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Molecular Analysis of Pattern Recognition Receptors Involved in Sensing of Human Rhinoviruses by Dendritic Cells

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Abstract

Human rhinovirus (HRV) infections are the most prevalent cause of the common cold but may also induce a predisposition for bacterial infections as well as exacerbations of asthma. The pathogenic mechanisms of HRV infections leading to such complications are still poorly understood.

Dendritic cells (DC) are the main professional antigen presenting cells of the immune system. With the help of viral pattern recognition receptors (VPRR) DC can recognize evolutionary highly conserved molecular patterns of viruses. Once activated, the resulting mature DC are capable of inducing adaptive immune responses.

HRV, are small, icosahedral, nonenveloped (+)-strand single-stranded RNA (ssRNA) viruses. We have previously shown that HRV14, a member of the major group rhinoviruses, specifically down-modulates the accessory function of monocyte-derived-DC (DC). HRV14-treated DC do not mature and show a strongly diminished T-cell stimulatory capacity due to the upregulation of inhibitory surface molecules.

HRV do not replicate in DC. However, DC are well equipped with different single-stranded viral RNA (ssvRNA) detecting VPRR. Therefore, we wondered whether the genomic RNA of HRV alone, as a potential danger signal, has the capacity to induce DC maturation.

This study shows that HRV ssvRNA is recognized by DC, causing a strong Type I interferon (IFN) response. A profound IFN induction was also observed in a luciferase reporter assay using RIG-I-transfected HEK cells, identifying RIG-I as a potential VPRR for HRV RNA. However, in DC, in contrast to HEK cells, ssvRNA failed to induce NFκB activation, which is required for proper maturation induction. Furthermore, markers of maturation like the surface molecule CD83 were absent, and the proinflammatory cytokines TNFα, IL-12 and IL-6 were not produced. In addition, HRV ssvRNA failed to induce an increased T-cell stimulatory capacity in an allo-mixed leukocyte reaction. Therefore, ssvRNA of

HRV is only a *semi-danger signal* for DC: Despite recognition, ssvRNA fails to induce proper DC maturation.

Zusammenfassung

Infektionen durch humane Rhinoviren (HRV) sind die häufigste Ursache für Erkältungen. Sie bedingen aber auch eine Prädisposition für bakterielle Infektionen sowie Verschlimmerungen von Asthmaanfällen. Die pathogenen Mechanismen von HRV Infektionen, die jene Komplikationen verursachen, werden hingegen schlecht verstanden.

Dendritische Zellen (DC) sind die wichtigsten Antigen-präsentierenden Zellen (APC) des Immunsystems. Mit Hilfe von *viral pattern recognition* Rezeptoren (VPRR) können DC evolutionär hoch konservierte molekulare Muster von Viren erkennen. Die nach solcher Aktivierung resultierenden reifen DC besitzen die Fähigkeit, eine adaptive Immunantwort einzuleiten.

HRV sind kleine, ikosahedrische, nicht-Membran-umhüllte (+)-Strang Einzelstrang RNS (ssRNA) Viren. Wir haben kürzlich gezeigt, dass HRV14, ein Mitglied der *major group HRV*, die akzessorische Funktion von DC herunterreguliert. HRV14 behandelte DC reifen nicht und weisen durch die Expression inhibitorischer Oberflächenmoleküle eine stark herabgesetzte Kapazität zur T-Zell Proliferationsstimulation auf.

HRV replizieren in DC nicht. DC sind aber mit unterschiedlichen VPRR ausgestattet, die virale ssRNA (vssRNA) erkennen. Aus diesem Grund war unsere Fragestellung, ob die genomische RNS von HRV alleine - als potentielles *danger signal* - die Kapazität hat, DC Reifung zu induzieren.

Diese Studie zeigt, dass HRV ssvRNA durch DC erkannt wird, was zu einer starken Typ 1 Interferon (IFN) Antwort führt. Diese IFN Induktion wurde auch in einem *Luciferase Reporter Assay* unter Verwendung von RIG-I transfizierten HEK Zellen gefunden. Dieses Ergebnis identifiziert RIG-I als potentiellen VPRR für HRV RNA. Dennoch konnten wir in DC, im Gegensatz zu HEK Zellen, keine NFκB Aktivierung, die für die DC Reifung wesentlich ist, durch ssvRNA feststellen. Weiters wurden typische Reifungsmarker wie CD83, sowie die Freisetzung der proinflammatorischen Cytokine TNFα, IL-12 und IL6 nicht induziert. Letzlich konnte ssvRNA in einer *allo-mixed leukocyte reaction* keine

erhöhte T-Zell stimulatorische Kapazität von DC bewirken. HRV ssvRNA ist also nur ein *semi-danger Signal* für DC: Trotz Erkennung, induziert ssvRNA keine DC Reifung.

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Abbreviations

APC	antigen presenting cell
ARD	ankyrin repeat domain
CD	cluster of differentiation
CLR	C-type lectin receptor
cSMAC	central supramolecular complex
Ctrl.	control
DC	dendritic cell
cDC	conventional dendritic cells
dephospho.	dephosphorylated
dsRNA	double-stranded ribonucleic acid
eIF	eukaryotic initiation factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HEK	human embryonic kidney cells
HRV	human rhinovirus
ICAM	intercellular adhesion molecule
IFN	interferon
IκB	inhibitor of κ-B
IKK	IκB kinase
IL	interleukin
IRES	internal ribosome entry site
IRF	interferon regulatory factor
IS	ummunological synapse
ISRE	interferon signaling response element
JAK	Janus kinase
LDL	low density lipoprotein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MDA-5	melanoma-differentiation-associated gene 5
md-DC	monocyte-derived dendritic cell

MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MNC	mononuclear cells
NFκB	nuclear factor κ-B
NK	natural killer cells
mRNA	messenger ribonucleic acid
NFW	nuclease free water
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cells
pI:C	polyriboinosinic:polyribocytidylic acid
PKR	protein kinase R
phosho.	phosphorylated
pSMAC	peripheral supramolecular complex
PRR	pattern recognition receptor
RHD	rel homology domain
RIG-I	retinoic acid-inducible gene I
RT	room temperature
Sn	sialoadhesin
ssRNA	single-stranded ribonucleic acid
ssvRNA	single-stranded viral ribonucleic acid
STAT	signal transducer and activator of transcription
Th	T helper cell
TGF	tumor growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	T regulatory cells
UTR	untranslated region
VP	viral protein
VPRR	viral pattern recognition receptor

1. Introduction

1.1. DC and their functions

The human immune system is constituted by an innate as well as an adaptive component. The innate immune system is the first line of host defense against pathogens, whereas the adaptive immune system is important for removing pathogens during later phases of infection. Only concerted actions of both systems allow an efficient host defense response against invading pathogens. Adaptive immunity is charaterized by the capability to rearrange immunoglobulin genes to produce a large repertoir of antigen-specific receptors and the establishment of an immunological memory. The innate immune system, on the other hand, expresses limited numbers of germ-line encoded PRR, which have evolved to recognize highly conserved molecular patterns, thereby providing a kind of *inherited memory function*.[1]

DC link these two parts of the immune system by capturing and presenting antigens resulting in the initiation of adaptive immunity.[2]

1.1.1. DC have immunogenic properties

DC are a heterogenous population of bone-marrow-derived immune cells. Multiple subtypes of DC with different life span and immune function have been identified. In the absence of infection, the steady state, plasmacytoid DC (pDC), which are specialized on the production of high amounts of Type I IFN, and conventional DC (cDC) are found in the nonlymphoid tissues, in the circulation, and the lymphoid tissues (thymus, spleen, lymph nodes). Owing to their heterogenicity, also the resident cDC in lymphoid tissues consist of phenotypically different subsets. According to their tissue localization, cDC can be classified into skin DC in the epidermis (Langerhans cells) or dermal DC in the dermis; lymphoid-tissue associated DC; mucosal tissue-associated DC; and interstitial DC located, for instance, in liver and lungs.[3]

Soon after the discovery of DC by Steinman et al. in 1972 ,[4] it became clear that this cell type plays a central immunogenic role as professional antigen presenting cell (APC). In an experimental system for graft rejection, the mixed leukocyte reaction (MLR), DC were found to be 100 times more potent in inducing allogeneic T-cell proliferation compared to other cell types like macrophages, B-cells or monocytes.[5,6] More importantly DC are the most versatile APC because they are the only cell type capable of inducing a primary immune response, resulting in the proliferation of naive T-cells and thereby the induction of an adaptive immune response. This finding defines DC as key players in immunogenicity, which is the capacity of inducing an immune response. [7-9]

1.1.2. The maturation model
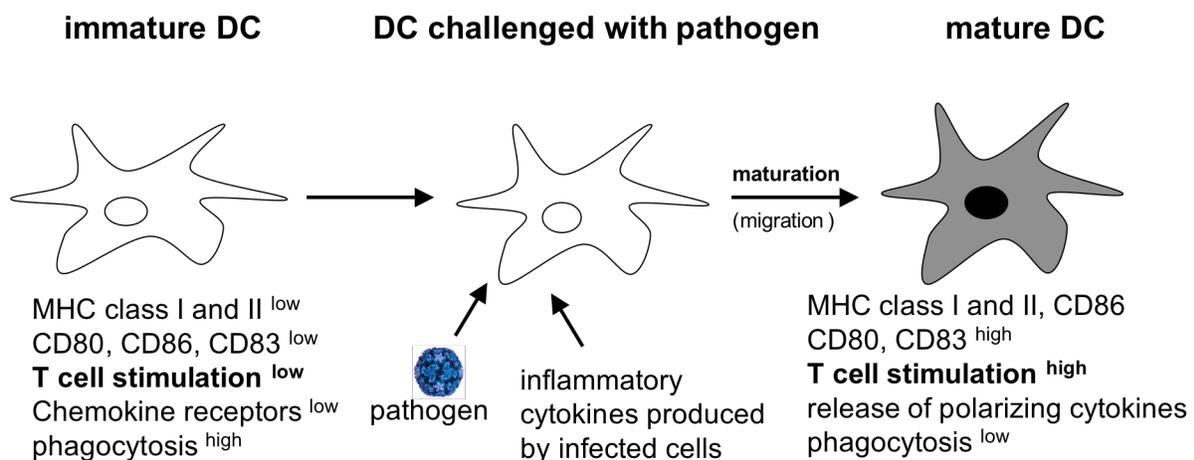
**Fig. 1 The maturation model (adapted from [10])**

DC activation

DC are present as immature precursors either in the peripheral tissues, blood or secondary lymphoid organs. The T-cell stimulatory function of DC is however not a constitutive property.[11] It is rather primed by inflammatory cytokines like TNF, IL-1, or interferons produced during infection. However, for the induction of DC

maturation exogenous stimuli like microbial products (f.i. LPS, CpG, dsRNA) are required additionally.[12,13] DC sense these danger signals by pattern recognition receptors (PRR) which are specialized on detecting a broad spectrum of pathogen-associated molecular patterns (PAMP). Once receiving these activation signals a maturation program is started, which co-ordinates many phenotypical and functional changes, involving antigen capture and presentation, migration, costimulation, production of T-cell polarizing cytokines and prolongation of DC life-span (see Fig. 1).[14,15]

Migration to the local lymph nodes

During maturation DC lose their responsiveness to inflammatory chemokines like CCL3, CCL5, or CCL20 through receptor downregulation or desensitization. On the other hand, the upregulation of CCR7, the lymph node-homing chemokine receptor, allows the response to homeostatic chemokines (CCL19, CCL21). As a consequence, those DC residing in the periphery or blood start migrating to the T-cell areas of local draining lymph nodes.[16] Furthermore, in the presence of GM-CSF, which levels rise during inflammation, monocytes residing in the blood can start to differentiate into monocyte-derived DC (md-DC) upon migration into the peripheral tissue.[17]

Decreased phagocytic capacity and enhanced antigen presentation

Immature DC show high levels of constitutive macropinocytosis and phagocytosis, which allow them to constantly sample the surrounding area for pathogen entry. Furthermore, they express endocytic receptors for pathogens like the mannose receptor, DEC-205, or DC-SIGN allowing their specific uptake. In contrast, mature DC, activated via PRR signaling, shut off their high endocytic rates and switch to antigen processing and presentation. The synthesis and transport of MHCII molecules ensures that DC can present degraded extracellular antigens in a MHCII-antigen complex to the antigen-specific T-cell receptor (TCR) on naive CD4+ T-cells. Furthermore, DC are capable of cross-presenting degraded exogenous antigens. This is achieved by releasing these

antigens into the cytosol, where they are degraded by the proteasome. After re-uptake into the phagosome, they can assemble with MHCI, which normally only presents intracellular endogenous antigens, and travel to the plasma membrane. These complexes can then interact with specific TCR on CD8+ T-cell. The low affinity interaction of MHC-antigen complexes with specific TCR is also refered to as signal 1 of antigen presentation. [9,18]

Costimulation and the immunological synapse (IS)

In naive T-cells low doses of antigen presented by MHC molecules on DC are not sufficient to trigger TCR signaling. For this purpose, costimulatory interactions (refered to as signal 2) between DC and T-cell are required. In order to allow sustained receptor stimulation and signal amplification, multiple DC and T-cell interactions are required.[9]

The original model, based on experiments with B-cell - T-cell interactions, proposed that an immunological synapse (IS) was needed to achieve this goal: The IS is a multimolecular complex which results from a polarized interaction between T-cell and APC. It organizes the recruitment and segregation of cell-surface receptors and intracellular adaptor and signaling molecules.[19]

The IS is formed of an outer ring, the peripheral supramolecular activation complex (pSMAC), allowing adhesion molecule interactions between ICAM-1 (on DC) and LFA-1 (on T-cells) providing cell-cell attachment and a central region, the central SMAC (cSMAC), enriched in MHC-antigen (DC) and TCR (T-cell) complexes. In addition, as a consequence of maturation, DC also express the costimulatory molecules CD80 and CD86 in this inner ring. These molecules bind to the T-cell surface molecule CD28 thereby boosting TCR signaling. [20]

Recently the model of a static IS required for T-cell activation by DC in contrast to B-cells has been challenged: It appears that DC induced TCR signaling occurs rather in peripheral microclusters allowing MHC-antigen-TCR and CD80/CD86-CD28 interactions. These microclusters, constituting a multifocal IS, are formed before the cSMAC is assembled. The latter, however, seems to act in TCR recycling rather than T-cell activation. This is also supported by the presence of

CD45, a potent phosphatase of effector molecules, in the cSMAC.[21] The cSMAC may also be required for secondary inhibitory signaling pathways which are upregulated after TCR triggering, including ICOS and CTLA-4 activation by the DC ligands ICOS-L and CD80 / 86.[22]

In support of this dynamic IS model in DC, segregation of CD80/86-CD28 interactions from MHC-antigen-TCR complexes was shown to enhance costimulation. This seems to be achieved by a dynamic regulation of the DC actin cytoskeleton, which acts to form selective barriers in the plasma membrane of DC. This mechanism may also prevent MHC-antigen-TCR clusters from migrating into the cSMAC region, thereby delaying inactivation of the signal cascade induced by MHC-antigen-TCR interactions. Taken together, T-cell activation by DC is achieved by sustained microcluster formation, which involves multiple MHC-antigen-TCR and costimulatory CD80/86-CD28 interactions.[21]

Recently it was found that costimulation is more complex than originally anticipated. Apart from the classical CD28-CD80/86 interaction, additional costimulatory interactions are required to allow effective long-lasting T-cell responses or the generation of memory T-cells. These additional interactions are carried out by members of the tumor-necrosis factor receptor (TNFR) family: The mainly inducible receptor / ligand pairs are thought to modulate the quantity, quality and duration of immune responses. Receptor / ligand pairs crucial for additional costimulation include CD40 ligand (CD40L) / CD40, OX40- OX40L, 4-1BB / 4-1BBL, CD27 / CD70, and herpes-virus entry mediator (HVEM) / LIGHT.[23]

