2010-08-19

Dissecting the Role of Innate Pattern Recognition Receptors and Interferon Regulatory Factor-5 in the Immune Response to Human Metapneumovirus and other Pathogens: A Dissertation

Zhaozhao Jiang

University of Massachusetts Medical School

DISSECTING THE ROLE OF INNATE PATTERN RECOGNITION RECEPTORS AND INTERFERON REGULATORY FACTOR-5 IN THE IMMUNE RESPONSE TO HUMAN METAPNEUMOVIRUS AND OTHER PATHOGENS

A Dissertation Presented

By

Zhaozhao Jiang

Submitted to the Faculty of the

University of Massachusetts Medical School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

AUGUST 19, 2010

INTERDISCIPLINARY GRADUATE PROGRAM

DISSECTING THE ROLE OF INNATE PATTERN RECOGNITION RECEPTORS AND INTERFERON REGULATORY FACTOR-5 IN THE IMMUNE RESPONSE TO HUMAN METAPNEUMOVIRUS AND OTHER PATHOGENS

A Dissertation Presented

By

Zhaozhao Jiang

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Katherine Fitzgerald, Ph.D., Thesis Advisor

Michelle Kelliher, Ph.D., Member of Committee

Daniel Libraty, M.D., Member of Committee

Egil Lien, Ph.D., Member of Committee

Amy Hise, M.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Neal Silverman, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
August 19, 2010

Acknowledgements

Graduate study at UMASS is definitely one of the most important and memorable periods in my life. I have been working with a group of brilliant people lead by Dr. Kate Fitzgerald. I have been extremely fortunate to have Kate as my advisor. She is not only an intelligent scientist but also a great director and teacher. Kate always has good ideas and is willing to test them with hard work. She is always ready to help guide us to find the solution for the questions and problems that come up in our research. I have learned from her to reach conclusions based on very solid data. She has also advised me to broaden my academic knowledge by reading more and given me suggestions for furthering my career.

It is an honor to have Drs. Neal Silverman, Michelle Kelliher, Daniel Libraty and Egil Lien on my TRAC committee and thesis committee. I have appreciated their comments and suggestions and, most importantly, the motivation they have given me. I am also grateful to Dr. Amy Hise for evaluating my dissertation.

Life in the lab has been enjoyable because of the great working environment provided by all the people I work with: Lisa Waggoner, Shruti Sharma, Joseph Conlon, Sandhya Ganesan, Vijay Rathinam, Brian Monks, Anna Cerny, Shenghua Zhou, Martina Severa, Mikayla Thompson, John Kaminski, Jennie Chan, Stefan Schattgen, Soren Jensen, Cara West, and all the members

in the Golenbock, Latz, Silverman, and Lien labs. I also thank them for their time spent reading my thesis.

In particular, I would like to thank Nadege Goutagny. From the first day I came to this lab, she has guided and helped me learn all the techniques. She is not only a collaborator, but also my good friend who makes my life brighter.

Finally, I would like to thank my family. Although my parents and brother are far away from me, their support is always around. My husband and my daughter(s) have been bringing me all the happiness I could enjoy in a family during my time here.

Table of Contents

List of publications

1. Marchlik E, Thakker P, Carlson T, **Jiang Z**, Ryan M, Marusic S, Goutagny N, Kuang W, Askew GR, Roberts V, Benoit S, Zhou T, Ling V, Pfeifer R, Stedman N, Fitzgerald KA, Lin LL, Hall JP. 2010. Mice lacking Tbk1 activity exhibit immune cell infiltrates in multiple tissues and increased susceptibility to LPS-induced lethality. *J Leukoc Biol.* [Epub ahead of print]

Zhaozhao Jiang performed experiment testing the activation of IRF3 and IFNβ in WT or TBK1 deficient macrophages upon LPS stimulation, as well as the reporter assay with different TBK1 expressing plasmids. (Figure 2C,D,E of the paper)

2. Rathinam VA, **Jiang Z**, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol.* 11(5): 395-402.

Zhaozhao Jiang performed experiments monitoring the IFNβ activation in splenocytes, and the reporter assay as well as WB testing inflammasome activation in 293T cells. (Figure 2A,B, 4A,E of the paper)

3. Goutagny N, **Jiang Z**, Tian J, Parroche P, Schickli J, Monks BG, Ulbrandt N, Ji H, Kiener PA, Coyle AJ, Fitzgerald KA. 2010. Cell type-specific recognition of human metapneumoviruses (HMPVs) by retinoic acid-inducible gene I (RIG-I) and TLR7 and viral interference of RIG-I ligand recognition by HMPV-B1 phosphoprotein. *J Immunol.* 184(3):1168-79.

*Goutagny N and **Zhaozhao Jiang** contributed equally.

4. Cole LE, Laird MH, Seekatz A, Santiago A, **Jiang Z**, Barry E, Shirey KA, Fitzgerald KA, Vogel SN. 2010. Phagosomal retention of Francisella tularensis results in TIRAP/Mal-independent TLR2 signaling. *J Leukoc Biol*. 87(2):275-81.

Zhaozhao Jiang prepared peritoneal macrophages for analysis of Fransicella responses.

5. Ablasser A, Poeck H, Anz D, Berger M, Schlee M, Kim S, Bourquin C, Goutagny N, **Jiang Z**, Fitzgerald KA, Rothenfusser S, Endres S, Hartmann G, Hornung V. 2009. Selection of molecular structure and delivery of RNA oligonucleotides to activate TLR7 versus TLR8 and to induce high amounts of IL-12p70 in primary human monocytes. *J Immunol.* 182(11):6824-33.

Zhaozhao Jiang performed quantitative PCR comparing the MDA5 expression levels in PDCs and monocytes. (Figure 2D middle panel of the paper)

6. Pandey AK, Yang Y, **Jiang Z**, Fortune SM, Coulombe F, Behr MA, Fitzgerald KA, Sassetti CM, Kelliher MA. 2009. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. *PLoS Pathog.* 5(7):e1000500.

Zhaozhao Jiang performed reporter assay testing the activation of IRF5 by RIP2 for type I IFN induction in 293T cells. (Figure 7B,C of the paper)

7. Chen W, Lam SS, Srinath H, **Jiang Z**, Correia JJ, Schiffer CA, Fitzgerald KA, Lin K, Royer WE Jr. 2008. Insights into interferon regulatory factor activation from the crystal structure of dimeric IRF5. *Nat Struct Mol Biol.* (11):1213-20.

Zhaozhao Jiang performed reporter assay to correlate structure-function relationships of IRF5 and IRF3 and implications to functional activity of these IRFs in terms of IFNβ activation. (Figure 2B,D,E of the paper)

8. Paun A, Reinert JT, **Jiang Z**, Medin C, Balkhi MY, Fitzgerald KA, Pitha PM. 2008. Functional characterization of murine interferon regulatory factor 5 (IRF-5) and its role in the innate antiviral response. *J Biol Chem*. 23;283(21):14295-308.

