséhez vezet, és a szövetközi vándorlásra nem képes állapot megőrzése követ;
- eredményeink szerint az RLR gének és fehérjék, valamint a kapcsolódó jelátviteli útvonalak elemeinek kifejeződése szignifikánsan magasabb a $CD1a^+$ DS-ekben, mint a fenotípusos tulajdonságaik és funkcionális aktivitásuk alapján is különböző $CD1a^-$ altípusban;
- a RIG-I és MDA5 gének csendesítésével végzett kísérleteink az RLR-ok által közvetített jelátvitel fontosságát igazolták a $CD1a^+$ DS-ek általi naïve $CD8^+$ T-sejtek aktiválásában;

- kísérleti eredményeink az RLR rendszer DS altípus-specifikus működését igazolták, és feltárták az ennek hátterében álló molekuláris mechanizmusokat, a $CD1a^+$ DS-eket egyedi funkciókkal rendelkező, gyulladásos sejttípusként azonosítottuk;
- azt is bebizonyítottuk, hogy a vándorló képességgel rendelkező $CD1a^+$ DS-ek emberi nyirokszövetekben is kimutathatóak;
- a két komponensű IC31® adjuváns a vérben keringő monocitákban, $MHC\text{-}II^+$ sejtekben és moDS-ekben halmozódik fel;
- az IC31® jelenlétében differenciálódó DS-ekben gátolt a $CD1a^+$ sejttípus differenciációja, a gyulladásos citokinek szekréciója, miközben a sejtek IFNβ termelése fokozódik;
- az IC31® adjuvánst olyan – a DS-ek funkcionális sajátságait módosító – oltóanyag komponensként azonosítottuk, amely az NF-κB és IRF3 jelátviteli útvonalak egyensúlyának befolyásolásával révén moDS-eben fokozza az IFNβ citokin termelését, miközben az NF-kB jelpálya és a gyulladáskeltő citokinek megjelenése gátolt;
- azt is igazoltuk, hogy az IFNβ termelést fokozó aktivitás a moDS-ek sejten belüli vezikuláris rendszerében halmozódó TLR-okon keresztül valósul meg.

Kutatási eredményeink a membránhoz kötött és a citoplazmában elhelyezkedő mintázatfelismerő receptorok közti funkcionális munkamegosztás jelentőségére hívják fel a figyelmet. Ennek gyakorlati jelentőségét a DS altípusok funkcióinak célzott módosítási lehetőségei és a sejtes immunválasz irányított szabályozása jelenti, aminek nagy jelentősége van a DS-ek megelőző és terápiás célú gyakorlati felhasználásában.

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7.2 Publication list prepared by the Kenézy Life Sciences Library

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Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

*1. Fekete, T., **Szabó, A.**, Beltrame, L., Vivar, N., Pivarcsi, A., Lányi, Á., Cavalieri, D., Rajnavölgyi, É., Réthi, B.: Constraints for monocyte-derived dendritic cell functions under inflammatory conditions.
Eur. J. Immunol. 42 (2), 458-469, 2012.
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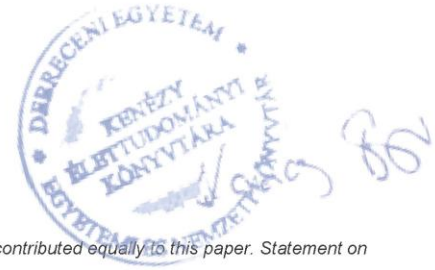

**The article dually serves as the basis of dissertation, These authors contributed equally to this paper. Statement on the partial use of article was issued on Mai 12, 2012*

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List of other publications

3. **Szabó, A.**, Osman, R.M., Bacskai, I., Kumar, B.V., Agod, Z., Lányi, Á., Gogolák, P., Rajnavölgyi, É.: Temporally designed treatment of melanoma cells by ATRA and polyI.
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DOI: http://dx.doi.org/10.1515/BC.2008.054
IF:3.035

Total IF: 15.317

Total IF (publications related to the dissertation): 10.095

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

16 October, 2012

8. KEYWORDS

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

TÁRGYSZAVAK

Dendritikus sejt, Toll-szerű receptor, RIG-I-szerű receptor, NF-κB, IRF3, jelátvitel, veleszületett immunitás

9. ACKNOWLEDGEMENTS

I would like to thank my supervisor Prof. Éva Rajnavölgyi for her continuous support, excellent professional mentorship, and inspiration during my entire Ph.D. and *pre-doc* period. I am grateful for the opportunity to train and work in her department of scientific excellence.

I am thankful for Dr. Árpád Lányi, Dr. Attila Bácsi, Dr. Bence Réthi, Dr. Szilvia Benkő, and Dr. Péter "Gogy" Gogolák for their valuable suggestions, constructive advices, and the inspiring technical and non-technical discussions during my years at the Department of Immunology.

Special thanks to Erzsike Nagyné Kovács and Ágota Veres, who were always extremely kind, eager to help, and provided me excellent technical assistance and work support.

I wish to thank my good friend and colleague Brahma V. Kumar for critically reviewing this dissertation, and especially my dear students, Rolah M. Lønning Osman (Gen. med.), Rafael Abulafia (Gen. med.), Krisztián Horváth (Gen. med.), Behzad Nadianmehr (Gen. med.), and Krisztián Bene (B.Sc. and M.Sc.) for their techincal help, "tea-times", and for all the happy moments.

I would like to thank the whole staff of the Department of Immunology (University of Debrecen, MHSC) for the supporting and friendly environment.

And last, but not least I am sincerely grateful to my family and to all of my dear friends helping me get through the difficult times, and for their emotional support and encouragement.