Notably CD40L expressed by activated T-cells is a key regulator of T-cell costimulation since interaction with increased levels of CD40 on mature DC upregulates the expression of costimulatory molecules like CD80/86, 4-1BBL, OX40L and CD70,[24] enhances antigen presentation and IL-12 production.[25]

On T-cells CD27 and HVEM are constitutively expressed, whereas the expression of OX40, 4-1BB, and CD40L is induced during T-cell activation. Noteably, HVEM is downregulated by T-cell activation and its ligand LIGHT on

DC is also no longer expressed by mature DC indicating a role during early activation of T-cells and APC.[23]

To summarize, members of the CD28-B7 costimulatory family cannot fully account for effective long-lasting T-cell responses. However, all costimulatory TNFR-family molecules are potentially available at the time of antigen encounter or within several days after. According to a recent model they control the absolute number of effector T-cells which accumulate during a primary response, thereby determining whether a protective T-cell response can occur.[23,24]

1.1.3 DC tune T-cell responses

DC, as the main APC inducing primary immune responses, do not only start adaptive immunity, they also shape T-cell polarity. The capability of DC to fine-tune T-cell responses allows DC to induce appropriate adaptive immune responses adjusted to the kind of invading pathogen.[26]

Specific T-cell responses are mainly achieved by the selective release of polarizing cytokines by DC. IL-12 induces a Th1 response required for fighting intracellular pathogens like viruses and bacteria whereas IL-4 release causes differentiation into Th2 cells, which are critically involved in immune responses against helminths and extracellular toxins.[27] Furthermore, TGFβ and IL-2 induces regulatory T-cells, which are essential for maintaining peripheral immune tolerance, and TGFβ in combination with IL-6 induce Th17 polarization. These cells have been shown to be involved in autoimmune diseases.[28,29]

1.1.4. DC are involved in tolerogenicity

There is strong experimental evidence that DC are also key players in the induction and maintainance of peripheral tolerance. It seems that in the steady state, which means in the absence of deliberate exposure to maturation signals, most DC fail to deliver adequate costimulatory signals which can tolerize peripheral CD4$^+$ and CD8$^+$ T-cells by inducing deletion, anergy or regulation. Thereby, DC keep immune responses in check and help to block the development of autoimmune diseases.[30,31] For instance, immature human DC pulsed with influenza matrix protein failed to induce CD8+ T-cell effector

functions monitored after immunization in contrast to mature antigen-pulsed DC.[32] Furthermore, T-cell tolerance may be induced by targeting resting DC with antigen in the absence of adjuvant causing T-cell unresposiveness. [33]

The term maturation was originally applied to DC to refer to the acquisition of immunogenicitiy. However, also DC expressing substantial levels of costimulatory molecules and other maturation markers may be tolerogenic rather than immunogenic. Ultimately, the distinction between an immunogenic and a tolerogenic DC might be in subtle quantitative differences in the expression of various known maturation markers[34], or even in DC longevity.[35]

1.1.5. DC influence other immune cells

In addition to their function as APC, activated DC may also affect cell types other than T-cells. DC can release high amounts of IL-12, IL-18, and IFNα during infections. These cytokines do not only activate NK cells [36] but also induce B-cell differentiation into plasma cells required for antibody production.[37,38]

1.2. Recognition of pathogens by PRR

DC, among other cells of the immune system, are equipped with a repertoir of evolutionary highly conserved proteins, specialized on the recognition of specific PAMP like carbohydrates, lipids or ribonucleases. These proteins called PRR, allow the sensitive detection of bacterial, fungal or viral pathogens before pathogen spread and intervention by the adaptive immune system occur.[1]

PRR differ according to their protein family and cellular localization. The Toll-like receptors (TLR), which are type I integral membrane glycoproteins, are localized at the plasma membrane as well as in the endosomes,[39,40] whereas the NOD-like receptors, detectors of bacterial components[41], as well as the DExD/H box RNA helicases[42] are found exclusively in the cytoplasm. Additionally DC express C-type lectin receptors (CLR) on their plasma membrane. These receptors are, in contrast to the other PRR, specialized on antigen binding and uptake. They

primarily act by modifiying PRR signaling induced by other PRR. Antigen uptake by CLRs alone rather causes tolerance than DC activation.[43]

PRR have distinct expression patterns which has important implications on the type of pattern that is recognized by a certain DC subtype.[39] Furthermore, expression of PRR can be additionally modified by the activation or tissue localization of the DC allowing tissue-specific responses to microbial stimulation.[44]

Finally there is increasing evidence that different PRR co-operate in the induction of DC maturation, enableing DC to fine-tune their reponse. For instance, CLR like the mannose receptor or the β-glucan receptors (f.i. dectin-1) participate in the uptake of microbial components, which can cause DC activation after PAMP recognition by cytoplasmic PRR.[44] The vast majority of experimental evidence comes, however, from TLR co-operation. For instance, combined TLR ligand stimulation of DC may cause synergistic induction of TNF, IL-1β, IL-6, IL-10, IL-12, and IL-23 compared to single agonist stimulation.[45,46] On the contrary, cytokine production can also be negatively regulated by simultaneous signaling through certain TLR. TLR2 activation can, for instance, block TLR3 or TLR4 ligand induced IL-12p35 expression by the increased production of IL-10.[47] The molecular mechanisms underlying co-operative activation of PRR signaling are just starting to be deciphered. However, knowledge about multiple PRR activations required for host resistance to pathogens has important implications for developing vaccination and immunotherapy strategies against infectious diseases. Recent studies highlighted the effectiveness of simultaneously applied TLR ligands as potent adjuvants.[48,49]

1.2.1. Viral pattern recognition receptors (VPRR)

DC activation is induced by viral PAMP recognized by VPRR belonging to two protein families: The TLR (TLR2, 4, 7, 8, 9) and the DExD/H box RNA helicases (MDA-5, RIG-I). Initially, VPRR were believed to become exclusively activated in a virus replication dependent way. MDA-5 and TLR3, for instance, respond to dsRNA replication intermediates. Recent findings, however, emphasize the

presence of VPRR which are activated by ssRNA or viral glycoproteins in the absence of a productive infection (TLR7/8, RIG-I, or TLR2,4) (for details see table 1). [40,50]

Furthermore, DC are also equipped with the dsRNA binding proteins protein kinase R (PKR) and 2`,5`-oligoadenylate synthetase, which are upregulated in an IFN-dependent fashion. Therefore, they are involved in amplifying the activation process induced by other VPRR.[51] In addition, DC also express CLR, like mannose receptor, DEC-205 and DC-SIGN, which are in contrast to other PRR, specialized on antigen binding and uptake. They primarily act by modifiying PRR signaling. Antigen uptake by CLRs alone does not cause DC activation.[43]

Since most VPRR are specialized on recognizing RNA, a molecular pattern not exclusively confined to the viral system, cellular compartimentalization of VPRR is a key determinant in foreign and self RNA discrimination. The endosomal located TLR are only challenged with RNA (of viral origin) under physiological conditions. This enables the cells to discriminate between foreign and self RNA.[52,53]

On the other hand, in the cytoplasm where cellular mRNA are abundant, foreign and self RNA distinction is mainly based on the presence of uncapped 5`-ends and unmodified nucleotides, a hallmark of many viral genomes. The uncapped 5`-end is usually absent in cellular mRNAs, which are capped [54], or is masked by bound cellular proteins.[55,56] Furthermore, cellular RNAs usually undergo conversion to contain modified nucleotides, which decrease the RNA`s VPRR stimulatory capacity.[57]

In addition, secondary structures of ssvRNA genomes as well as non-methylated CpG DNA from DNA viruses may be specifically recognized.[1,40,58]

As with PRR in general, also for VPRR there are cell-type and DC subtype specific expression patterns, which has functional consequences: in vitro generated md-DC in contrast to pDC, for instance, neither express TLR9 nor TLR7, which renders them incapable of detecting unmethylated CpG DNA and makes TLR7 usage impossible (also see table 1).[39]

DC subsets may also differ according to their uptake mechanisms. pDC compared to md-DC retain endocytosed components in their endosomes for a longer time period, which may potentiate TLR signaling.[59,60]

Name of the receptor	Protein family	Expressed by	Cellular localization	Recognized PAMP	Artificially activated by
TLR2	Toll-like receptors	md-DC	plasma membrane	viral glycoproteins (f.i. measles virus)	zymosan
TLR3	Toll-like receptors	md-DC	endosome	dsRNA	pI:C
TLR4	Toll-like receptors	md-DC	plasma membrane	viral glycoproteins (f.i. RSV, MMTV)	LPS
TLR7	Toll-like receptors	pDC	endosome	ssvRNA, synthetic ssRNA rich in GU, siRNA[53,61,62]	R-848, 3M-002 [63,64]
TLR8	Toll-like receptors	md-DC	endosome	ssvRNA, synthetic ssRNA rich in GU [53,62]	R-848, 3M-002 [63,64]
TLR9	Toll-like receptors	pDC	endosome	CpG DNA [65]	CpG oligo-nucleotides [66]
RIG-I	DExD/H box RNA helicases	pDC, md-DC	cytoplasm	5`-triphosphorylated ssRNA/dsRNA, secondary structures in ssvRNA [57,58,67]	see recognized PAMP
MDA-5	DExD/H box RNA helicases	pDC, md-DC	cytoplasm	dsRNA replication intermediates [68]	transfected pI:C [69]

Table 1: Overview about VPRR which differ according to their protein family, expression, localization and recognized PAMP [1,39,40]

1.2.2. VPRR Signaling

Despite the presence of several VPRR, VPRR activation by their specific PAMP has evolved to apply highly conserved signaling transduction pathways which converge on the activation of type I IFN as well as the transcription factor family nuclear factor κB (NFκB) (Fig.2). [1,40]
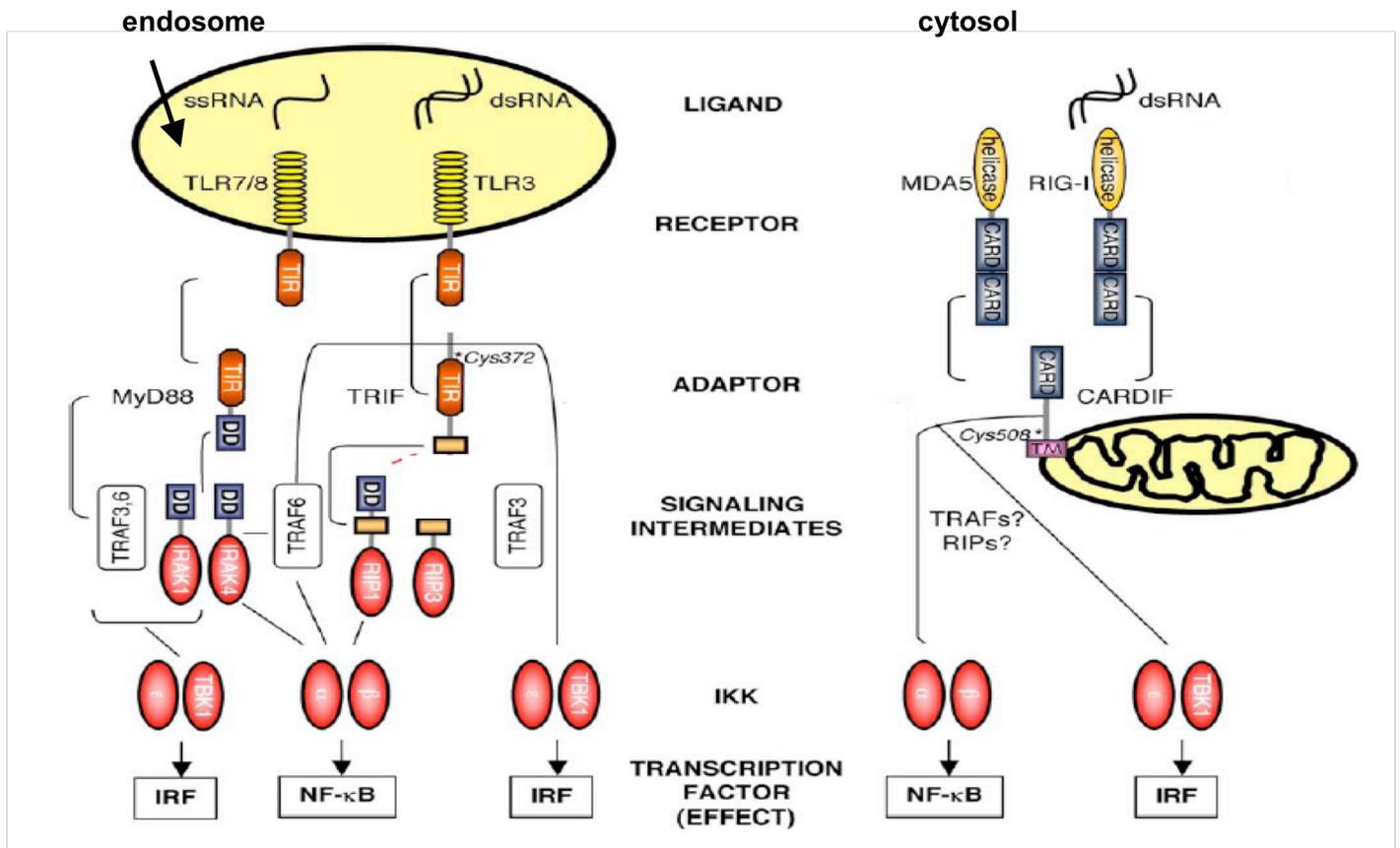

Fig. 2 Signal transduction after VPRR activation (adapted from [70])

Type I IFN activation and function

The IFN response is an early host defense mechanism, which occurs prior to an immune response and is required for the establishment of an antiviral state (see below). Although IFN actions are pleiotropic, their antiviral activity has been

studied the most. In humans all Type I IFN including IFNα, IFNβ, and IFNω have antiviral activity. Humans express just one IFNβ and IFNω, but 13 different isoforms of IFNα.[51]

In the host cell, Type I IFN induce the activation of interferon regulatory factors (IRF). These proteins bind to conserved consensus sequences, the so called IFN signaling response elements (ISRE), thereby inducing the expression of a broad repertoire of antiviral proteins. These proteins exert their function by blocking viral replication and thereby induce an antiviral state.[51]

The best characterized known IFN-inducible gene products with antiviral activity are the Mx GTPases. The Mx1 gene was originally identified in inbred mice which showed an extraordinary high degree of resistance against influenza A virus infections. Mx proteins are inducible by IFNα/β but not by IFNγ. In humans two Mx GTPases, MxA and MxB, are expressed, but only MxA has an intrinsic antiviral activity. MxA is located in the cytoplasm and acts by blocking viral nucleocapsid transport into the nucleus, a process which involves GTPase activity.[51]

Another Type I IFN-induced enzyme with antiviral activity is APOBEC, a deaminase, which impairs retrovirus replication by introducing additional mutations.[40]

Yet, the RNA-dependent PKR is another example of an IFN-induced protein. PKR activation initiated by binding of RNA with ds character, induces a multitude of downstream effects. For instance, PKR is responsible for eukaryotic initiation factor 2α (eIF-2α) and inhibitor of κB (IκB) phosphorylation, which causes a block in mRNA translation and NFκB activation, respectively.[51]

Furthermore, Type I IFN also induce a "2-5A response" leading to degradation of RNA. This involves two enzymes, 2`,5`-oligoadenylate synthetase (OAS) and RNase L. dsRNA activated OAS catalyzes the synthesis of oligoadenylates (abbreviated 2-5A), which in turn activate RNase L, a potent endoribonuclease. RNase L then starts to degrade both viral and cellular RNAs. The degradation of self-RNA was recently shown to amplify antiviral innate immunity by inducing IFNβ expression.[71]