Zhaozhao Jiang performed experiments examing the role of IRF5 in inflammatory cytokine and type I IFN responses in different immune cell subsets. (Figure 6C,D,F of the paper)

List of tables

List of figures

List of abbreviations

2-5A	2’-5’ linked AMP-oligomers
A1R	HMPV-A1 virus recovered from cDNA
APB	A1 virus expressing the P protein from B1 virus
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF2	Activating transcription factor 2
Atg	Autophagy-related protein
BDCA-2	Blood DC antigen 2
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
CARD	Caspase activation and recruitment domain
CBP	cAMP response element-binding (CREB)-binding protein
CDC/mDC	Conventional/myeloid DC
CIAP	Calf intestinal alkaline phosphatase
CMV	Cytomegalovirus
DAMP	Danger-associated molecular pattern
DBD	DNA binding domain
DC	Dendritic cell
dsRNA	Double strand RNA
EBOV	Ebola virus
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
HCV	Hepatitis C Virus
HEK	Human embryonic kidney
HMPV	Human metapneumovirus
HSV	Herpes simplex virus
IAD	IRF-associated domain
IAV	Influenza A virus
iE-DAP	D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
IFNAR	Interferon-α/β receptor
IKKε	I kappa B kinase
IL	Interleukin
IPAF	ICE Protease-Activating Factor
IPC	IFN-producing cell
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	Interferon Stimulated Gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KO	Knock-out

LGP2	Laboratory of genetics and physiology 2
Lm	*Listeria monocytogenes*
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
Mal	MyD88-adapter-like
MAP	Mitogen-activated protein
MAVS	Mitochondrial antiviral signaling protein
MBP	Myelin basic protein
MDA5	Melanoma differentiation-associated gene-5
MDP	Muramyl peptide
MEF	Mouse Embryonic Fibroblast
MHC	Major histocompatibility complex
mPDCA-1	Mouse PDC antigen 1
Mtb	*Mycobaterium tuberculosis*
MyD88	Myeloid differentiation primary response gene 88
NALP	NACHT, LRR and PYD domain-containing protein
NDV	Newcastle Disease Virus
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	NOD like receptors
NOD	Nucleotide-binding oligomerization domain containing
NS	Non-structural
OAS	Oligo-adenylate synthetase
ODNs	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PDC	Plasmacytoid dendritic cell
PGN	Peptidoglycan
PKR	dsRNA-dependent protein kinase
PolyIC	Polyriboinosinic polyribocytidylic acid
PRRs	Pattern recognition receptor
PYD	Pyrin domain
rhMPV-ΔG	A recombinant hMPV lacking the G protein
RIG-I	Retinoic acid-inducible gene-I
RIG-IC	RIG-I helicase domain only
RIP	Receptor-interacting serine-threonine kinase
RLR	RIG-I like receptor
RNAP	RNA polymerase
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLE	Systemic Lupus Erythematosus
ssRNA	Single strand RNA
STAT	Signal transducer and activator of transcription

STING	Stimulator of IFN gene
SV	Sendai virus
TBK1	TANK-binding kinase 1
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TYK2	Tyrosine kinase 2
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WNV	West Nile virus
WT	Wild type

Abstract

The Innate immune system is the first line of defense against invading microbial pathogens. It is a fast-acting and non-antigen-specific defense system, which employs germline encoded surveillance systems capable of responding to a broad-spectrum of pathogens. The innate immune system involves a variety of immune cells, which express different profiles of surveillance or detection receptors. Upon sensing pathogens, these receptors trigger cell signalling to turn on transcription of inflammatory cytokines, chemokines, anti-microbial peptides and type I Interferons. These effectors have direct effects on the control of pathogen load and also activate the adaptive immune system, which is ultimately required to clear infections. The type I interferons (IFNs) are the principal cytokines strongly induced during infection with viruses and are required for direct control of viral replication and modulation of cells of the adaptive immune response. The signalling pathways induced in order to activate type I IFNs are dependent on the interferon regulatory factors (IRFs). Striving for survival, microbes have evolved various strategies to subvert/impair these critical defense molecules.

In this thesis work, I have used Human Metapneumoviruses (HMPVs), a relatively newly described family of paramyxoviruses as model viruses to explore the role of pattern recognition receptors (PRRs) and the IRF family of transcription factors in the innate immune response. These studies revealed that the recognition of HMPV viral pathogen-associated molecular patterns (PAMPs)

by immune cells is different in different cell types. Retinoic acid-inducible gene-I (RIG-I), a cytosolic RNA helicases senses HMPV-A1 virus for triggering type I IFN activation by detecting its 5'- triphosphate viral RNA in most human cells, including cell lines and primary monocytes. An exception to these findings was plasmacytoid dendritic cells (PDCs), where Toll-like receptor (TLR)-7 is the primary sensor involved in detecting HMPV viruses. By comparing the innate immune response to two HMPV strains, we found that these two closely related strains had very different immune stimulatory capabilities. HMPV-1A strain triggered type I IFNs in monocytes, PDCs and cells of epithelial origin. In contrast, a related strain, HMPV-B1 failed to trigger IFN responses in most cell types. Our studies suggested that the phosphoprotein (P) of HMPV-B1 could prevent the viral RNA from being detected by RIG-I, thus inhibiting the induction of type I IFN production in most cell type examined. This finding adds to our understanding of the mechanisms by which viruses are sensed by surveillance receptors and also unveils new means of viral evasion of host immune responses.

Although IRFs are extensively studied for their role in regulating type I IFN activation, especially in TLR and RIG-I like receptor (RLR) signalling pathways upon viral infection, a clear understanding of how this family of transcription factors contributes to anti-viral immunity was lacking. Studies conducted as part of this thesis revealed that in addition to IRF3 and IRF7, which play a central role in anti-viral immunity downstream of most PRRs (e.g. TLRs, RLRs, DNA

sensors), the related factor IRF5 was also an important component of innate anti-viral defenses. Using IRF5-deficient mice we studied in detail the role of IRF5 in coordinating antiviral defenses by examining its involvement in signalling downstream of TLRs. These studies led us to examine the role of IRF5 in the regulation of type I IFNs as well as inflammatory cytokines in different cell types. While most TLRs that induced IFNβ showed normal responses in IRF5-deficient mice, CpG-B-induced IFNβ production in CD11c+CDCs isolated from mouse spleen but not those generated *in vitro* from bone marrow required IRF5. This was in contrast to responses with lipopolysaccharide (LPS) or polyriboinosinic polyribocytidylic acid (polyIC), ligands for TLR4 and 3, respectively. Moreover, we found that in contrast to IRF3 and/or IRF7, IRF5 was important in coordinating the expression of inflammatory cytokines such as TNFα downstream of some TLRs. In addition to our studies to examine the requirement for IRF5 in TLR signaling, we also showed that muramyl peptide (MDP) from *Mycobaterium tuberculosis* (Mtb) could activate type I IFNs via IRF5. This was the first evidence linking IRF5 to a non-TLR-driven pathway. IRF5 activation in this case was downstream of a novel nucleotide-binding oligomerization domain containing (NOD)-2/receptor-interacting serine-threonine kinase (RIP)-2 signaling pathway.

Collectively, the studies outlined in this thesis have assisted in providing a framework to understand the role of TLRs, RLRs and IRFs in the immune response to paramyxoviruses and have unveiled new mechanisms of activation

of the IRFs as well as new mechanisms by which pathogens subvert or evade these important innate defense mechanisms.

Chapter I Introduction

1.1 The Innate immunity

1.1.1 Introduction

The integrated human immune response comprises two major parts: the innate and the adaptive arms. The innate immune system is the first line of host defense against microbial infection after the physiological barriers (such as intact skin, tears, and mucous membranes) have been breached. Thinking that only vertebrates developed adaptive immunity while other organisms only have innate immune system to survive from pathogen attack, it is obvious that the innate immune response plays a very important role in protecting the host from infection or injury. The innate immune system is known as a non-antigen-specific defense system, which employs germline-encoded receptors and signaling components to enable it to respond to a broad-spectrum of pathogens. The response is fast-acting, beginning within minutes of pathogen exposure. While the innate immune response has no memory or lasting protection, it plays a critical role in activating the subsequent adaptive immune response, including the ability to evoke memory such that the response reacts quickly upon re-exposure to the same pathogen. In contrast to the innate immune system, the adaptive immune system is much slower acting and highly antigen specific as it is shaped by the antigen through somatic recombination of T and B lymphocytes. Memory T and B cells are subsequently developed for immunological memory.

In contrast to the adaptive immune system, which mainly employs T and B lymphocytes, the innate immune system involves a wide variety of cells of both lymphoid and myeloid lineages including monocytes, macrophages, dendritic cells (DCs), mast cells, neutrophils, natural killer (NK) cells, and NK T cells. Besides the cellular defenses, which also produce cytokines and chemokines to enhance the cell-mediated immune response, the innate immune response also contains an important humoral component with effector molecules, such as complement system proteins and antimicrobial peptides being involved for direct action on invading pathogens.

1.1.2 Cellular players

Monocytes. Circulating in the blood under steady state, monocytes are precursor cells for macrophages and dendritic cells. Microbial infection rapidly triggers monocytes to produce inflammatory cytokines and chemokines. There are two major subsets of human monocytes (CD14+ and CD16+) (1) and murine monocytes (Ly6C+ and CX3CR1+) (2). They express different levels of adhesion receptors and respond to distinct trafficking cues, and as a consequence differentiate into DCs or macrophages.

Macrophages. Macrophages are the most active and efficient phagocytes, which differentiate from circulating monocytes. F4/80 (mouse)/EMR1(human) is the most useful specific antigen marker to identify macrophage populations (3, 4). Macrophages can be found in many tissues, participating in innate response to pathogens as phagocytic cells. They are

equipped with a wide range of receptors for phagocytosis including opsonic receptors (complement receptors and Fc Receptors) (5, 6) and some pathogen recognition receptors (scavenger receptors, c-type lectins etc) (7, 8). When macrophages ingest a pathogen, an internal membrane compartment called the phagosome is formed, which then fuses with a lysosome, in which, enzymes and peroxides digest the pathogen. However some intracellular pathogens, for example *Listeria monocytogenes* (Lm), can escape from the phagosome into the cytosol to proliferate (9). Macrophages are also equipped with recognition molecules including TLRs (10) and cytosolic RNA helicases (11) that are not responsible for phagocytosis, but rather act as sensors which recognize microbial products and trigger signaling pathways leading to the production of cytokines and chemokines to enhance the immune response.

Dendritic cells. Dendritic cells (DC) were first identified in 1868 in the human epidermis by Paul Langerhans (12). It was not until 1973 when murine DCs were identified by Steinman and Cohn in mouse spleen (13). The term “dendritic cells” was coined based on the unique morphology of these cells with numerous dendrites extending from the main cell body. DCs constitute a heterogeneous population of cells that are derived from hematopoietic precursors and share common physical, phenotypic and functional characteristics. They develop from bone marrow-derived hematopoietic stem cells, which diverge into lymphoid and myeloid lineages at an early stage of hematopoietic development. Most DC subpopulations are derived from myeloid progenitors, which give rise to

committed DC progenitors in the bone marrow. These DC precursors then migrate from the bone marrow to the lymphoid organs or non-lymphoid organs through the blood to give rise to both conventional DCs (also referred to as myeloid DCs, CDC/mDC) and PDCs (14). In addition, PDCs can also be derived from lymphoid progenitors and therefore differ from conventional lymphoid and myeloid cells (15).

Different DC populations are characterized by their distinct sets of surface markers, see Table 1.1 for the examples of surface markers expressed on murine DCs and human blood DCs. The CDC/mDC is a heterogeneous group of DC of myeloid lineage that can be separated in three distinct groups according to their expression of surface markers CD4 and CD8: the $CD8^+$CDC, the $CD8^-$ $CD4^+$CDC and the $CD8^-CD4^-$CDC (double negative, DN). Recently, PDC-specific markers have been identified and include blood DC antigen 2 (BDCA-2) for human PDCs (16) and mouse PDC antigen 1 (mPDCA-1) for murine PDCs (17).

Commonly, DCs are divided into PDC and CDC/mDC based on their functions. PDCs, which consist of only 0.2-0.8% of human blood cells, were first identified as the natural type I IFN-producing cells (IPC) (18), as they can secrete 100-1000 times more type I IFN than other blood cell populations upon viral infection (19). PDCs produce type I IFN as early as 4hrs following viral infection and produce most of their IFNα within 24hrs (15). The quick and robust production of type I IFN by PDCs appears to be a direct consequence of their constitutively high expression level of IRF7. As such their production of type I

Table 1.1 Surface markers expression on murine DCs and human blood DCs.

	murine DC			
	CD8a+	CD4+	DN	pDC
CD4	-	+	-	+/-
CD8a	+	-	-	low
F4/80	low	+	low	-
B220	-	-	-	+
mPDCA1	-	-	-	+
Ly6G/c	-	-	-	+
CD11C	+	+	+	low
CD11b	-	+	+	-

	human blood DC	
	mDC	pDC
BDCA1	+	-
BDCA2	-	+
BDCA3	+	-
BDCA4	-	+
CD11C	+	-
CD11b	+	-
CD123	-	+
CD4	+	+
HLA-DR	+	+
lin (CD3/14/19/56)	-	-

IFNs does not depend on the positive feedback regulation (discussed below) required to amplify IFN production and achieve high levels required for efficient control of virus infection (20). PDCs also differ from conventional DCs and monocytes in the profile of the TLRs that they express. As detailed later in this thesis and of particular relevance for my studies, PDCs express high levels of TLR7 and TLR9, two TLRs, which are expressed in the endosomal compartment where they recognize single strand viral RNA and CpG DNA, respectively. Consequently, PDCs are specialized to respond to microbial nucleic acids, particularly those from viruses, which access the endosomal compartment. The low acidic pH within endosomes enables viral uncoating, conditions, which can expose viral genomes to endosomal TLRs.