Type I IFN can also exert their action in an autocrine as well as paracrine fashion. All released isoforms bind to the Type I IFN receptors IFNAR1 and IFNAR2. The activated receptors form heterodimers eliciting a Janus kinase (JAK) – signal transducer and activator of transcription (STAT) mediated signal transduction pathway which amplifies Type I IFN production. On the other hand, secreted Type I IFN harbor a protective antiviral effect on other cell types, limiting viral replication and spread.[51] Furthermore, they signal danger to NK cells, amplify DC maturation and facilitate the induction of adaptive immune responses.[72]

NFκB activation and function

The family of NFκB proteins is a central mediator of inflammatory responses and immune function. The vast majority of the inflammatory gene expression program is NFκB dependent. Therefore, these dimeric transcription factors control hundreds of genes. Examples of proteins relevant for DC maturation controlled by NFκB activity include the cytokines IL-1β, IL-6, IL-10, and IL-12 or the surface molecules CD40, CD83, and CD86.[73]

In vertebrates the highly conserved NFκB transcription factors are constituted by homo- and heterodimers composed of five different proteins: p50, p52, c-Rel, RelA (also called p65) and RelB. All of them have a Rel Homology Domain (RHD) at their N-terminus which is responsible for DNA binding, dimerization, inhibitor binding, and nuclear localization.[74]

In resting cell, NFκB is usually located in the cytoplasm. NFκB nuclear localization and DNA binding activity is tightly controlled by the family of IκB proteins. These proteins are a subfamily of the large ankyrin repeat domain (ARD) containing superfamily. The ARD domain is used to mask the nuclear localization signal of NFκB dimers thereby hindering nuclear import of these transcription factors. Cellular stimulation causing PRR activation results in specific phosphorylation, ubiquitination and proteasome-mediated proteolysis of IκB proteins which frees NFκB dimers. This specific activation of NFκB is

mediated by different types of IκB kinases (IKK). The canonical pathway is induced by IKKβ and utilizes IκBα, IκBβ, and IκBε as inhibitors. The non-canonical pathway is activated by IKKα and utilizes the p52 precursor protein p100, whose C-terminal domain can function like an IκB protein to sequester NFκB dimers in an inactive state.[74,75]

NFκB activation causes translocation into the nucleus where NFκB assembles with other transcription and co-factors. DNA binding of these complexes is mediated via the RHD domain to highly degenerate consensus sequences in the promoter of NFκB target genes. Upon these many target genes also the IκB genes are found. This means that IκB metabolism is dynamically regulated not only by stimulus induced degradation but also by feedback re-synthesis.[74,75]

Because of their closely interwoven interactions the small network of IKK, IκB, and NFκB proteins has been termed the NFκB signaling module.[76,77]

1.3. Human rhinoviruses (HRV)

HRV infections are the most prevalent cause of the common cold but may also induce a predisposition for bacterial infections as well as exacerbations of asthma. The pathogenic mechanisms of HRV infections leading to such complications are still poorly understood. [78-80]

HRV, family members of the *Picornaviridae*, are small, icosahedral, nonenveloped (+)-strand ssRNA viruses. So far more than 100 serotypes of HRV, which are classified according to their receptor binding specificities, are known: The vast majority of HRV belong to the major group rhinoviruses, which bind to human ICAM-1 [81,82], whereas minor group rhinoviruses make use of the low density lipoprotein receptor family,[83] Notably HRV are host specific exclusively infecting humans.

1.3.1. HRV cell entry and structure of the viral genome

Viral cell entry is mediated rather by receptor-mediated endocytosis than by direct transfer of the viral RNA into the cytosol.[84,85] Once inside the cell, viral protein translation can start from the positive strand genomic RNA, which has a

highly conserved structure: Genes encoding viral capsid proteins are followed by genes for non-structural proteins. This coding region is flanked by untranslated regions (UTRs) at the 5` and 3` end. These UTRs form extensive secondary structures which are required for translation and viral replication.[86]
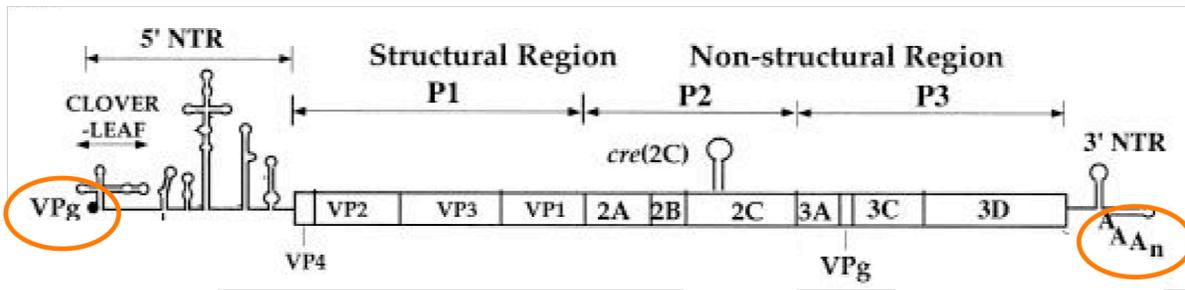

Fig.3 The genome of HRV (adapted from [87])

VP = viral protein, NTR = non-translated region, extensive secondary structures at the 5`NTR represent IRES, AAAn = polyA tail,

A unique feature of the picornavirus genomic RNA is the presence of a covalently attached viral protein, VPg, at the 5` end as well as a polyA tail at the 3` end (Fig.3). Since HRV lack a cap-structure at the 5`end of the RNA, viral translation is initiated at an internal ribosome entry site (IRES) located upstream of the single open reading frame (ORF).[88] Therefore, viral proteins are synthesized by a cap-independent mechanism leading to the formation of a single polyprotein, which is cleaved into smaller entities mainly by the virus encoded proteinases 3C^{pro} and 2A^{pro}.[89,90] Both proteinases have also been shown to cleave cellular proteins.[91] During a productive infection this causes cleavage of the eukaryotic initiation factors eIF4GI and eIF4GII, which leads to "host cell shutoff". This means that translation of the capped cellular mRNAs is blocked during the initiation step, whereas un-capped viral RNA is translated efficiently via a cap-independent mechanism. This enables the virus to use the host cell for its own purposes since viral in contrast to cellular protein synthesis is unaffected by this cleavage. [92,93]

1.3.2. HRV – modulators of the immune system

HRV mainly infect the ciliated epithelial cells of the upper respiratory tract. However, histological examinations of virus-infected nasal epithelium demonstrated no cytopathic effects. Instead, the release of proinflammatory mediators during HRV infections could be observed. These soluble factors are considered to induce proliferation, chemotaxis, and activation of inflammatory cells, which causes an amplification of the inflammatory process.

Despite the limiting coding capacity of HRV, these viruses have evolved sophisticated ways of evading immune activation and slowing down immune responses.

Neutralizing antibodies, for instance, are produced relatively late during HRV infection. Currently there are also no reports about a CD8+ cytotoxic T-cell response, which is usually central in fighting non-cytopathic viruses, during HRV infections. [94]

On the other hand, major group HRV actively manipulate mononuclear phagocyte adhesiveness, thereby possibly retaining these cells at the site of inflammation and hindering emigration to the lymphoid tissues. [95]

Furthermore, HRV has been shown to increase IL-10 release by monocytes, which has been hypothesized to decrease the local inflammatory reaction.[96]

In addition, major group HRV also manipulate DC, by inducing the upregulation of the inhibitory surface molecules, B7-H1 and sialoadhesin (Sn):[97]

B7-H1 is a family member of the B7 molecule family. It was originally identified as costimulatory receptor promoting T-cell proliferation. However, it was shown recently that B7-H1 is an accessory molecule which exerts its inhibitory effects through the PD-1 receptor on T-cells. Furthermore, it is critically involved in T-cell anergy induction in IL-10 treated DC. Although mature DC also express high levels of B7-H1, its inhibitory function seems to be overwhelmed by abundantly expressed costimulatory molecules like CD80 and CD86. Interestingly B7-H1 is also expressed by a variety of tissue cells. Thereby, its expression is believed to be important for maintaining peripheral tolerance.[98,99]

Sn, on the other hand, is a member of the sialic acid binding lectin family of I-type lectins which preferentially bind to sialylated carbohydrate structures.[100] Sn is a macrophage marker which is not expressed by monocytes, lymphocytes and DC.[101] Recently CD43 has been described as the receptor for Sn on T-cells. CD43 is usually removed from the IS to the distal pole of T-cells to allow DC-T-cell interaction during antigen presentation. Since HRV14 treated DC show high levels of Sn, its interaction with CD43 may play a significant role in disturbing proper IS formation.[97]

Taken together HRV treated DC may induce a deep anergic state in T-cells by the upregulation of inhibitory surface molecules.[97]

On the basis of these findings, it has been hypothesized that the unique functional modulation of immune cells by HRV may cause adverse effects on local immunity, thereby predisposing affected individuals to secondary infections.[94]

2. Aims of this work

DC, as the main players linking innate and adaptive immunity, are well equipped with VPRR to detect viral entry and consequently prevent virus replication and spread. A recent study has added up to our understanding of how HRV manipulate the accessory function of these cells.[97] However, the initial steps of HRV recognition and the influence of these processes on DC maturation are unknown.

HRV do not replicate in immune cells, in contrast to epithelial or endothelial cells.[96,102,103] Therefore, this work focused on the effects of ssvRNA from major and minor group HRV on DC. In particular, we were aiming to find out whether HRV ssvRNA, as a potential danger signal, can induce DC maturation independent from its serotype as a consequence of VPRR activation. For this purpose, we intended to study early signal transduction pathways induced by HRV genomic RNA, as well as the outcoming immune stimulatory capacity of these DC.

In addition, luciferase reporter assays in HEK cells were performed to identify a potential receptor for HRV RNA.
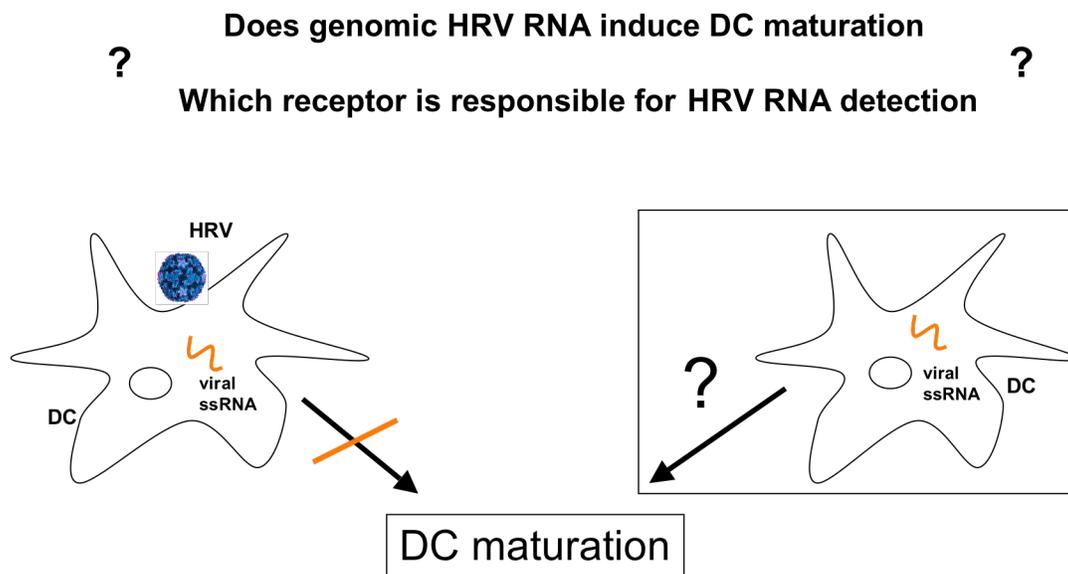

Fig. 4 Aims of this study

3. Results

3.1. HRV genomic RNA is recognized by DC independent of its phosphorylation status

In contrast to many cell lines, HRV do not replicate in immune cells (see Fig.5). [96,102] Therefore, we wondered whether the ssvRNA on its own can induce DC maturation, and the intact virus has evolved some kind of evasion strategy to interfere with this recognition process, thereby blocking DC maturation.

A

B
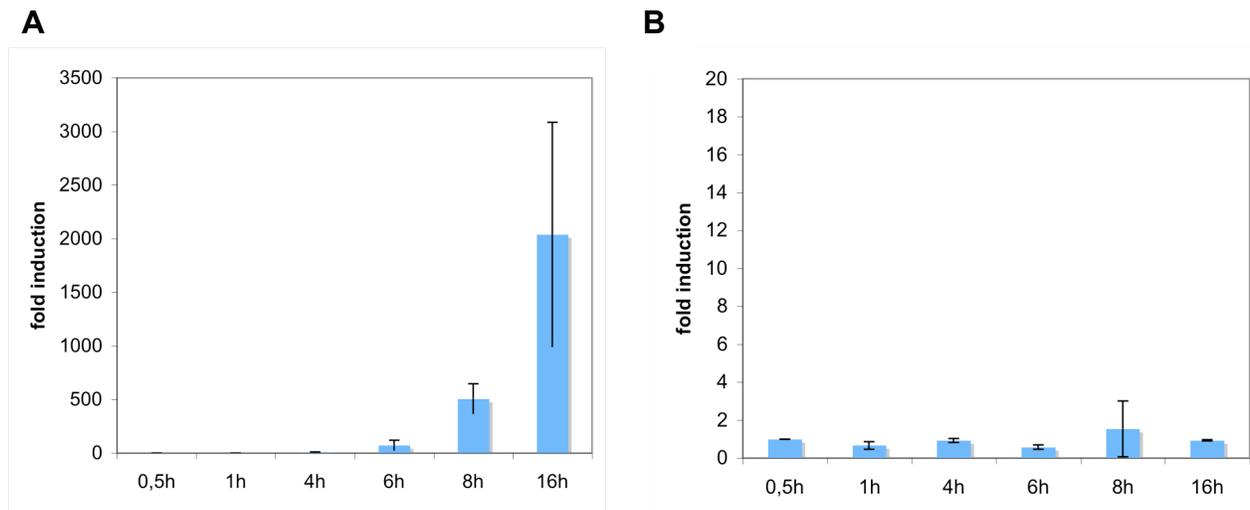
Fig.5 HRV14 does not replicate in DC

RNA from HRV14 treated Hela cells **(A)** or DC **(B)** was isolated after the indicated time points. cDNA was generated and used for the real time PCR reaction. Test results were normalized to the message of the housekeeping gene G3DPH. The results shown represent the fold inductions of the HRV14 positive strand compared to the 30 minutes incubation step. One representative experiment out of two is shown. Mean values of duplicate determinations +- standard deviation are shown.