CDC/mDCs differ from PDCs phenotypically and functionally. mDCs express different surface markers compared to PDCs. For example, CD11c is highly expressed on mDCs and is either not expressed or expressed at very low levels on PDC. Distinct subtypes of CDC also have differenct functions. For instance, the $CD8^+$CDCs appear to be specialized at cross-presentation of exogenous antigen to CD8 T cells (21). mDCs also express different sets of TLRs than PDCs: TLR7 and TLR9 are highly expressed on PDCs, while mDCs express TLR1, 2, 3, 4, and 8, which allows them to recognize a broader spectrum of pathogens (15). Upon viral infection, CDCs produce type I IFN, interleukin (IL)-12 and other proinflammatory cytokines, although type I IFN levels are much lower than those produced by PDC. Besides TLRs, CDCs also express a much

wider selection of PRRs, these include the RNA helicases RIG-I and melanoma differentiation-associated gene-5 (MDA5), which recognize cytosolic viral RNA to activate the type I IFNβ as well as a wide range of cytosolic sensors for DNA. In most cell types the type I IFN, IFNβ is induced first as a result of IRF3 activation. Upon IFNβ release, IFNα subtypes are then induced as a result of IFNβ signaling through its receptor, the IFNα/βR which signals via a JAK/STAT pathway to turn on Interferon stimulated genes. One such ISG, IRF7 is upregulated and upon activation by many of the same molecules, which activate IRF3, subsequently primes further IFNα and β production. The production of IFNα by CDCs is dependent on this IFNβ positive loop since IRF7 is not constitutively expressed in these cell types (22).

DCs can also be classified based on their location and function: (i) $CD11c^{hi}MHC\text{-}II^{+}$ CDC/mDCs in lymphoid organs (spleen, bone marrow, thymus, lymph nodes etc) are mainly resident DCs where they function to present antigens to T cells (23). (ii) $CD11c^{hi}MHC\text{-}II^{+}$ non-lymphoid-organ (skin, intestine, kidney, liver etc.) CDC/mDCs are migratory DCs and have the ability to take up antigen from the periphery and migrate to the draining lymph node for antigen presentation (24). (iii) PDCs are present in both lymphoid organs and non-lymphoid organs with $CD11c^{int/-}MHC\text{-}II^{-/int}$ surface markers. They are professional antigen-presenting and IFNα-producing cells after encountering pathogens (25, 26).

To study DCs in the laboratory, two major approaches are used. The first involves the generation of DCs *in vitro* from precursor cells. In the case of human cells, monocytes are differentiated into monocyte-derived DCs, while in the mouse, bone marrow cells are the precursor cells utilized. The second approach involves the isolation of terminally differentiated DCs from mouse tissues. These two techniques are detailed further below.

1) Monocyte-derived DCs are a mixed cell population generated when human monocytes or murine bone marrow cells are cultured with GM-CSF resulting in a cell population containing phagocytes such as macrophages, as well as DCs (14). These DCs have the characteristics of *in vivo* DCs in both morphology and function. While cultured for several days in recombinant cytokines, one caveat to the use of these DCs is the fact that they are somewhat primed or preactivated during this procedure. For example, bone marrow derived dendritic cells (BMDCs) can be stimulated to secrete nitric oxide while DCs isolated from mouse spleens do not appear to possess this activity. Moreover, high numbers of BMDCs can inhibit T cell proliferation while DCs isolated by other means often fail to do so (27). More recently, Flt3L has been used to culture bone marrow cells to produce PDCs. Flt3L treatment results in a mixed population of cells with both myeloid DCs and PDCs.

2) DCs isolated from lymphoid organs: splenic and thymic DCs each contain these subtypes: PDCs, the $CD8^+$CDC, the $CD8^-CD4^+$CDC and the $CD8^-CD4^-$CDC. Whether from spleen or thymus, they express different sets of

chemokine receptors (CCRs) and TLRs and produce different chemokines (28). Recent reports have quantitatively showed differential expression of PRRs between DC subtypes (29) and as a consequence these different cells can have differential abilities in viral recognition (30).

Natural killer cells. NK cells are granular lymphocytes with natural cytotoxicity as well as cytokine-producing functions. NK cells can kill virally infected or transformed cells by direct cell contact. NK cells use two major pathways to kill target cells by apoptosis: i) exocytosis of toxic granules (perforin, granzymes etc.) and ii) engagement of death receptors on target cells with their ligands expressed on NK cell surfaces (e.g. Fas/FasL, TRAIL-R/TRAIL). Besides killing, NK cells also secrete cytokines and chemokines, including IFNγ, which restricts viral infection as well as stimulates adaptive immunity (31). NK cells play an important role in immunity to a wide range of viral infections (32). Due to the powerful ability of NK cells to quickly kill target cells without requiring antigen-specific recognition, the activation of NK cells is tightly regulated by a balance between activating and inhibitory receptors. Inhibitory receptors (mostly MHC class I-specific receptors) mediate “self-tolerance” while activating receptors give NK cells the signal to eliminate the target cells (33). The function of NK cells is also regulated by the cytokine microenvironment surrounding them, including type I IFN and IL-12 (32).

1.2 Pattern recognition receptors

As the first line of defense against invading microbes, the innate immune system initiates the immediate recognition of invading pathogens. In contrast to the adaptive immune system, which develops a broad repertoire of antigen-specific receptors, the innate immune system responds to a broad spectrum of pathogens with only a limited repertoire of germline-encoded receptors. The innate immune receptors can recognize three categories of signals: i) The exogenous common biological components shared by groups of pathogens, so called PAMPs; ii) the endogenous metabolic molecules produced during cell damage upon infection, which is called the danger-associated molecular patterns (DAMPs), such as alarmins; iii) the inhibitory signals expressed by healthy cells but not by infected cells or non-self pathogens, such as major histocompatibility complex (MHC) class I molecules, which usually inhibit the innate immune response to the cells expressing these signals (34).

Microbial PAMPs include lipids (e.g. LPS) as well as proteins such as flagellin. These ligands can be recognized by different groups of receptors to induce appropriate effector responses such as cytokines and chemokines immediately upon infection. In addition to classic PAMPs and DAMPs, nucleic acids are a particularly potent trigger of innate immune receptors. PAMPs, DAMPs and nucleic acids are usually detected by PRRs such as TLRs, RLRs, NOD-like receptors (NLRs) and cytosolic DNA sensors (Figure 1.1).

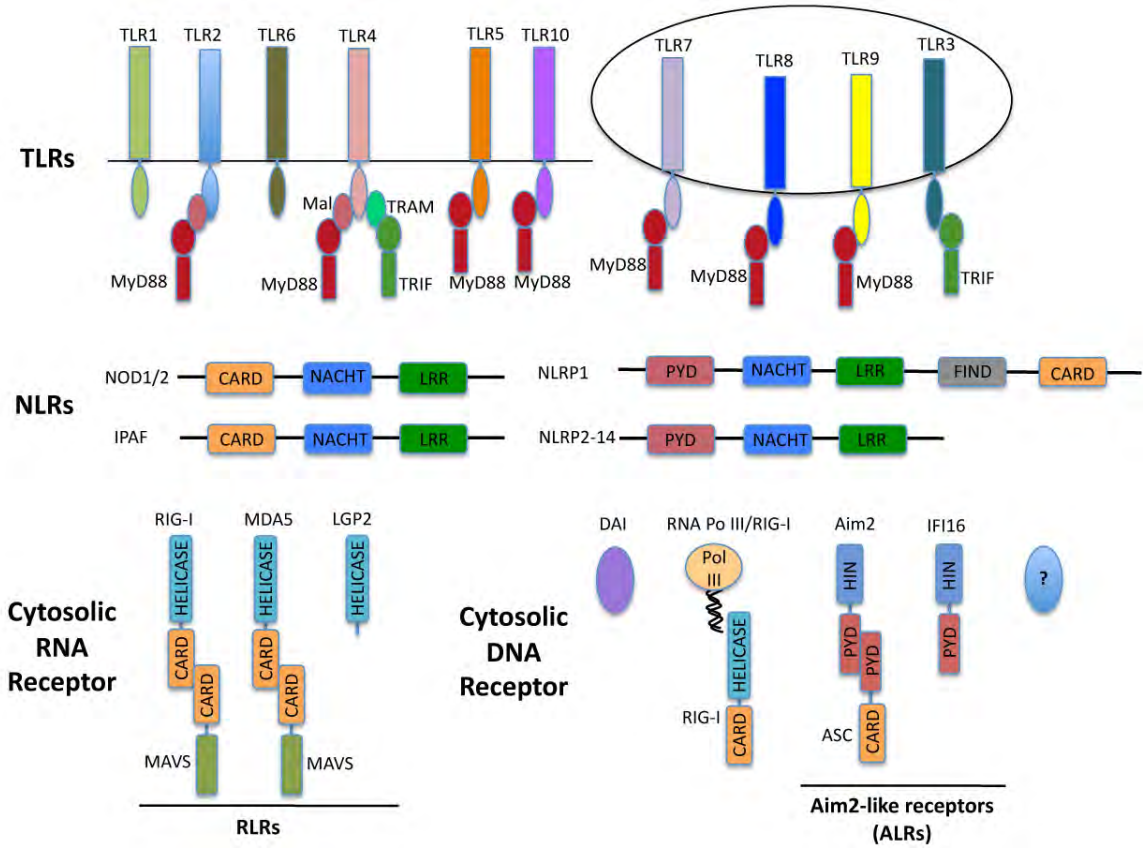


Figure 1.1 Pattern recognition receptors. (i) TLRs: TLR1, 2, 4, 5, 6, and 10 appear to localize at the cell surface, while TLR3, 7, 8 and 9 are localized inside endosomes. Most TLRs employ MyD88 as the adaptor protein for downstream signaling while TLR3 uses TRIF.TLR4 uses both MyD88 and TRIF, as well as two additional adapters Mal/TIRAP and TRAM. (ii) NLRs: the NLR family members are cytosolic receptors containing a central NACHT domain for activation of signaling, a C-terminal LRR domain and N-terminal CARD or PYD domain for downstream signaling. Some members also have Baculoviral inhibition of apoptosis protein repeat domain (BIR, not shown) (iii) cytosolic RNA receptors are also called cytosolic RLR family contains RIG-I, MDA5 and LGP2. RIG-I and MDA5 recruit the common adaptor protein MAVS through CARD-CARD interaction for signaling, while LGP2 only contains a helicase domain. (iv) cytosolic DNA receptors: DAI was the first identified cytosolic receptor. The Pol III/RIG-I system converts polydAdT into a RIG-I ligand. Recently, two more receptors, the PYHIN proteins, Aim2 and IFI16 were also identified as DNA sensors. AIM2 forms an inflammasome while IFI16 induces IFN.

1.2.1 TLRs

TLR family members are well conserved from Drosophila to mammals. They are type I transmembrane glycoproteins, with extracellular domains containing leucine-rich-repeat (LRR) motifs for ligand recognition and intracellular Toll/IL-1R (TIR) homology domains responsible for signaling (35). TLRs work as dimeric proteins, capable of both homo- or hetero-dimerization, to recognize components from bacteria, fungi, parasites, and viruses. To date, 13 murine and 10 human TLRs have been identified which are expressed at different levels in different immune cell types (Table 1.2). Working together, TLRs enable the host to detect most types of infection.

1). Endosomal TLRs: TLR3, TLR7/8 and TLR9

When microbes enter the cells by receptor mediated endocytosis, the acidic environment of the phagolysosome leads to their degradation and unveiling of their genomes inside endosomes, where the nucleic acid will be detected by endosomal TLRs. TLR3 recognizes double stranded (ds)-RNA including the replication intermediates of viruses, TLR 7/8 recognize single stranded (ss)-RNA, and TLR 9 recognizes unmethylated CpG DNA motifs. TLR3 is expressed in myeloid cells including conventional/myeloid DCs and monocytes as well as on some epithelial cells, but not in PDCs. Moreover the expression level of TLR3 can be upregulated by type I IFN. The synthetic dsRNA analog, polyIC, can be recognized by TLR3 to induce type I IFN. TLR3 deficient mice

Table 1.2 Expression profile of TLRs and RLRs in immune cell types (15, 38).

phenotype	pDC	CD11c+ mDC	Monocytes	NK cell
TLRs				
TLR1	+	+	++	++
TLR2	-	+	+++	+
TLR3	-	++	-	+
TLR4	-	+	++	-
TLR5	-	+	+	+
TLR6	+	+	+	+
TLR7	++	-	-	-
TLR8	-	++	+	-
TLR9	++	-	-	-
TLR10	+	+	-	-
RLRs				
RIG-I	-	+	+	?
MDA5	-	+	+	?
function				
phagocytosis	-	++	++	-
type I IFN	+++	+	+	?

- means low
+ means high

showed impaired response to polyIC and resistance to polyIC induced shock (36). While TLR3 was the first PRR implicated in the immune response to RNA viruses, TLR3 deficient mice do not display increased susceptibility to most RNA viruses. TLR3 does play an important role in influenza A virus (IAV) and West Nile virus (WNV) infection. TLR3 expression levels are increased in IAV infected pulmonary epithelial cells. TLR3 deficient mice have lower levels of inflammatory cytokines, such as Rantes, IL6 and IL12p40/p70 and have longer survival (37). In contrast, WNV has been shown to benefit from TLR3 for entry into the brain and TLR3 deficient mice are more resistant to lethal WNV infection (39). The generation of TLR3 knockouts (KOs) and their analysis in models of viral pathogenesis indicated that additional receptors for control of viruses remained to be revealed (see below).

In humans, TLR7 and TLR9 are predominantly expressed in PDCs, while TLR8 is expressed in monocytes and myeloid DCs (15, 38). TLR7 can recognize single strand viral RNA as well as imidazoquinoline derivatives such as R848 and other synthetic ssRNA. TLR7 is one of the most sensitive receptors sensing viruses in PDCs. The deficiency of TLR7 in PDCs abrogated the induction of IFNα by several RNA viruses, such as influenza virus, Dengue virus and vesicular stomatitis virus (VSV) (40-42). It has been reported that human TLR8 senses GU-rich RNA (43), while murine TLR8 can be activated by a combination of poly T oligodeoxynucleotides (ODNs) as well as the TLR agonist imidazoquinoline (44). TLR9 recognizes unmethylated CpG DNA motifs that are

present in viral and bacterial but not in vertebrate DNA. The induction of IFNα by DNA viruses, such as mouse cytomegalovirus (CMV), herpes simplex virus **(**HSV)-1, and -2 as well as murine gammaherpesvirus 68 (MHV-68) are dependent on TLR9 in PDCs (45-48).