To get hold of HRV genomic RNA, ssvRNA was purified by two methods: First the genomic RNA was directly isolated from a viral suspension (Fig.6 A), but due to low yield, we switched to in vitro transcription, which provided high yields of good quality RNA. This RNA was derived from an in vitro transcription plasmid

encoding the whole genomic RNA including the untranslated regions and the polyA tail (Fig.6 B, C).
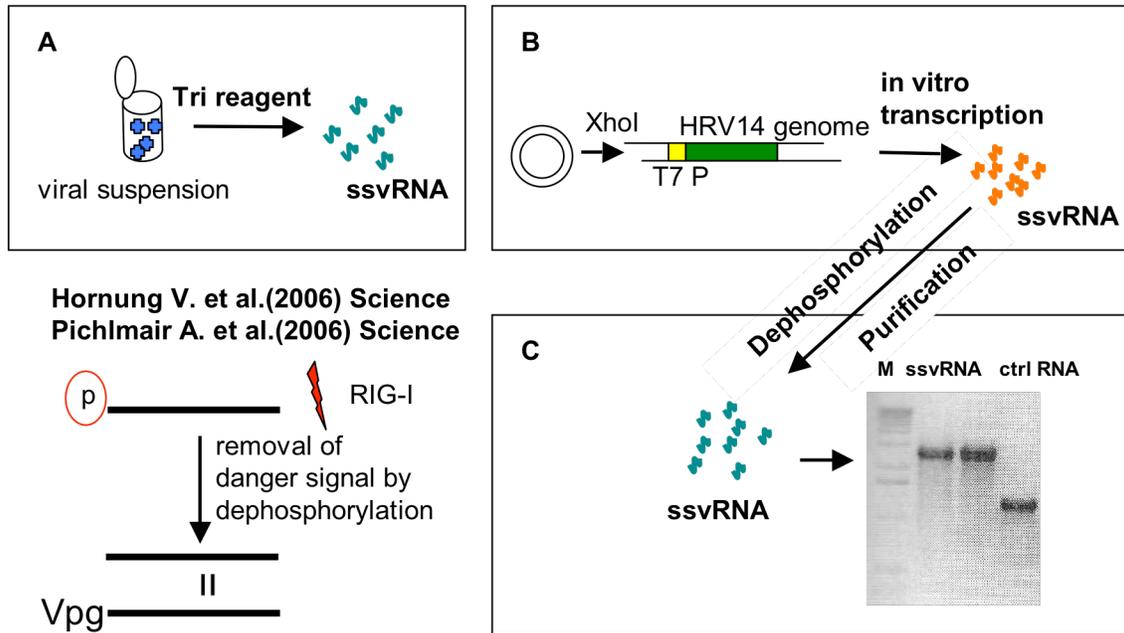
Fig.6 Purification of HRV genomic RNA

HRV2/14 genomic RNA was isolated either from a viral suspension using Tri reagent **(A)** or by in vitro transcription from linearized T7 promoter in vitro transcription plasmids encoding full lenght genomic RNA of HRV2/14 including the untranslated regions and the poly A tail **(B).** After DNA digestion and purification, the in vitro transcription reaction gave rise to high yields of degradation free, highly pure viral RNA as shown on the agarose gel **(C).** Part of the RNA was dephosphorylated by calf intestine alkaline phosphatase to generate RNA lacking the 5`-triphosphate which may be sensed by the VPRR RIG-I. [57,67] This RNA species resembles better the natural occuring genomic RNA, which lacks a 5`-triphosphate and is capped by the viral protein Vpg **(see left panel at the bottom)**. As a control for the in vitro transcription and dephosphorylation reaction the control (ctrl.) RNA for elongation factor 1α from xenopus was treated exactly the same as the viral RNA.

In nature HRV genomic RNA is capped at the 5`-end by the viral protein VPg, thereby blocking recognition by the VPRR RIG-I. Since in vitro transcribed RNA has a triphosphate instead of a cap at the 5`-end, this potential danger signal can be effectively removed by calf intestine alkaline phosphatase.[57,67] (also see Fig.6)

To control the efficiency of this dephosphorylation process, a control RNA from Xenopus (ctrl RNA) was synthesized and processed identically to the viral RNA.
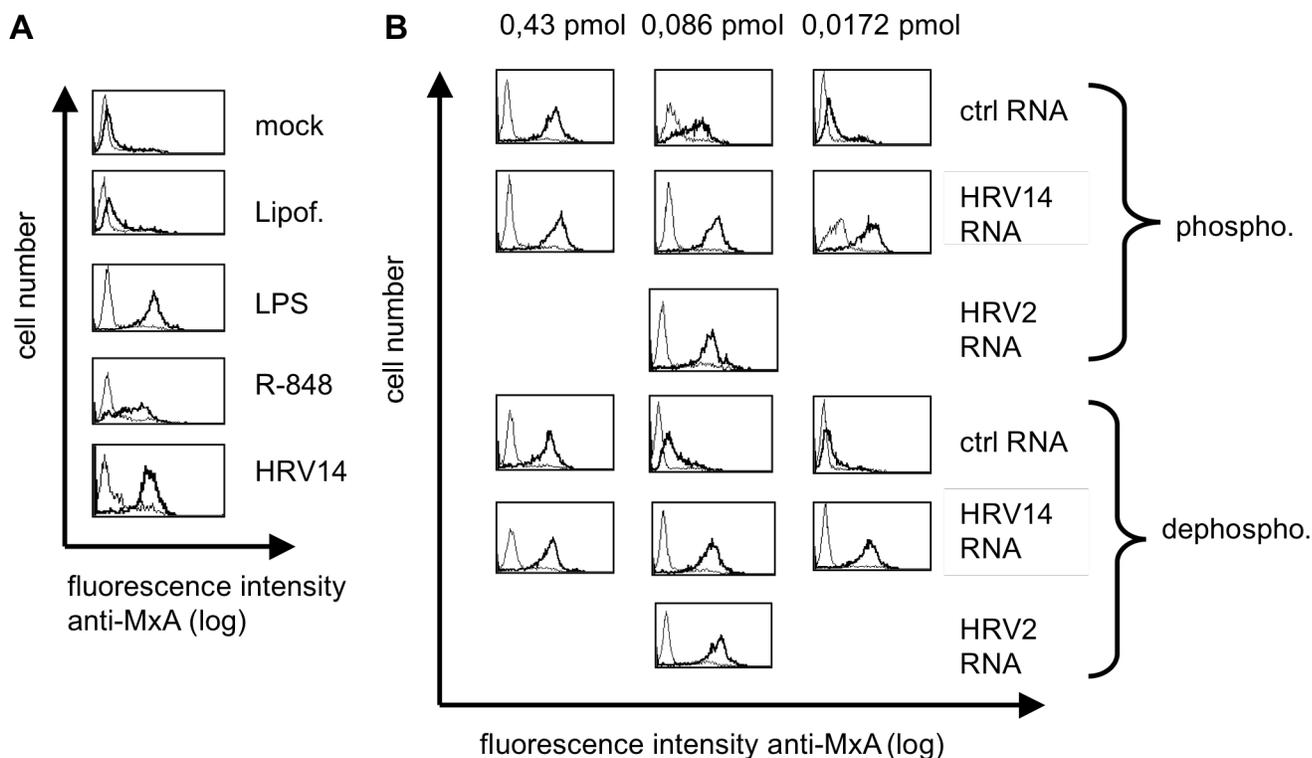
Fig.7 HRV genomic RNA induces strong MxA expression independent from its phosphorylation status

(A) DC were stimulated either with LPS (100ng/ml), R-848 (2 µg/ml), HRV14 (5 TCID$_{50}$), Lipofectamine (0,5 µl) or medium. Cytoplasmic MxA expression was assessed after two days of incubation by FACS analysis. The thin line in the histograms represent binding of the isotype control antibody, whereas the thick line shows the reactivity of the monoclonal anti-MxA antibody with the stimulated DC.

(B) DC were transfected with ctrl, HRV2 or HRV14 RNA at different molarities using lipofectamine. Part of the RNA had been dephosphorylated by calf intestine alkaline phosphatase after in vitro transcription. MxA expression was detected as described in **(A).**

Staining from one representative experiment out of at least 4 experiments with different donors is shown.

Since a Type I IFN response is a hallmark of virus recognition [40], we performed cytoplasmic FACS stainings of MxA, a type I IFN induced protein,[104] as a read out system. As shown in Fig.7 B DC either transfected with in vitro transcribed

ctrl RNA or HRV RNA strongly upregulated MxA expression, comparable to HRV14 or LPS treated cells (Fig.7 A). In contrast, dephoshorylated HRV RNA was still recognized by DC, whereas dephosphorylated ctrl RNA was only inducing MxA expression at high concentrations. On the other hand, for RNA amounts of 0,086 pmol and below, DC recognized only HRV RNA independ of its phosphorylation status, whereas the ctrl RNA was only sensed as a danger signal in its phosphorylated form.
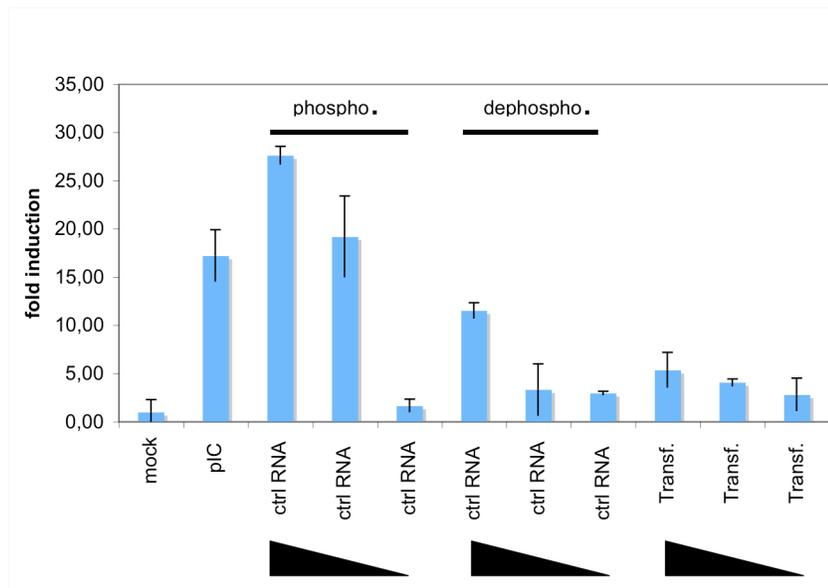
Fig.8 Dephosphorylated RNA has a strongly diminished stimulatory capacity on RIG-I
HEK cells were transiently transfected with plasmids encoding RIG-I and ß-galactosidase along with an ISRE reporter plasmid from which luciferase protein is synthesized upon RIG-I activation. Cells were either mock treated or transfected with different amounts of phosphorylated / dephosphorylated in vitro transcribed ctrl RNA (1,68 pmol, 0,336 pmol, 0,067 pmol) or transfectin alone. Luciferase activity was measured by a luminometer and the ratio between this value and the ß-galactosidase activity was calculated. Shown are the relative mean values of duplicate measurements as fold inductions of mock treated cells +- standard deviation.

The efficiency of the dephosphorylation reaction was also controlled with an independent experiment. In this luciferase reporter assay HEK cells transiently transfected with RIG-I and an IFN signaling induced luciferase reporter were either transfected with phosphorylated or dephosphorylated ctrl RNA. In this

assay, as expected, only phosphorylated ctrl RNA was causing a strong IFN response, whereas this induction was significantly diminished after dephosphorylation (Fig.8).

3.2. RIG-I is a potential VPRR for HRV RNA

Surprisingly, the HRV14 RNA was also specifically upregulating the IFN response in RIG-I transfected HEK cells, independent of its phosphorylation status (Fig.9 A). These data fit to the results of the cytoplasmic FACS stainings, however, they suggest in addition that RIG-I is a potential VPRR for HRV recognition.

Furthermore, it should be noted that similiar results were obtained from a luciferase reporter assay using a NFκB luciferase reporter: The ctrl RNA showed increased luciferase activity due to RIG-I activation only in its phosphorylated form. The HRV RNA, however, also activated NFκB signaling in its dephosphorylated form (Fig.9 B). These findings are in accordance with reports from the literature stating that activated RIG-I induces both IFN and NFκB signaling.[40,42]

From these results we concluded that the dephosphorylation reaction had been performed efficiently and that other features of HRV RNA than its 5`-phosphorylation status are crucial for recognition. Furthermore, we could show that both 5`-phosphorylated ctrl RNA and dephosphorylated HRV14 RNA are specifically recognized by RIG-I. This recognition process involved both IFN and NFκB signaling in RIG-I transfected HEK cells.
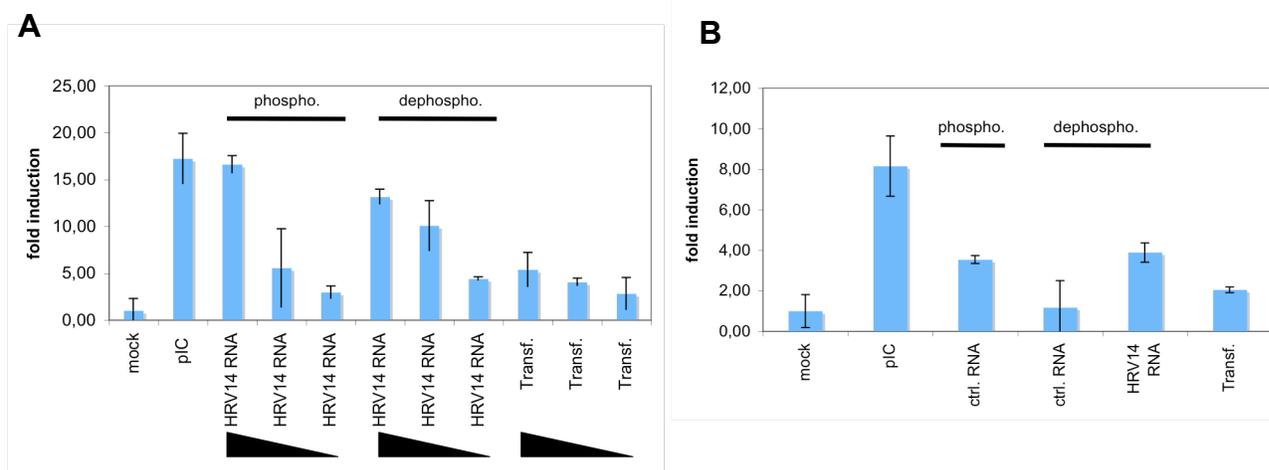
Fig.9 RIG-I is a potential VPRR for HRV RNA

HEK cells were transiently transfected with plasmids encoding RIG-I and ß-galactosidase along with an ISRE **(A)** or NFκB **(B)** reporter plasmid from which luciferase protein is synthesized upon RIG-I activation. Cells were either mock treated, transfected with 1ug pIC, transfected with different amounts of phosphorylated / dephosphorylated in vitro transcribed HRV14 RNA (0,43 pmol, 0,086 pmol, 0,0172 pmol) **(A)**, equimolar amounts of phosphorylated / dephosphorylated in vitro transcribed ctrl RNA or dephosphorylated HRV14 RNA (0,43 pmol each) **(B)** or transfectin alone. Luciferase activity was measured by a luminometer and the ratio between this value and the ß-galactosidase activity was calculated. Shown are the relative mean values of duplicate measurements as fold inductions of mock treated cells +- standard deviation.

3.3. HRV genomic RNA neither induces NFκB activation nor inhibits its activation by maturation stimuli

The results from the HEK luciferase assay suggested that RIG-I can specifically detect HRV14 RNA, which induces both IFN and NFκB signaling. Therefore, we wanted to confirm the second hallmark intimately linked to viral pattern recognition by assessing NFκB activation in DC.

Activation of DC with maturation stimuli like LPS or the TLR7/8 agonist R-848 causes degradation of IκB, a NFκB inhibitor, which can be easily assessed by Western blot analysis. Degradation of IκB starts early after stimulation and is characterized by a feedback loop causing enhanced IκB production.[74] Although we could find this type of response after LPS or R-848 stimulation, we were

neither able to detect IκB degradation after treatment with HRV14 nor after transfection with its genomic RNA (see Fig.10 A). This is reminiscent of an electrophoretic mobility shift assay (EMSA) performed with the intact virus: over a time range of 9 hours, no NFκB binding to the NFκB consensus sequence could be detected (Fig.10 B).