2). Cell surface TLRs, TLR2, TLR4 and TLR5

TLR2 forms heterodimers with TLR1 or TLR6 to recognize a variety of bacterial components, including lipoproteins, peptidoglycan (PGN) and lipoteichoic acid. TLR2 deficient mice are highly susceptible to *Staphylococcus aureus* infection (49) and fail to clear spirochetes after *Borrelia burgdorferi* infection (50). TLR4 associates with MD-2 to recognize the bacterial cell wall component LPS. The TLR4-null mice strains, for example C3H/HeJ, show reduced response to Gram-negative bacteria and higher susceptibility to *Salmonella typhimurium* infection (51). TLR5 senses flagellin, a component of bacterial flagella (52). TLR2 and TLR4 were also shown to recognize viral envelope proteins. For example, TLR2 can be activated by Measles virus hemagglutinin protein (53), HSV-1 (54) and human CMV (55). TLR4 can be activated by Respiratory syncytial virus (RSV) fusion protein (56), and mouse mammary tumor virus envelope protein (57).

3) TLR signaling

The activation of TLRs is initiated by the engagement of their ligands. The extracelluar LRR domain is responsible for the recognition of the PAMPs from diverse microbial pathogens. The crystal structures of TLR1-2-Pam_3CSK_4, TLR3-dsRNA and TLR4-MD2-LPS complexes that have been determined recently (58-60) revealing key insights into the activation mechanism for these receptors. The ligand binding to the horse-shoe-like LRRs induces the dimerization of TLRs to form an “m” shaped dimer, which brings the intracellular TIR domains close together to trigger signaling.

Activated TLRs recruit 2 major adaptor proteins through TIR domain interactions: myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). MyD88 is employed by all known TLRs except TLR3, which is totally dependent on the adaptor protein TRIF. In TLR2 and TLR4 signaling, an additional adaptor named MyD88-adapter-like (Mal) (also called TIRAP) is also used as a bridging receptor to recruit MyD88 to the receptor. TLR4 signals through both MyD88 and TRIF pathways, and a fourth adapter protein, TRIF-related adaptor molecule (TRAM), is used to recruit TRIF to the receptor. MyD88 and TRIF trigger distinct signaling pathways for the production of inflammatory cytokines and type I IFNs. For inflammatory cytokine production, MyD88 associates with interleukin-1 receptor-associated kinase (IRAK)-4 and IRAK-1, which in turn activate TNF-receptor-associated factor (TRAF)-6 to trigger nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and Mitogen-activated protein (MAP) kinase pathways. Type I

IFN production upon TLR7/9 signaling in PDCs also require MyD88. Binding of nucleic acids to TLR7/9 leads to the formation of a protein complex with IRAK4, IRAK1, TRAF6, TRAF3 and IRF7, in which IRF7 activation is critical for IFNα production. In the case of TLR3/4, type I IFN production relies upon the TRIF pathway. TRIF interacts with TRAF6, TRAF3 and RIP1. TRAF3 is responsible for the activation of the TANK-binding kinase 1 (TBK1)/IκB kinase (IKK)-ε and downstream IRF3 leading to IFNB gene induction (the activation of IRF3 is detailed below), while TRAF6 and RIP1 are responsible for the activation of NFκB and MAP kinase, leading to the production of inflammatory cytokines (51).

1.2.2 NLRs and inflammasome

Over the last decade several families of cytosolic pattern recognition receptors have been identified. These include the NLR family of proteins, which were first identified as important receptors of bacterial components. The NLR family members have a central NACHT domain for activation of signaling *via* ATP-dependent oligomerization, a C-terminal LRR domain for ligand recognition and N-terminal Caspase recruitment domain (CARD) or pyrin domain (PYD) for downstream signaling through protein-protein interactions (61). Among this large family, the functions of several members have been more thoroughly studied. NOD1 and NOD2 can recognize cytosolic breakdown products of bacterial cell walls during bacterial infection, D-glutamyl-meso-diaminopimelic acid (iE-DAP) and MDP respectively. Upon ligand binding, NOD1/2 oligomerizes and recruits

RIP2 to activate NFκB signaling or CARD9 to trigger MAP kinase signaling. Mutations in NOD2 were reported to associate with human inflammatory diseases including Crohns disease (62-64). Some other NLRs, such as NACHT, LRR and PYD domains-containing protein (NALP)-1, NALP3 and ICE Protease-Activating Factor (IPAF), upon activation, regulate capase-1 activity through a protein complex called the inflammasome.

The Inflammasome is a protein platform formed upon infection or stress to activate the maturation of proinflammatory cytokines, such as IL-1β and IL-18. Inflammasome complexes comprise one or more NLR proteins as well as caspase-1, and in most cases the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC). Of all the NLRs, the NALP3 inflammasome is the best characterized. It contains NALP3, caspase-1 and ASC, which interacts with NALP3 and caspase-1 through PYD-PYD or CARD-CARD interactions, respectively. The stimuli described to trigger NALP3 inflammasome activity include crystals such as uric acid crystal (65-67), silica (68-70), fungi (*Candida albicans)* (71)*,* bacteria *(Lm*, *Staphylococcus aureus)* (72) and viruses (Sendai virus (SV), influenza virus, and adenovirus) (73, 74). Although the mechanism underlying the activation of NALP3 inflammasome is still not clear, recent reports indicate that stimuli induce either cellular stress/danger signals including potassium efflux or lysosomal damage, or the generation of reactive oxygen species (ROS) to activate NALP3 inflammasome (75). IPAF inflammasome consists of IPAF and caspase-1, which interact with each other

through CARD-CARD interactions, although in some cases ASC is required for maximal activity. IPAF inflammasome can be activated by gram-negative bacteria, such as *Salmonella typhimurium, Shigella flexneri, Legionella pneumophila* and *Pseudomonas aeruginosa* (76-81). The activation of inflammasomes results in the activation of caspase-1, which is a cysteine protease responsible for the maturation of IL-1β and IL-18 from the inactive pro-form of these cytokines. IL-1β and IL-18 play important roles in the regulation of immune responses, so the integrated regulation of both the production of pro-form and the activation of caspase-1 through inflammasomes are well controlled.

1.2.3 Cytosolic RNA receptors

When viruses, especially RNA viruses infect cells, dsRNA and RNA with uncapped 5'-triphosphate ends are generated during viral transcription and viral replication. These viral products can be sensed by the cytosolic RLR family, which contains RIG-I, MDA5 and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 contain two N-terminal CARD domains for downstream signaling, and a DExD/H RNA helicase domain with ATPase activity. RIG-I also contains a C-terminal regulatory domain for inhibiting the function of RIG-I by masking the CARD domains in the absence of stimulation (82). Upon viral RNA binding, RIG-I will be activated by conformational changes to expose the CARD domains, which in turn interact with a CARD domain containing adaptor mitochondrial antiviral signaling protein (MAVS) (also called IPS-1, VISA or

CARDif) (83-86) through CARD-CARD interaction. Activated MDA5 leads to the same signaling pathways as RIG-I by recruiting the same adaptor MAVS. MAVS is localized at the mitochondria and in peroxisomes (87), and can recruit TRAF3 to activate TBK1/IKKε kinases for IRF activation as well as TRAF6 to activate IKKα/β for NFκB activation (88). There is also some evidence that MAVS can recruit stimulator of IFN gene (STING) for IRF3 activation (89).

Recent reports indicate that RIG-I and MDA5 selectively recognize different RNA motifs. RIG-I recognizes short dsRNA, 5'-triphosphate-ssRNA as well as short synthetic dsRNA polyIC (<1kb) while MDA5 recognizes long dsRNA and long synthetic dsRNA polyIC (>1kb). RIG-I and MDA5 were thereby described to sense different type of viruses. Using RLR deficient mice or cells, reports have shown that RIG-I is mostly involved in IAV, VSV, SV, and Newcastle Disease Virus (NDV) recognition (90) while MDA5 mainly senses *picornaviridae* (such as Encephalomyocarditis virus (EMCV)) (91) and some murine viruses (such as murine norovirus) (92). Several viruses can be detected by both RIG-I and MDA5 for different viral RNA products, for example short segments of *Reoviridae* segmented dsRNA genome are substrate of RIG-I, while long segments are substrate of MDA5 (91, 93).

As mentioned above, LGP2 is also a member of the RLR family. LGP2 has a helicase domain like that found in RIG-I and MDA5, but does not contain a CARD domain. LGP2 was reported to negatively regulate RIG-I function in *in vitro* assays (94, 95). Recently, LGP2 deficient mice have shown enhanced type I

IFN production in response to polyIC and VSV, while the same deficient mice also showed a defect in type I IFN production in response to EMCV (96). Additional KO studies from another group, however, suggested that this molecule was a positive regulator of the RIG-I, MDA-5 pathways (97). Thus, the function of LGP2 in innate immune response to viral infection is still a little unclear.

1.2.4 Cytosolic DNA receptors

The accumulation of cytosolic DNA during infection or after tissue damage has also emerged as an important trigger of innate immunity. Several receptors have been identified, which activate distinct functional outcomes with different mechanisms. DNA-dependent activator of IRFs (DAI, also called DLM-1 or ZBP1) was the first identified candidate cytosolic DNA sensor (98). DAI contains at its N-terminus two Z-DNA binding domains that can bind B-DNA. *In vitro* studies using siRNA have shown that DAI is important for type I IFN induction by various DNA sources including that from HSV. Although downregulation of DAI expression in L929 cells impaired type I IFN response to dsDNA, mouse embryonic fibroblasts (MEFs) appeared normal when expression of DAI was knocked down (99). Moreover, DAI deficient cells showed normal immune responses to poly(dA-dT)·poly(dA-dT) stimulation and DAI-deficient mice had normal responses to a DNA vaccine (100). These results indicate that the immune response to cytosolic DNA stimulation is cell type dependent, and DAI may only play a partial or redundant role in cytosolic DNA sensing. Recently two

groups suggested that transfected poly(dA-dT)·poly(dA-dT) could be transcribed into RNA ligands for RIG-I by polymerase III in the cytosol, which provides an alternative cytosolic DNA recognition mechanism (101, 102). However, the type I IFN production in response to poly(dA-dT)·poly(dA-dT) is not RIG-I dependent in mouse myeloid dendritic cells (101), which suggested the existence of additional DNA sensors, the identity of which remain unknown. Most recently, a new receptor for this pathway, IFI16, was identified. IFI16 is a PYHIN (PYD- and HIN domain) protein, which can bind to viral DNA and turn on IFNB gene transcription (Bowie et. al, Manuscript in press, Nat. Immunology).

While initial studies on cytosolic DNA receptors mostly focused on the induction of type I IFN. Recently it was shown that cytosolic DNA delivery could also active the inflammasome and thereby induce IL-1β release. Four independent groups identified Absent in melanoma 2 (AIM2) as a cytosolic DNA receptor to induce inflammasome activation for IL-1β maturation (103-106). AIM2, like IFI16, is a member of HIN-200 family, containing a C-terminal HIN domain for DNA binding, and an N-terminal PYD domain to associate with adaptor protein ASC to recruit caspase-1. AIM2 can sense cytosolic dsDNA poly(dA-dT)·poly(dA-dT) as well as DNA PAMPs from viruses or bacteria. AIM2 deficient cells lost inflammasome activation in response to *Francisella tularensis* live vaccine strain (LVS), vaccinia virus (VV) and mouse CMV. In addition, inflammasome activation by Lm is partially dependent on AIM2 signaling (72, 107-110). Given the broad-spectrum of DNA pathogens as well as aberrant self

DNA that can arise during certain situations (e.g. Systemic Lupus Erythematosus (SLE)), it is not surprising that redundant cytosolic DNA sensors exist and lead to different signaling pathways.

1.3 Type I IFN production and regulation

When PRRs detect invading pathogens, they initiate signaling pathways leading to the production of multiple cytokines and chemokines for host defense. The type I IFNs are the principal cytokines strongly induced during infection by viruses and bacteria. They are well known for their antiviral functions by inhibiting viral replication either directly or indirectly or by promoting apoptosis of infected cells through induction of IFN stimulated genes. Type I IFNs signal through IFN receptors in an autocrine or paracrine manner. The enhanced susceptibility of type I IFN receptor KO mice to virus infection have shown the critical importance of type I IFN for antiviral defenses (111, 112). Besides, type I IFN plays important roles in modulating the adaptive immune system. For example, IFNα and β can upregulate class I MHC molecules and promote DC maturation, thus facilitate antigen presentation (113). Type I IFNs comprise a growing family of IFN proteins, among these, IFNα/β are the best studied and will be referred to when talking about type I IFN.

1.3.1 The induction of type I IFN

The promoter of the IFNB gene contains at least 4 important positive regulatory elements called PRDI to IV (114). PRDI and III have overlapping binding sites for interferon regulatory factors (IRFs), PRDII is the binding site for NFκB and PRDIV for activating transcription factor 2 (ATF2)/c-Jun activated downstream of the c-Jun N-terminal kinase (JNK) and p38 MAP kinases. Activation of the IFNβ promoter requires activation of all these transcription factors and formation of a protein complex called the enhancesome (115) that also contains the co-activators such as cAMP response element-binding (CREB)-binding protein (CBP) or p300. The IFNA gene promoter only has binding sites for the IRFs and lacks NFκB binding sites (116). In most cell types, IFNα is induced by a positive feedback mechanism involving IRF7, which is itself an Interferon Stimulated Gene (ISG) (117, 118) (detailed below). Thus, IRFs are essential transcriptional factors required for the induction of type I IFN.

IRFs

The IRFs are a family of transcriptional regulators with multiple functions, such as modulation of innate and adaptive immune responses and immune cell development. The IRF family contains 9 members, IRF1 through 9. All of them have well conserved N-terminal DNA binding domains (DBDs), which is characterized by several 5-tryptophan-repeats. The DBD forms a helix-loop-helix motif recognizing GAAA and AANNNGAA sequences. The C-terminal part of the IRFs is less conserved. IRF3-9 contain IRF-associated domain (IAD)-1, and IRF1

and IRF2 contain IAD2. These domains mediate protein-protein interactions between IRFs and interaction with other proteins such as CBP/p300 (119, 120).