A

B
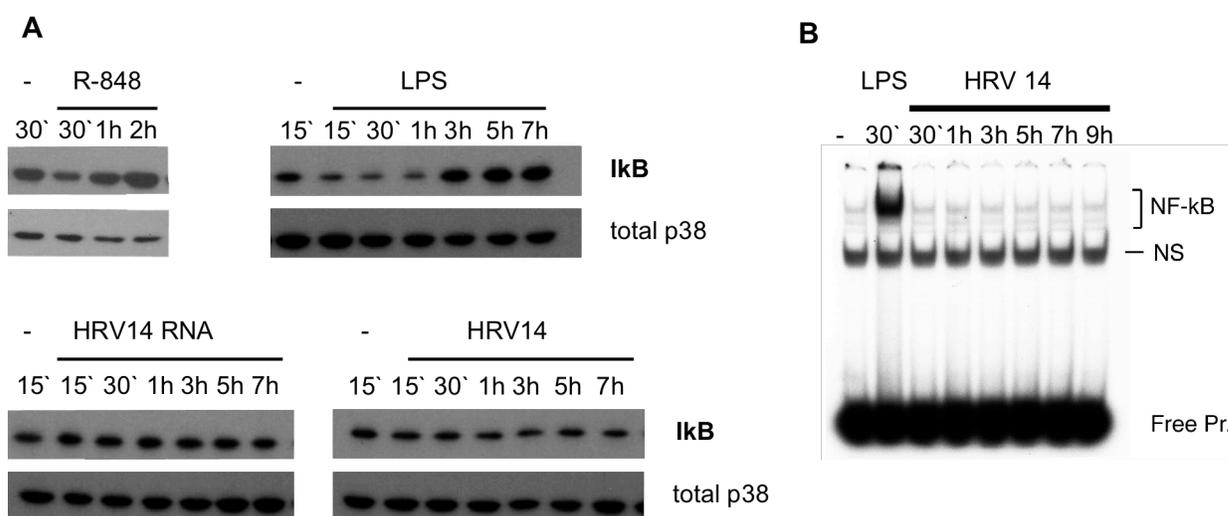
Fig.10 Both HRV14 and its RNA fail to induce NFκB activation in DC

(A) DC were stimulated with either R-848 (2 μg/ml), LPS (100 ng/ml), HRV14 (5 $TCID_{50}$) or transfected with 0,086 pmol HRV14 RNA. Cells were harvested after the indicated time points and subjected to SDS-PAGE. Specific signals for IκB-α were detected by Westernblot analysis. Loading controls were determined with an antibody against total p38 after stripping the membranes. One representative experiment out of three is shown.

(B) Nuclear extracts from DC stimulated either with medium, LPS (100 ng/ml) or HRV14 (5 $TCID_{50}$) were purified after the indicated time points and incubated with radiolabelled NFκB consensus sequence. Specific binding to this sequence causes a shift during the EMSA (electrophoretic mobility shift assay) experiment. NFκB = specific binding of NFκB to its consensus sequence, NS= nonspecific binding, free Pr.= free radiolabelled primers

Since HRV are positive strand RNA viruses, viral proteins can be translated from the genome once having entered the cytosol. Although there is a lack of replication, low concentrations of proteins with inhibitory functions on the NFκB

pathway may be present. To exclude that HRV14 has evolved a NFκB blocking mechanism, DC were pretreated with the virus for 1h to allow viral entry and then stimulated with known inducers of the NFκB pathway and DC maturation. As shown in Fig.11 HRV14 neither blocks TLR3 nor TLR8 mediated IκB degradation, thereby rendering it unlikely that HRV14 is actively blocking NFκB activation in DC.

Taken together, despite a strong Type I IFN induced MxA expression in HRV RNA transfected and HRV14 treated DC, the second hallmark of viral pattern recognition, namely NFκB activation, was absent in DC which is in sharp contrast to the situation in HEK cells. However, the missing NFκB activation in DC was not a consequence of active viral inhibition.
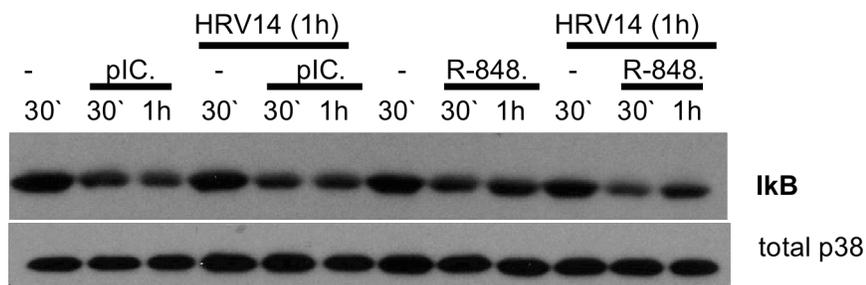
Fig.11 HRV14 is not actively blocking IκB degradation induced by TLR agonists

DC were either left untreated or were pre-incubated with HRV14 (5 $TCID_{50}$) for 1 hour prior to stimulation with either pI:C or R-848 (both 2 µg/ml). Cells were harvested after the indicated time points and subjected to SDS-PAGE. Specific signals for IκB-α were detected by Westernblot analysis. Loading controls were determined with an antibody against total p38 after stripping the membranes.
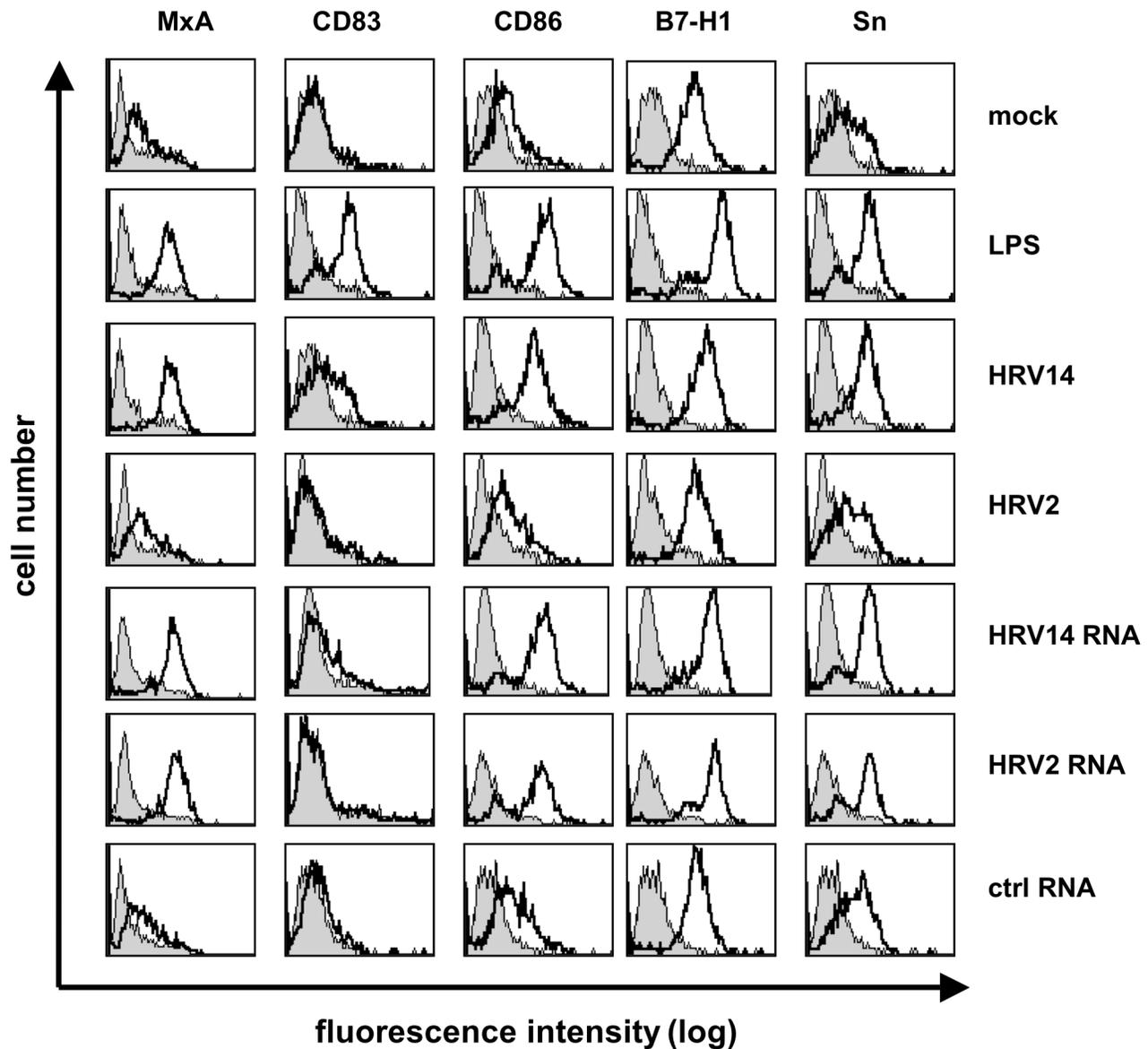
Fig.12 HRV RNA transfected DC lack CD83 upregulation, whereas CD86 and inhibitory surface markers are induced

The expression pattern of the surface molecules CD83, CD86, B7-H1 and Sn on DC was compared to the presence of cytoplasmic MxA after two days of stimulation. Antibodies recognizing surface molecules or cytoplasmic MxA (after permeabilization) were visualized by an Oregon Green-conjugated goat anti-mouse IgG antibody and detected by FACS analysis. Grey histograms represent the negative controls (non-binding mAb VIAP), whereas the thick lines show the specific binding of the respective mAb.

3.4. HRV genomic RNA fails to induce proper upregulation of the DC maturation marker CD83

Although the presence of high numbers of maturation markers on the surface of DC is not exclusively an indicator for immunogenicity, it is however a pre-exquisite.[14] Maturation markers which are typically upregulated during maturation are CD83 and CD86. The expression of both surface proteins was clearly increased upon LPS stimulation as shown by FACS analysis. Although CD86 expression was also induced by HRV14 treatment as well as transfection with ssvRNA, for these stimuli CD83 remained at the level of unstimulated cells (Fig.12).

3.5. Transfection with HRV ssvRNA induces the enhanced expression of inhibitory surface molecules

The ability of DC to deliver signal 2 to activate T-cells depends on the delicate balance of costimulatory as well as inhibitory surface molecules. Therefore, we also assessed the expression of the latter after DC stimulation.

We have previously shown that HRV14 in contrast to HRV2 treatment of DC induces the expression of B7-H1 and Sn.[97] For this reason, it was surprising to find that ssvRNA from both HRV serotypes iduced the expression of these inhibitory molecules (Fig.12). Furthermore, the upregulation of Sn strongly correlated with the MxA inducing potential of the stimulus. (compare MxA and Sn levels of HRV2 treated with HRV14 treated DC in Fig.12)

3.6. HRV ssvRNA transfected DC are poor producers of TNFα, IL-6, IL-12, and IL-10

During antigen presentation a defined cocktail of cytokines is required for the proper induction and fine-tuning of an adaptive immune response. The presence of cytokines released by DC upon stimulation therefore provides important information about the functional capability of these cells.
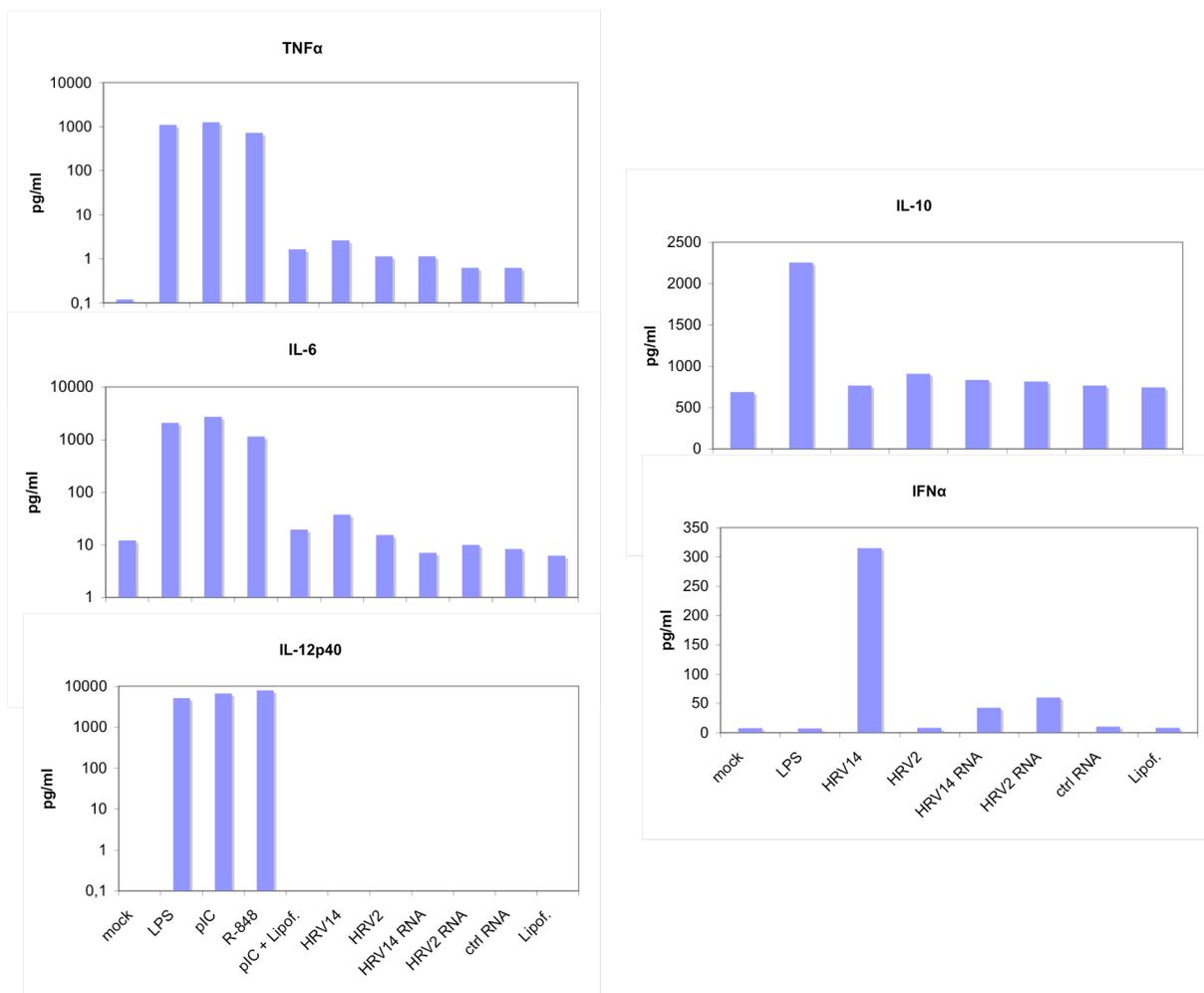
Fig.13 HRV RNA neither induces the release of proinflammatory cytokines nor of IL-10
The supernatants of stimulated DC were analyzed for their content of released cytokines after two days of culture with the indicated stimuli. TNFα, IL-6, IL12-p40, and IL-10 concentrations were determined by Luminex, whereas IFNα levels were assessed by ELISA. TNFα, IL-6, and IL-12p40 concentrations are depicted on a log scale.

Whereas typical maturation inducers like LPS, pI:C and R-848 boosted the release of TNFα, IL-6, and IL-12p40, neither HRV nor its genomic RNA managed to do so. On the other hand, we could also not observe virus induced release of the anti-inflammatory cytokine IL-10 (Fig.13).

This secretion profile was in sharp contrast to the release of IFNα measured by ELISA: HRV14 treated DC secreted relative high amounts of IFNα, and also HRV14 / 2 ssvRNA transfected DC were found to release IFNα (Fig.13).

It should be noted that transfection with RNA did not increase cell death compared to lipofectamine treated cells (Fig.14).
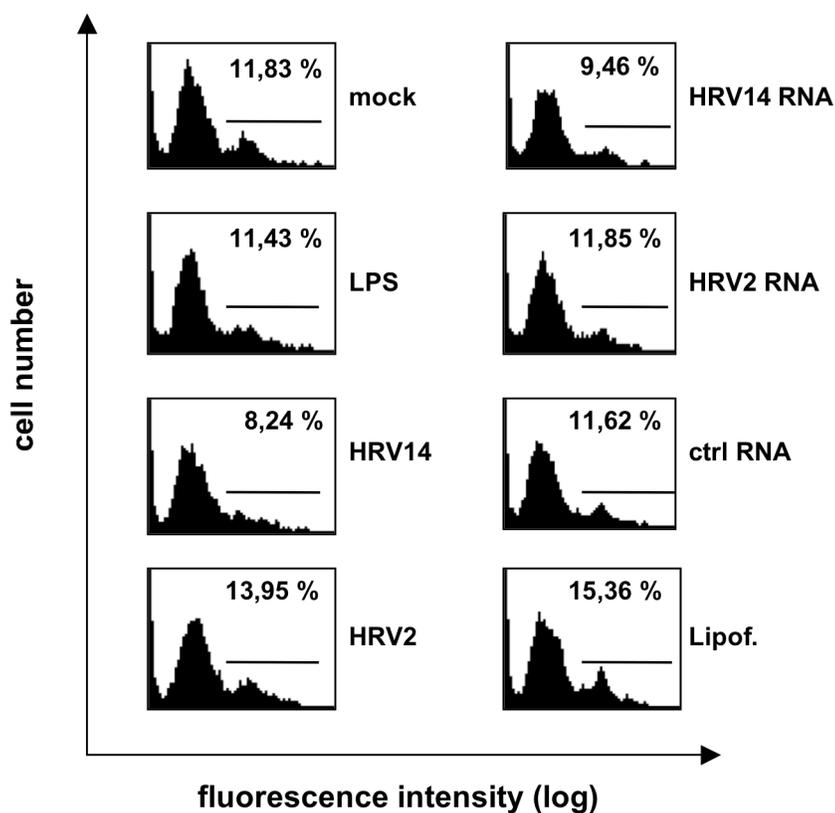
Fig.14 Transfection with Lipofectamine complexed RNA does not increase cell death in DC
DC treated with medium (mock), LPS (0,1 µg/ml), HRV14/2 (5 TCID$_{50}$) or transfected with 0,086 pmol of HRV14 / HRV2 / ctrl RNA complexed to Lipofectamine or Lipofectamine alone were incubated for 2 days. Cell death was monitored by adding propidium iodide to DC prior to FACS analysis. The proportion of dead cells for each condition is expressed as a percentage at the right corner.

3.7. HRV ssvRNA transfected DC show a different T-cell stimulatory capacity compared to LPS or HRV-treated DC

Maturation inducers like LPS typically induce an enhanced T-cell stimulatory capacity in DC compared to mock treated DC in an allo-mixed leukocyte reaction (MLR) (Fig.15). This capacity is evident at different DC - T-cell ratios and has been extensively used as an in vitro model system for testing DC capability of inducing an adaptive immune response after stimulation.
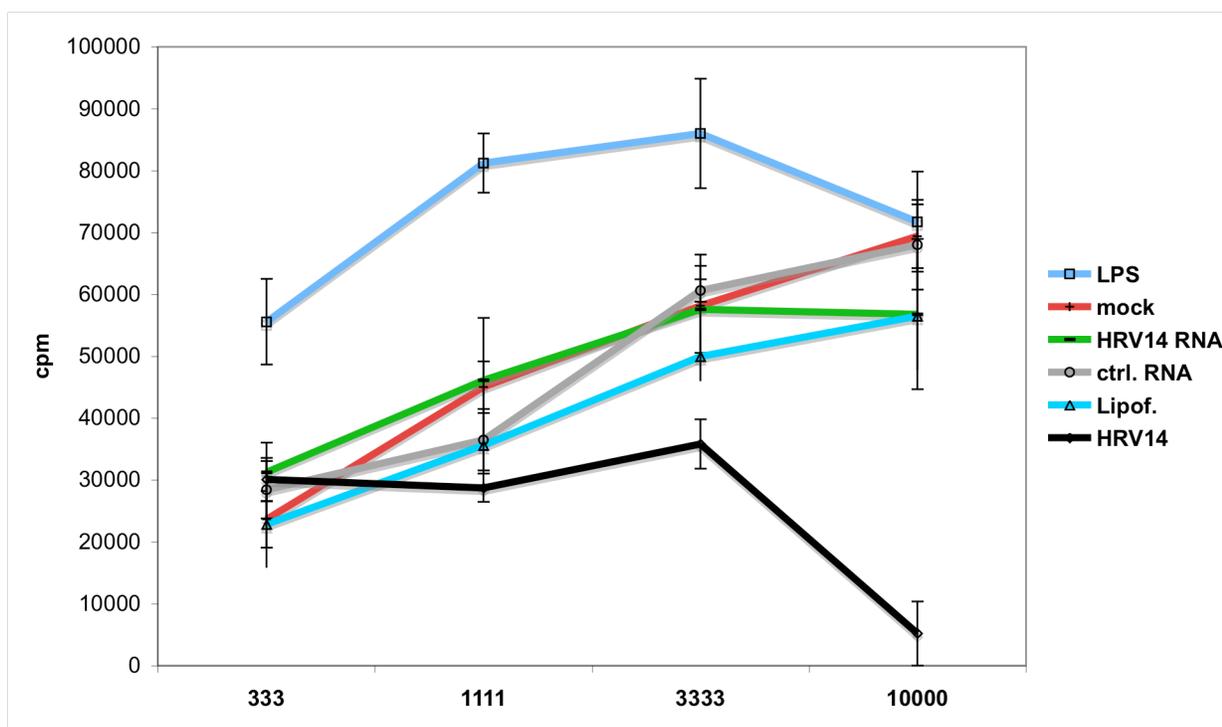
Fig.15 DC transfected with HRV14 RNA show no enhanced T-cell stimulatory capacity
Purified T-cells were stimulated with graded numbers of allogeneic DC inoculated with either HRV14 (5 $TCID_{50}$) or transfected with HRV14 RNA (0,086 pmol) for 48h. As controls allogeneic DC transfected with ctrl RNA (0,086 pmol) or Lipofectamine as well as mock or LPS (100 ng/ml) treated DC were included. Proliferation of T-cells was monitored on day 5 of co-culture by adding methyl-^{3}H-TdR followed by measuring its incorporation 18h later. The results of one representative experiment of three independent experiments is depicted. Shown are the relative mean values of triplet measurements +- standard deviation.

In contrast to LPS stimulated DC, HRV14-treated DC showed a strongly reduced T-cell stimulatory capacity as we have previously published.[97] DC transfected with HRV14 ssvRNA showed, however, neither an enhanced nor a diminished T-cell stimulatory capacity. The observed T-cell proliferation was rather comparable to mock or lipofectamine treated DC. Similiar results were also obtained with ctrl RNA transfected DC.

Taken together, transfection of DC with HRV ssvRNA does not recapitulate treatment with the intact HRV14, because these cells show no reduced T-cell proliferation in an allo-MLR. Consequently, as previously postulated,[97] signaling events downstream of ICAM-1 could be responsible for this discrepancy.
Furthermore, our results support the idea that neither major nor minor group HRV genomic RNA, despite recognition and a resulting prominent Type I IFN response, has the potential to induce DC maturation.

4. Discussion

For a long time, it was thought that viruses are only sensed by DC after replication. However, the finding that the VPRR TLR7/8 as well RIG-I detect ssRNA in a replication independent manner,[40] shed more light on the sophisticated alternative virus detection mechanisms evolved by DC.

In the past years, the major goal concerning VPRR activation in DC was to identify the PAMPs recognized by these receptors. Most experiments were done in the murine immune system. As a consequnce data about signal transduction and receptor activation were primarily based on studies with (knock out) mice or cell-lines.

Recently, the identification of ssRNA as a potential agonist for TLR and DExH/D helicase signaling, has caused many research groups to focus on the immune stimulatory role of RNA: For instance, transfections of murine DC with GU-rich or viral ssRNA were shown to induce strong IFNα responses as well as the release of proinflammatory cytokines.[53,57,61,67] Furthermore, transfections of DC with tumor derived RNA or immunostimulatory RNA oligonucleotides were shown to induce DC maturation.[105,106]

In contrast, little is known about VPRR activation in human DC, especially in monocyte-derived-DC. Some studies showed that transfection of this cell type with mRNA encoding viral proteins or tumor antigens can induce DC maturation.[107,108] However, so far there are no reports dealing with the question whether these cells can be functionally matured by ssvRNA transfection. – However, it should be considered that md-DC are routinely used in DC-based cancer therapy trials.[109] Therefore, data adding up to a more detailed view about activation of this cell type will be beneficial.

Furthermore, we have previously shown that HRV14, a member of the major group rhinoviruses, specifically down-modulates the accessory function of DC: HRV14-treated DC do not become mature and show a strongly diminished T-cell stimulatory capacity due to the upregulation of inhibitory surface molecules.[97] Since DC are equipped with different ssvRNA detecting VPRR, we wondered

whether the genomic RNA of HRV14 alone, as a potential danger signal, has the capacity to induce DC maturation.

In this study, we show that DC transfected with HRV14 ssvRNA react to this stimulation by a profound increase in cytoplasmic MxA, an IFN induced antiviral protein.[104] Since similar results were obtained independent of the ssvRNA`s phosphorylation status, a PAMP other than the presence or absence of a triphosphate at the 5`end seems to play a role for recognition. Identical results found for HRV2 ssvRNA also show that the recognition process is independent from the receptor binding specificities of the HRV serotype. This is in contrast to the situation when DC are treated with intact HRV: Here only HRV14 induced a strong MxA induction. Therefore, PAMP involved in IFN induction are possibly highly conserved structures common to different HRV serotypes, for instance the non-translated regions (NTR) at the 3`or 5`-end of the genome.[86] This idea is supported by a recent finding that conserved secondary structures in the NTR of the ssRNA genome of hepatitis C virus are specifically recognized by RIG-I.[58]

Since a type I IFN response is a hallmark of VPRR activation and often discussed as a consequence of NFκB activation, it was surprising to find this second hallmark missing after either transfecting DC with ssvRNA or treating them with HRV14. However, a recent report claims that Type I IFN production is possible even in the absence of NFκB isoforms, thereby providing an explanation for this discrepancy.[110] Furthermore, we have also shown that the missing NFκB activation is not a consequence of active viral interference, which is a major concern when dealing with positive strand viruses.[111]

Given the fact, that for all known VPRR, activation of NFκB as a consequence of receptor activation has been described,[1] it is puzzling not being able to see NFκB activation in DC after ssvRNA transfection. There are several possible explanations for this phenomenon: First, VPRR signal transduction has been mainly studied in murine immune cells or cell-lines. Therefore, these pathways may operate differently in human DC. Second, a yet unidentified RNA binding receptor specialized on IFN induction rather than known VPRR could be

activated by ssvRNA in DC. And finally, it is also possible that NFκB activation was occuring below the detection limit of the method used.

The lack of NFκB activation, which has been known as a master switch of cytokine gene regulation for a long time, probably also entails the missing upregulation of the maturation marker CD83 as well as the lacking release of the proinflammatory cytokines TNFα, IL-12, and IL-6, all of which are known to be transcribed from NFκB regulated genes, in ssvRNA transfected DC.

On the other hand, we were surprised to find, that the ssvRNA on its own can induce the expression of the inhibitory molecules B7-H1 and Sn in DC. Former studies indicated that the HRV14 receptor ICAM-1 was responsible for this phenomenon, since HRV2 treated DC failed to induce their expression.[97] However, as shown here, transfected HRV2 ssvRNA can also very effectively induce upregulation of both molecules in contrast to the intact virus. This phenomenon may be explained by a low LDL receptor expression on DC which makes entry of the HRV2 ssvRNA difficult if treated with the intact virus, thereby avoiding RNA detection. In addition, the strong correlation between MxA induction and Sn is in line with previous experimental data showing that the ICAM-1 specific antibody RR1/1 did not trigger Sn expression[97] possibly due to the missing IFN response.

Finally, it was not unexpected to find that HRV ssvRNA transfected DC exhibit no functionally mature phenotype in an allo-MLR. However, the observed low capacity to induce T-cell proliferation, was still not as profound as for HRV14-treated DC. As we have shown previously, similar low capacities for the induction of T-cell proliferation were obtained with HRV14- or UV-treated HRV14 (which was still capable to bind to DC) DC.[97] Therefore, we believe that signaling via ICAM-1 rather than signals derived from HRV ssvRNA might be critically involved in the downregulation of the T-cell stimulatory function of DC.

The profound IFN response after HRV14 treatment or ssvRNA transfection, shows that the genomic RNA of HRV is specifically recognized by DC. -Studies with MDA-5 knock out mice have led to the hypothesis that MDA-5 was important for recognizing picornaviruses.[69] Furthermore, it has been reported that TLR3

mediates antiviral activity against HRV in human bronchial epithelial cells.[103] However, activation of both VPRR requires viral replication, which is absent in DC treated with HRV. Therefore, it is more likely that HRV are recognized by a ssRNA binding receptor like TLR8 or RIG-I.

The results of our luciferase reporter assay show that HRV RNA can indeed be specifically recognized by RIG-I, which can activate IFN as well as NFκB signaling in HEK cells transiently transfected with this VPRR. This finding is in contrast to the situation in DC, where we could not detect any NFκB activation. It is tempting to speculate about cell-type specific differences in signaling between HEK cells and DC. Furthermore, it should be noted that the HRV RNA still had some residual replication capacity in HEK cells in contrast to DC. This low concentration of HRV dsRNA may account for NFκB activation in HEK cells and might suggest that the presence of dsRNA is required for NFκB activation but is dispensable for IFN signaling. This idea would be in contrast to the common model, which describes activated VPRR receptors as potent inducers of IFN and NFκB signaling.[1,42] However, it is in accordance with preliminary data showing that DC transfected with in vitro transcribed phosphorylated ctrl RNA showed no IκB degradation inspite of a prominent MxA response (data not shown). This observation was made although phosphorylated RNA is a well known activation stimulus for RIG-I.[57,67]

So far, we were not able to find a similiar response after treatment of RIG-I transfected HEK cells with HRV14 compared to after transfection with HRV14 RNA. (data not shown) A possible explanation might be that the concentration of virus used was too low to activate the reporter. However, higher concentrations of virus would have overriden the protective effect of the anti-apoptotic compound zVAD, which was recently shown to also block HRV replication.[112]

In the future, it will be crucial to perform additional luciferase reporter assays with replication deficient HRV RNA in RIG-I or TLR8 / MDA-5 (as negative controls) transfected HEK cells. Such experiments could help to find out whether low dsRNA concentrations are required for HRV RNA induced RIG-I activation or not.

Furthermore, transfections with replication deficient HRV RNA would also better mimick the situation in DC, where no HRV replication occurs.

In addition, it will be necessary to perform knock down experiments of the candidate VPRR for HRV14 in DC as a proof of principle. It would also be interesting to find out which parts of the genomic RNA are detected by DC. This could be tested by transfecting selected in vitro transcribed stretches of RNA into DC and HEK reporter cells.

Taken together, this study shows that the ssvRNA of HRV is only a *semi-danger signal* for DC: The ssvRNA of HRV is specifically recognized by DC, causing a strong IFN response. However, ssvRNA fails to induce NFκB activation, which is required for proper maturation induction. In addition, maturation markers are absent, proinflammatory cytokines are not produced and HRV ssvRNA fails to induce an increased T-cell stimulatory capacity in an allo-MLR.

The results from the HEK luciferase reporter assays suggest that the ssvRNA activates RIG-I in HEK cells. Upcoming evaluation of these data in DC will help to clarify HRV recognition processes and VPRR signaling in DC.

5. Material & Methods

5.1. Monoclonal antibodies

Specificity	Clone	Isotype	source
B7-H1	DF272	IgG1	Otto Majdic, Institute of Immunology, Vienna
Calf intestine alkaline phosphatase	VIAP	IgG1	Otto Majdic
CD1a	VIT6b	IgG1	Otto Majdic
CD11b	VIM12	IgG1	Otto Majdic
CD14	MEM18 /VIM13	IgG1 / IgM	Otto Majdic
CD16	3G8	IgG1	Caltag, Burlingham, CA
CD19	BU12	IgG1	G. Moldenhauer, Heidelberg, Germany
CD33	4D3	IgG2	Otto Majdic
CD40	G28-5	IgG1	ATCC, Rockville, ML
CD54	5-341	IgG2a	Otto Majdic
CD80	7-480	IgG1	Otto Majdic
CD83	HB15	IgG2b	Caltag
CD86	BU63	IgG1	Caltag
CD169 (Sialoadhesin)	7-239	IgG1	Otto Majdic
MHCI	W6/32	IgG2a	ATCC
MHCII	CLII-1.47	IgG2a	Otto Majdic
MxA	383-7D4	IgG1	Otto Majdic

Table 2: Monoclonal antibodies (mouse) used in this study

5.2. Isolation of peripheral blood mononuclear cells (PBMC)

Heparin-medium: 500ml RPMI 1640 (+ 10% FCS +100U/ml penicillin + 100ug/ml streptamycin and 2mM L-glutamine) + 10 U/ml Heparin (stock: 5000U/ml, Baxter, Vienna)

MACS-buffer: 1000 ml 1x PBS def. + 25 ml HSA (stock: 20%, Centeon, Vienna) + 10 ml EDTA (stock: 0,5M; Gibco, Invitrogen GmbH, Lofer, Austria); sterile filtered

Peripheral blood mononuclear cells were separated from whole blood of healthy donors by density gradient centrifugation using Ficoll-Plaque (Amersham, Little Chalfont, UK). Blood was diluted 1:2 to 1:3 with heparin-medium. For density gradient centrifugation a layer of heparinized blood was carefully pipetted onto 15 ml of Ficoll-Plaque. The cells were spun 30 minutes at 1750 without brake. After centrifugation granulocytes and erythrocytes gathered at the bottom of the tube, whereas PBMC formed a ring in the interphase. This ring was transferred into a new tube and spun down (5 minutes, 1750 rpm). The pelleted cells were washed twice with MACS-buffer: The pellet was resuspended in a few ml of buffer, filled up to 50 ml and spun down (5 minutes, 1750 rpm). After discarding the supernatant, the washing step was repeated. The number of PBMC was determined by the Coulter particle count & size analyzer (Beckman Coulter, Inc., Fullerton, CA) and the cells were used for isolating monocytes and T-cells.

5.3. Magnetic cell sorting (MACS)

Magnetic cell sorting (MACS) is a method for selective enrichment or depletion of cells expressing a surface protein distinct for their cell type. For this purpose, cells can be labelled with biotin-conjugated antibodies directed against the specific molecule. These labelled cells can be targeted by a secondary antibody directed against the biotin residues on the primary antibody. Since the secondary antibody is coupled to paramagnetic beads, specific cells in a mixture can be selectively retained in the iron mesh of the column once a strong magnetic field is applied. While nonlabelled cells pass through the column, retained cells may be eluted by removing the magnetic field.

Material:

Straptavidin-Microbeads

Magnetic cell separator VarioMACS

Separation column CS

(all from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)

5.3.1. Isolation of monocytes from PBMC

Monocytes were positively selected by MACS from PBMCs. For the selection the monocyte marker CD14 was used.

Around 1×10^9 PBMCs were used for one MACS separation column. The cells were resuspended in 750 µl MACS-buffer and 250 µl biotinylated anti-CD14 mAb (VIM13 and MEM18, both 15 µg/ml, Otto Majdic) were added (15 min, 4° C). After washing twice with MACS-buffer the pellet was resuspended in 750 µl MACS-buffer and 250 µl streptavidin-microbeads were added (15 min, 4° C).

To wash the column, it was assembled and placed in the magnetic field. Buffer was injected through the 3-way stopcock using the side syringe. The cell suspension was pressed through a 70 µm strainer and loaded onto the column. Buffer was added untill 40 ml had passed through. A 0,8 µm needle was used to control the flow rate. This first fraction contained T-, B- and NK-cells. For the second fraction the column was removed from the magnetic field and the buffer was added in 10 ml portions alternating through the side syringe or loaded on the top of the column. After each addition step the column was put back into the magnetic field and about 10 ml were let run through. For this fraction a 0,9 µm needle was used. The steps were repeated until 50 ml had passed through.

The third and monocyte containing fraction was collected after removal of the column from the magnetic field. Buffer was filled several times into the reservoir of the column. After each step the liquid was taken out through the lateral valve using a syringe. These steps were repeated until a volume of 40 ml had been collected. The number of monocytes was determined after centrifugation (5 min, 1750 rpm) and resuspension.

The purity (> 95%) of the isolated monocytes was controlled by immunofluorescence analysis. Double stainings for surface markers were performed with the directly labelled antibodies CD4-PE / CD3-FITC, CD8-PE / CD3-FITC, CD19-PE / CD3-FITC, CD56-PE / CD3-FITC, HLA-DR / CD3-FITC, HLA I-FITC, CD14-PE / HLA-D-FITC.

5.3.2. Isolation of T-cells

The first fraction of the monocyte isolation protocol was used for the selection of T-cells by depletion of CD11b (mAb: LM2, 15 µg/ml), CD14 (MEM18, 15 µg/ml), CD16 (3G8, 15 µg/ml), CD19 (BU12, 15 µg/ml), CD33 (CD33-4D3, 30 µg/ml) and MHC II (VID1, 15 µg/ml) positive cells.

After centrifugation (5 min, 1750 rpm), the cells were resuspended in 750 µl MACS-buffer and 250 µl biotinylated mAb-mix (anti-CD11b, CD14, CD16, CD19, CD33 and MHCII mAb) were added (15 min, 4° C). After washing twice with MACS-buffer, the pellet was resuspended in 750 µl MACS-buffer and 250 µl streptavidin-microbeads were added (15 min, 4° C).

To wash the column, it was assembled and placed in the magnetic field. Buffer was injected through the 3-way stopcock using the side syringe. The cell suspension was pressed through a 70 µm strainer and loaded onto the column. Buffer was added until 50 ml had passed through. For the negative depletion, a 0,7 µm needle was used to control the flow rate.

The collected fraction was spun down and the cells were counted. The purity (>95%) was controlled by immunofluorescence analysis. Double stainings for surface markers were performed with the directly labelled antibodies CD4-PE / CD3-FITC, CD8-PE / CD3-FITC, CD19-PE / CD3-FITC, CD56-PE / CD3-FITC, HLA-DR / CD3-FITC, HLA I-FITC, CD14-PE / HLA-D-FITC.

5.4. Generation of md-DC

md-DC were generated by culturing purified peripheral blood CD14+ monocytes for 5-7 days in RPMI1640 (Gibco, Invitrogen GmbH, Lofer, Austria) supplemented with 10% FCS (Sigma-Aldrich, St. Louise, MO), 2 mM L-glutamine (Gibco, Invitrogen GmbH, Lofer, Austria), 100 U/ml penicillin, 100 U/ml streptamycin (Sigma-Aldrich, St. Louise, MO), 50 ng/ml GM-CSF and 100 U/ml IL-4. Cells were incubated at 37° C, 5 % CO_2.

5.5. Freezing & thawing of cells

Mammalian cells can be stored in liquid nitrogen for prolonged periods of time with minimal loss of viability in subsequent cell culture. For that purpose, cells were spun down, counted and resuspended in freezing medium (RPMI1640 supplemented with 20% FCS, 100U/ml penicillin, 100 μg/ml streptamycin, 2mM L-glutamine and 10% DMSO) to a concentration between $1 – 5x \, 10^7$ cells/ml. 1 ml aliquots were filled into cryotubes (Nalgen Nunc Internation, Roskilde, Denmark) and stored overnight at -80° C in a freezing box filled with isopropanol prior to transfer to liquid nitrogen.

For thawing, frozen cells were warmed with lukewarm tap-water until a clump of frozen cells was left in the tube. After 5 minutes, the cell suspension was transferred to a 20 ml tube and droplets of supplemented cell culture medium where added in an interval of 1 min: Starting with 5 drops at 0`, 10 drops at 1`etc. up to 5 minutes. After two washing steps, the cell number was determined by the Coulter particle count & size analyzer (Beckman Coulter, Inc., Fullerton, CA).

5.6. Stimulation of DC

DC were cultured at a density of $1x10^6$ /ml (most experiments) or $2x10^6$ /1.5ml (for RNA isolation) in supplemented RPMI (+10% FCS +100U/ml penicillin + 100

µg/ml streptamycin and 2mM L-glutamine). Stimulations were carried out at the indicated concentrations (see table).

Stimulus	Concentration /ml	Source
LPS	0,1-1 µg	Sigma-Aldrich
pI:C	2 µg	Sigma-Aldrich
R-848	2 µg	Alexis Biochemicals
HRV14	5 $TCID_{50}$	Joachim Seipelt
HRV2	5 $TCID_{50}$	Joachim Seipelt
pI:C + Lipofectamine2000	0,2 µg + 0,5 µl	Sigma-Aldrich
HRV14 RNA + Lipofectamine2000	0,0172 / 0,086 / 0,43 pmol + 0,1 / 0,5 / 2,5µl	In vitro transcription
HRV2 RNA+ Lipofectamine2000	0,0172 / 0,086 / 0,43 pmol + 0,1 / 0,5 / 2,5 µl	In vitro transcription
Control RNA + Lipofectamine2000	0,0172 / 0,086 / 0,43 pmol + 0,025 / 0,125 / 0,625 µl	In vitro transcription
Lipofectamine2000	0,1 / 0,5 / 2,5µl	invitrogen

Table 3: Overview about reagents used for DC stimulation

5.7. Virus preparations

Virus preparations were obtained from the lab of Prof. Joachim Seipelt. Virus stocks prepared in Hela Ohio cells (as described in [96]) were further purified using sucrose gradients. Virus was pelleted by centrifugation at 100 000 g for 2 h and resuspended in small volume „virus buffer" (50 mM Tris (pH 7,5), 2 mM $MgCl_2$). It was then treated with DNase I (5 mg/ml, Fermentas, St. Leon-Rot, Germany) and RNase A (5 mg/ml, Fermentas, St. Leon-Rot, Germany) for 10 min at RT and further digested with trypsin (0,5 mg/ml, Dipro) for 5 min at 37° C. After addition of N-laurylsarcosin (0,1 %, Schuchardt, Munich, Germany) digestion was continued overnight at 4° C. After removal of the low-speed centrifugation, the sample was centrifuged on a sucrose gradient (7,5 – 45 % sucrose in virus buffer) for 2 h at 155 000 g. Virus-containing fractions are visible as a turbid band in the middle of the gradient and were collected with a syringe. Virus was

concentrated by pelleting and resuspension in virus buffer. Virus stocks had a concentration of 1,61 x10^9 (HRV14) and 6 x10^8 (HRV2) / TCID$_{50}$ /ml and were stored at -80° C until use. Virus stocks were tested for LPS contaminations via FACS analysis of maturation markers after DC stimulation with virus or heat-inactivated virus and compared to LPS and heat-inactivated LPS treated DC.

5.8. Measurement of cytokines from culture supernatants

The Fluorokine® MultiAnalyte Profiling (MAP) system (R&D Systems Inc., Minneapolis, MN), provides a tool to simultaneously measure multiple cytokines in a single sample. Analyte-specific antibodies are pre-coated onto color-coded beads. Standards and samples are pipetted into the wells and analytes of interest are bound by the immobilized antibodies. After washing away any unbound substances, biotinylated antibodies specific for the analytes of interest are added. Following a wash, captured analyte is detected using streptavidin-phycoerythrin (S-PE). The Luminex^{100} analyzer is a dual laser, flow-based, sorting and detection platform. One laser is bead-specific and determines which cytokine is being detected. The other laser determines the magnitude of PE-derived signal, which is in direct proportion to the amount of analyte bound. Supernatants from various experiments after 48h of DC stimulation were measured via the Luminex^{100} System for their content of IL-10, IL-12p40, IL-6 and TNFα.

5.9. IFNα enzyme-linked immunosorbent assay (ELISA)

IFNα concentrations in the supernatants of stimulated DC (48h) were determined by human IFN-α ELISA kit (PBL InterferonSource, NJ).

5.10. Linearization of DNA templates used for in vitro transcription

T7 Polymerase in vitro transcription plasmids encoding full-lenght genomic HRV14 / HRV2 RNA including the poly A tail (plasmids provided by Prof. Joachim Seipelt) were linearized by restriction enzyme digestion. For this purpose, 10 µg of DNA from maxiprep (Qiagen, Germantown, MD) were incubated with 2 µl BSA 100x, 5 µl appropiate 10x reaction buffer (both New England Biolabs, Frankfurt am Main, Germany), either 3 µl MluI (HRV2, 10 000U/ml, New England Biolabs Frankfurt am Main, Germany,) or 3 µl KpnI (HRV14, 10 U/µl, Fermentas, St. Leon-Rot, Germany) and nuclease free water (Gibco, Invitrogen GmbH Lofer, Austria) in a total volume of 50 µl (overnight, 37° C).

Successful linearization was checked by agarose gel electrophoresis by comparing linearized and circular templates (1% agarose in 1xTAE, 90 V, 1,5 h). The linearization reaction was stopped by adding 1/10 5M NH_4 acetate stop solution and 2 volumes of 96% ethanol (1h, -20 C). The pelleted DNA (40 min, 13000 rpm, 4° C) was washed with 200 µl 70% ethanol and centrifuged once more (15 min, 13 000 rpm, 4° C). The dried pellets (20 min, RT) were resuspended in 20 µl nuclease free water (all reagents except ethanol provided with MEGAscript High Yield Transcription Kit, Ambion, Austin, TX).

5.11. RNA preparation by in vitro transcription

To generate high amounts of viral RNA the MEGAscript High Yield Transcription Kit (Ambion, Austin, TX) was used: 1 µg of linear DNA template was mixed with 8 µl of NTP mix, 2 µl of 10x reaction buffer, 2 µl T7 polymerase enzyme mix and 7 µl of nuclease free water (4h, 37° C). The DNA template was digested by addition of 1 µl of TURBO DNase (15min, 37° C). The in vitro transcribed RNA was precipitated by adding 30 µl of LiCl precipitation solution and 30 µl of

nuclease free water (30 min, -20° C). After centrifugation (15 min, 13000 rpm, 4° C) the RNA was washed with 70% ethanol. Pelleted (15min, 13000 rpm, 4° C) and dried (5 min, RT) RNA was resuspended in 50 µl nuclease free water. RNA concentrations were determined at 260 nm with a BioPhotometer (Eppendorf, Germany).

To obtain dephosphorylated RNA part of the RNA was treated with 1 U / 20 pmol RNA of calf intestine alkaline phosphatase (30min, 37° C, Fermentas, St. Leon-Rot, Germany). Purified (de-) / phosphorylated RNA (RNeasy Mini Kit, Qiagen, Germantown, MD) was eluted with 30 µl of nuclease free water and the RNA concentration was determined once more. RNA was stored in aliquots at -80° C until use. Size, integrity and purity of RNA was determined by agarose electrophoresis (1% agarose in 1xTAE, 90V, 2h) after denaturing RNA in 5x RNA loading buffer (16 µl saturated aqueous bromophenol blue solution, 80 µl 500mM EDTA pH 8.0, 720 µl 37% formaldehyde, 2 ml 100% glycerol, 3,084 ml formamide and 4 ml 10x FA gel buffer containing 200 mM MOPS, 50 mM sodium acetate and 10 mM EDTA pH 7.0) at 65° C for 3-5 minutes.

As a control, 1µg of linearized control template (provided with the kit) encoding the 1,89 kb Xenopus elongation factor 1α gene was included in each in vitro transcription reaction and part of it was also dephosphorylated as described above.

5.12. Isolation of total cellular RNA

Total cellular RNA / viral RNA was isolated using TRI reagent (Sigma, St. Louis, MO). Up to 1x 10^7 pelleted cells or 200 µl of viral suspension were resuspended in 1ml TRI reagent (5min, RT; optional: -20° C until need). To remove proteins 200 µl of chloroform were added, vortexed (15 s) and incubated for 2-15 min at RT. After centrifugation (15 min, 13 000 rpm, 4° C) the aqueous phase was transferred into a new tube and mixed with 500 µl isopropanol. After 5-10 min at RT the samples were centrifuged (10 min, 13 000 rpm, 4° C) and the pellets were washed with 1 ml 75% ethanol. After a final centrifugation (10 min, 13 000rpm, 4°

C) the RNA pellet was air-dried for 5-10 min at RT and resuspended in 30 µl (viral RNA) or 12 µl (for cDNA) nuclease free water (Gibco, Invitrogen GmbH, Lofer, Austria).

5.13. Generation of cDNA

To generate cDNA 800-1000 ng of isolated total RNA from stimulated cells was reverse transcribed with Revert Aid MuLV-RT (Fermentas, St. Leon-Rot, Germany) using Oligo (dT)18 primers according to the manufacturer`s protocol. 800-1000 ng of RNA were mixed with 1 µg oligo (dT)18 primers and NFW was added up to 11µl. After incubation at 70° C for 5 minutes the samples were chilled on ice. To each reaction 4 µl of 5x MuLV-RT buffer, 2ul dNTP mix (10mM each), 0,5 µl RiboLock RNase Inhibitor (20 U), 1 µl Revert Aid MuLV reverse transcriptase (200 U), and NFW to a final volume of 20 µl were added. The mixture was incubated for 5 minutes at 37° C, followed by incubation at 42° C for 60 minutes. cDNA was stored at -20° C until use.

5.14. Real-Time PCR (RT-PCR)

Quantitative Real-Time PCR (RT-PCR) was performed by the Mx3005P QPCR system (Stratagene, La Jolla, CA) using SYBR Green detection. In all assays, cDNA was amplified using a standard program (2 min at 50° C, 10 min at 95° C, 40 cycles of 15 sec at 95° / 15 sec at 60° C / 30 sec at 72° C, 1 min at 95° C, 30 sec at 54° C and 1 min at 95° C).

Each well of the MicroAmp 96well plate (Applied Biosystems, Foster City, CA) was loaded with 0,34 µl cDNA, 1 µl primer mix (5 mM each primer), 5 µl Sybr Green Power PCR Mix (Applied Biosystems, Foster City, CA) and 3,66 µl nuclease free water (Gibco, Invitrogen GmbH, Lofer, Austria) to give a total volume of 10 µl. Relative quantification of target gene expression was performed by the MxPro QPCR software from Stratagene (housekeeping gene: G3DPH).

The testing of primer specificity included melting point analysis and agarose gel eletrophoresis of the PCR products.

Primer sequences:

IFN-beta:

Forward: 5`-TGCATTACCTGAAGGCCAAGG-3`

Reverse: 5`-GCAATTGTCCAGTCCCAGAGG-3`

HRV14:

Forward: 5`-GGCGCCATATCCAATGGTGT-3`

Reverse: 5`-TCCACCTGATCGAACGTCCA-3`

5.15. Electrophoretic mobility shift assay (EMSA)

EMSA were performed by Karl Stuhlmeier at the Franz Joseph Spital, Vienna.[113] For this purpose, $5x10^6$ md-DC were mock-treated, stimulated with 100ng/ml LPS for 30minutes or 5 $TCID_{50}$/cell HRV14 for 30 minutes, 1h, 3h, 5h, 7h and 9h. Stimulation was stopped by adding ice-cold PBS. Cells were transported on ice.

5.16. Flow cytometry

In a flow cytometer, single cells move past the excitation source and the light hitting the cells is either scattered or absorbed and then re-emitted (fluorescence). This scattered or re-emitted light is collected by a detector. Scattered light is the consequence of a light beam making contact with a cell, resulting in either reflected or refracted light reaching the detector. The pattern of light scattering is dependent on cell size and shape, giving relative measures of these cellular characteristics as cells flow through the beam. This can be quite useful, as cells can be sorted on the basis of size or shape to different collection tubes using a technique called electrostatic deflection, which employs charged plates to change the path of the cell. Fluorescence-based detection depends on

the absorption of light by the cell and the subsequent re-emission of this light at a different wave-lenght.

Flow cytometers make use of this technology by employing filters to block the original light source from reaching the detector, while the fluorescence emission is allowed through the detector, which allows only a very low background of stray light to reach a detector. In flow cytometry experiments, fluorescence is often achieved by the deliberate labelling of a cellular component using a fluorescent marker, usually a type of dye, covalently bound to an antibody. These dyes emit light only if light of the appropriate wavelenght (specified by the frequency of the laser) hits them, causing the emission of secondary light at a different wavelenght. Detection of the second wavelenght is used as a measure of the presence of the antigen recognized by the antibody on the cell.

5.16.1. Immunofluorescence of surface proteins

Binding of mAbs to Fcγ -receptors was blocked by incubation of cells with human immunoglobin (Beriglobin). As a negative control VIAP, a non-binding calf intestine alkaline phosphatase specific antibody was used. For secondary labelling, we applied an oregon green-conjugated goat anit-mouse antibody. Dead cells were excluded from the analysis by staining with propidium iodide.

PBS/BSA: 1x PBS def. + 1% BSA

Beriglobin (Aventis Behring GmbH, Vienna) diluted 1:8 in PBS/BSA to final concentration of 20mg/ml

Primary antibodies: 20 µg/ml

Secondary antibodies: oregon green-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, Oregon), 20 µg/ml in PBS/BSA

Propidium iodide: 1ug/ml in PBS/BSA

Pelleted cells ($5x10^5$/Assay, 5min, 1750 rpm) were resuspended in 50 µl beriglobin/assay and kept on ice for 20min. 50 µl of cell suspension were added

to 20 µl primary antibody in micronic-tubes (after mixing 30min at 4° C). Each assay was washed twice with PBS/BSA (5 min, 1750 rpm). Cells were incubated with 20 µl of secondary antibody/assay (30min, 4° C).

After washing cells twice with PBS/BSA, dead cells were labeled with propidium iodide (1:100) and mixed with 25-50 µl sheath fluid (BD FACS Flow, BD Bioscience, San Jose, CA).

The tubes were kept at 4° C in the dark until analyis by flow cytometry using the FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA).

5.16.2. Immunofluorescense of cytoplasmic proteins

Fixation medium
Permeabilization medium
Both from (Bio Research GmbH, An der Grub, Kaumberg, Austria)

Pelleted cells (5×10^5/Assay, 5min, 1750 rpm) were resuspended in 50 µl beriglobin/assay and kept on ice for 20min. 50 µl of cell suspension were added to 50 µl of fixation medium (20 min, RT). After washing twice (5min, 1750 rpm) cells were incubated with 20 µl primary antibody (20 µg/ml) and 50 µl permeabilization medium in micronic-tubes (after mixing 20min at RT). Each assay was washed twice with PBS/BSA (5 min, 1750 rpm). Cells were incubated with 20 µl of secondary antibody (20 µg/ml)/assay (20min, RT).

After washing cells twice with PBS/BSA they were mixed with 25-50 µl sheath fluid (BD FACS Flow, BD Bioscience, San Jose, CA).

No propidium iodide was added prior analysis by flow cytometry using the FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA).

5.17. HEK Luciferase Reporter Assay

For the HEK Luciferase Reporter Assay a luciferase reporter plasmid was transfected into HEK293 cells along with an expression plasmid for a given viral

VPRR. Production of the reporter protein luciferase is only induced upon VPRR activation which causes IFN production and NFκB activation required for reporter gene expression. The quantification of the reporter protein activity after stimulation, normalized to mock treated cells, allows to identify stimuli which are specifically recognized by certain VPRR.

HEK293 cells were seeded in 96 flat bottom well plates at 100 000 cells / well in 100 µl supplemented IMDM (IMDM, 10% FCS +100 U/ml penicillin + 100 µg/ml streptamycin and 2 mM $_L$-glutamine). 24 hours later, cells were transiently transfected by $CaCl_2$ transfection. For this purpose, 1 µg ß-galactosidase (ß-gal) plasmid, 1 µg VPRR plasmid and 0,04 – 0,2 µg reporter plasmid were mixed with nuclease free water to give a final volume of 45 µl. After incubation with 5 µl 2,5M $CaCl_2$ (5min, RT), 50 µl of 2x HBS buffer pH 6.95 were added during vortexing. After 1 minute rest, 10 µl of the stock were pipetted to each well.

20 hours after transfection, medium was changed to supplemented IMDM lacking antibiotics. Stimulations with Transfectin (BIORAD, Hercules, CA) complexed pI:C (1 ug) / HRV14 or control RNA (between 0,0172 – 1,68 pmol) were carried out for 12-18 hours. After stimulation the medium was carefully aspirated and cells were lysed with 25 µl / well 1x CCLR lysisbuffer (Promega, Madison, WI) for 1 minute. Plates were stored at -20° C overnight or at -80° C for a maximum of 3 days.

Luciferase activity was asssesed by transfering 10 µl of lysate to 96 well Microlite 1+ plates and measuring light emission after addition of 50 µl / well luciferase assay reagent (Promega, Madison, Wisconsin) using the Labsystems Luminoskan RS luminometer (lag time 2 sec, total time 12 sec, Labsystems, Finland).

ß-gal activity, as a measure of transfection efficiency, was assesed by addition of 100 µl ß-gal substrate solution (0,1 M $NaPO_4$ pH 7.5, 1 mM $MgCl_2$, 45 mM ß-mercaptoethanol, 1 mg/ml ONPG) / well to the remaining lysate. After 5-10 minutes incubation (37 C, 5% CO_2) the absorbance at 405 nm compared to 650 nm was measured using the THERMOmax microplate reader (Molecular Devices, Toronto, Canada).

Insert	Plasmid	Supplied by	
flag**RIG**-**I** full lenght	pEF-BOS	Takashi Fujita	Laboratory of molecular genetics Kyoto University (Japan)
Luciferase	pISRE-TA-Luc	Promega	Madison, Wisconsin
Luciferase	p NFκB -TA-Luc	Promega	Madison, Wisconsin

Table 4: Overview about plasmids used for the HEK luciferase reporter assay

5.18. Preparation of cell lysates

For preparing cell lysates 1×10^6 DC were pelleted (3min, 3000rpm) and resuspended in 75 µl 2x Laemmli buffer containing 50 µl ß-mercaptoethanol / ml (both BIORAD, Hercules, CA). Samples were frozen in liquid nitrogen and transfered to -80° C for long term storage.

The samples were sonicated (15 s) and denatured (5 min, 95° C) before SDS-PAGE.

5.19. SDS-polyacrylamide electrophoresis (SDS-PAGE)

Under denaturing conditions SDS masks the charged residues of proteins. SDS-PAGE therefore allows the eletrophoretic separation of proteins according to their size. Protein size is determined by using a protein standard of defined molecular weights.

The gel caster (Hoefer Scientific Instruments, San Francisco, CA) was prepared by assembling ethanol-cleaned glass / aluminium oxide plates and spacers. The running gel was prepared, cast up to 2 cm below the upper edge of the glass plate and covered with 96% ethanol (20min). After removing residual ethanol, the stacking gel was added and a comb carefully placed on top (20min).

After successful polymerization the chambers in the gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) were filled with 1x running buffer. Samples (10-12 µl) were loaded along with 5 µl SeeBlue Plus 2 Prestained Standard (Invitrogen GmbH, Lofer, Austria). Empty slots were filled with Laemmli

buffer. Gels were run constant at 60 V until the running gel was reached. Then the voltage was set to 120 V till proper separation.

Running gel (10%, for 2 gels):

4,8 ml aqua dest.

3 ml Tris HCl 1.5 M pH 8.8

4 ml Rotiphorese Gel 30 solution (Roth, Karlsruhe, Germany)

100 µl 10% SDS

100 µl 10% APS

8 µl TEMED

Stacking gel (4%, for 2 gels):

2 ml aqua dest.

840 µl Tris-HCl 0,5 M pH 6.8

400 µl Rotiphorese Gel 30 solution (Roth, Karlsruhe, Germany)

35 µl 10% SDS

35 µl 10% APS

4 µl TEMED

5.20. Westernblot analysis

Blotting buffer (1x)

100 ml stock (25 mM Tris, 192 mM glycine)

100 ml methanol

800 ml aqua dest.

PBS-T (wash solution):

1x PBS + 0,05% Tween-20

Blocking solution:

5% milk in PBS-T

After electrophoresis, SDS-gels were separated from the glass plates and marked at one end. The PVDF membrane (Immobilon-P, 0.45 µm, Millipore, Billerica, MA) and filterpapers (blotting paper 703, VWR, Darmstadt, Germany) were cut to fit the size of the gel. The PVDF membrane was shaken with methanol (1 min) and equilibrated together with the gel in blotting buffer (5 min). Finally the sandwich was assembled by putting membrane and gel between 3 buffer soaked filter papers at each side. The proteins were transfered onto the membrane at a constant voltage (15 V, 1h) using the Semi-Phor blotting apparatus (Hoefer Scientic Instruments, San Francisco, CA).

To prevent antibodies from unspecific binding to the membrane, it was shaken with blocking solution (1 h, 4° C). Incubations with the primary antibody were carried out in a 50 ml falcon tube (Sarstedt, Nümbrecht, Germany) with a minimum of 4 ml antibody solution diluted in blocking solution (7 rpm, overnight, 4° C). After washing (3x 10 min) membranes were incubated with the secondary HRP conjugated antibody dilution (blocking buffer) in a 50 ml falcon tube (7 rpm, 1 h, 4° C). Before ECL, membranes were given a final wash (3x 10min).

Specificity	Clone	Isotype	Species	Dilution	Company
IkBα	Sc-371	IgG	Rabbit	1:1000	Santa Cruz Biotech
p38	-	IgG	Rabbit	1:1000	Cell Signaling
Rabbit IgG	-	IgG-HRP	Goat	1:2000	Dako

Table 5: Polyclonal antibodies used for westernblot analysis

5.20.1. Development of blots by enhanced chemiluminescence (ECL)

ECL solution (200 ml, stored in the dark):

20 ml 1 M Tris pH 8.8

1 500 µl aliquot p-coumaric acid (340 mg in 26 ml DMSO, Sigma-Aldrich, St. Louise, MO)

1 1 ml aliquot Luminol (2,26 g in 51 ml DMSO, Fluka, Sigma-Aldrich, St. Louise, MO)

180 ml aqua dest.

For ECL, membranes were incubated with ECL solution mixed with 3 µl / ml 3% hydrogen peroxide for 1 minute, placed between two plastic overhead sheets, covered with a Kodak Biomax XAR film (Sigma-Aldrich, St. Louise, MO) and placed into a film casette. After exposure from 1 sec up to 1 hour specific bands were visible on the film where light had been emitted during HRP enzyme activity after developing the film in a developing machine.

5.20.2. Stripping of membranes for reuse

After ECL, membranes were either stored at 4° C or washed 3x 5min with PBS-T to remove ECL solution. To allow incubation with antibodies detecting proteins of similiar size, bound antibodies were removed by incubation of the membranes in 40 ml stripping buffer (6,25 ml 1 M Tris pH 6.8, 20 ml 10% SDS up to 100 ml with aqua dest. + 704 µl ß-mercaptoethanol) at 65° C for 20min.
To remove ß-mercaptoethanol, the membranes were washed 20x with dH_2O and finally blocked with blocking solution (shaking, 1 h, 4° C).

5.21. Allo mixed leukocyte reaction (Allo MLR)

The MLR is a system to simulate a graft-versus-host reaction: lymphocytes from a potential donor are mixed with stimulator cells from a potential recipient. If the two cell populations are not compatible in their MHC (allogeneic), proliferation of the T-cells occurs. This expansion is determined by measuring the methyl-^{3}H-thymidine incorporation into the DNA.
Graded numbers of DC (333, 1111, 3333 and 10000 cells) were mixed with 10^5 responder T-cells from a different donor in supplemented RPMI 1640 in 96 well round bottom plates under standardized culture conditions (37° C, 5% CO_2, humidified atmosphere). On day 5, 25 µl of 1:20 diluted methyl-^{3}H-thymidine (1 µCi / well) (ICN Pharmaceuticals Inc., Irvine, CA) was added to the cells. After 18

h incubation, the cells were lysed and their DNA collected on filter-plates (Perkin Elmer, Wellesley, MA). To each well on dried plates (37° C, 1 h) 25 µl of microscint scintillation mix (Packard, Meriden, Conneticut) was added. Radioactive emission was quantified on a Packard microplate scintillation counter (Packard, Meriden, Conneticut). For each setting triplicates were used.

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