1) IRF3 and IRF7

IRF3 and IRF7 share significant sequence homology to each other, and they are the key IRFs regulating type I IFN production during viral infection. TLR and RLR activation turn on distinct downstream signaling that result in TBK1 and IKKε kinase activation, which in turn phosphorylate IRF3 at its C-terminal regulatory region. Phosphorylation of IRF3 alleviates the auto-inhibitory domain of IRF3 and releases its transactivation domain, which permits IRF3 dimerization and nuclear translocation. IRF7 undergoes a similar activation procedure, and forms heterodimers with IRF3 or homodimers to activate type I IFN transcription together with coactivator, such as CBP and p300 (see below Figure 1.3). In addition to being activated by RLR and TLR, IRF3 and 7 are also important in cytosolic DNA sensing. Type I IFN activation by cytosolic DNA ligand requires IRF3 and 7. Moreover, the new adaptor protein in DNA signaling pathway, STING (also called MITA), can activate IRF3 (89, 121).

The induction of IFNα and IFNβ in most cell types (except in PDCs, which detailed above in “1.1.2 Cellular players – Dendritic cells”) is regulated differentially because of the expression level of IRF3 and IRF7. IRF3 is constitutively expressed in the cytosol of these cells, acts as a potent activator of IFNβ and IFNα4. While IRF7 can activate both IFNA and IFNB genes, its expression level is very low but can be induced by type I IFNs. Thus IFNβ and

IFNα4 are strongly induced in early response, while the other IFNα subtypes, such as α2, α6 etc. are induced in a delayed manner through a positive feedback regulation. The initial expression of IFNβ and IFNα4 trigger the type I IFN signaling through the interferon-α/β receptor (IFNAR) (detailed below) to induce the expression of IRF7, which then acts on type I IFN genes for the production (122) (Figure 1.2).

2) IRF5

IRF5 was discovered based on its homology to IRF3 and IRF7. In contrast to IRF3 and IRF7, which are activated by most if not all viruses, the involvement of IRF5 in the innate response to viral infection was less widespread as only certain kinds of viruses such as VSV, NDV and HSV activated IRF5 (123, 124). Previous work from our lab had implicated IRF5 in the regulation of IFNs downstream of TLR7 (125). Surprisingly, however, when IRF5-deficient mice were generated it was shown that rather than being a major regulator of IFN production, IRF5-deficient cells failed to turn on inflammatory cytokines downstream of a wide selection of TLRs. Other viruses such as SV that activate IRF3 and IRF7 do not appear to activate IRF5, which indicates that IRF5 is activated by different signaling pathways from those acting on IRF3/7 (126, 127).

IRF5 can interact with MyD88 and TRAF6 and is involved downstream of TLR-MyD88 pathway for inflammatory cytokine production. In IRF5 deficient DC and macrophages, inflammatory cytokines such as IL-6, TNFα, and IL-12 were

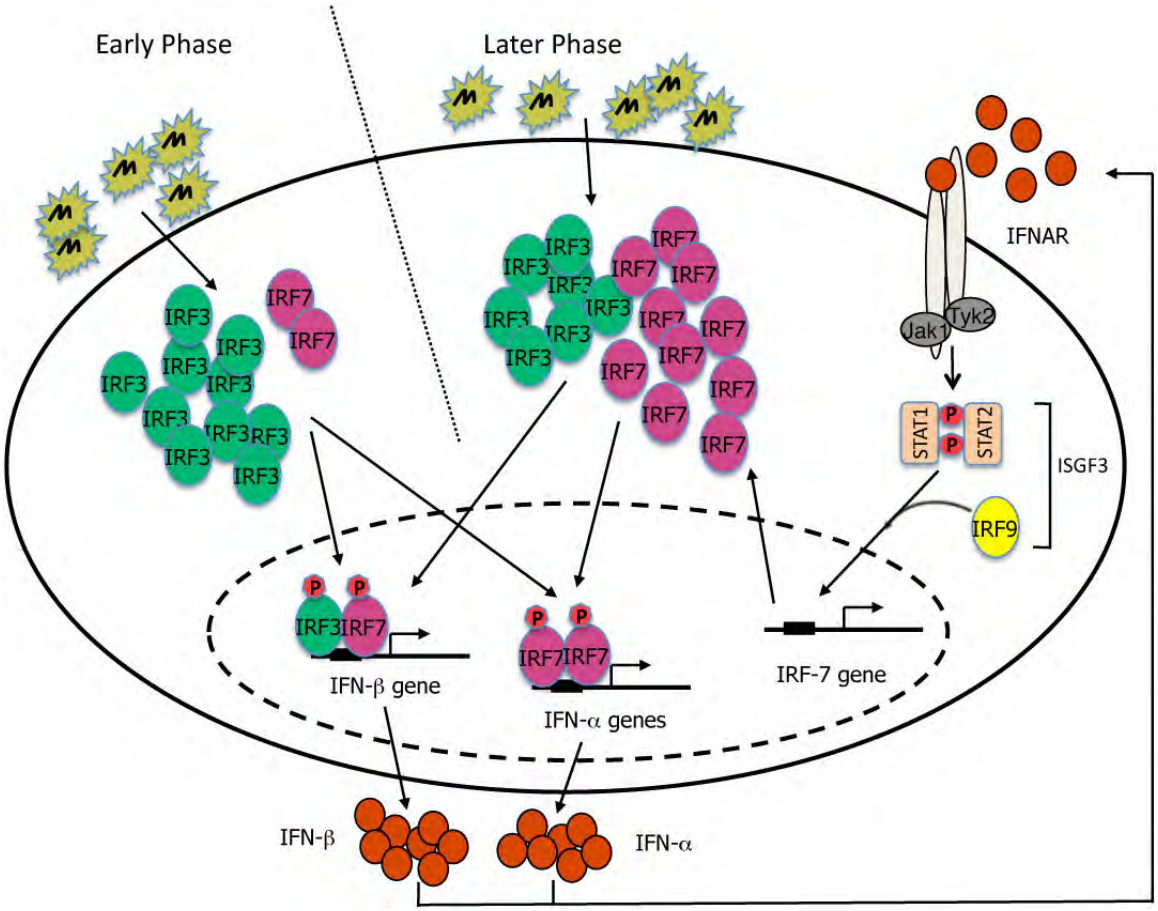


Figure 1.2 Type I IFN signaling and positive feedback regulation of type I IFN gene expression. IRF3 is constitutively expressed in the cytosol, while IRF7 is expressed at very low levels. Upon infection, IRF3 is the first factor to be phosphorylated and activated leading to IFNβ and IFNα4 induction, which then trigger the type I IFN signaling pathway through the interferon-α/β receptor (IFNAR). IFNα/β binding to the receptor promotes the IFNAR1/IFNAR2 dimerization, facilitates the cross-phosphorylation of TYK2 and JAK1 for activation, which in turn phosphorylate STAT1 and STAT2. After being activated, STAT1 and STAT2 heterodimers recruit IRF9 (p48) to form the transcriptional complex called ISGF3, which translocates into the nucleus to activate ISGs, including IRF7. Thus in the late phase of infection, the abundant IRF3 and IRF7 can form heterodimers or homodimers to turn on type I IFN genes leading to amplification of antiviral responses.

severely impaired upon TLR-mediated stimulation. Consistently, IRF5 deficient mice show resistance to CpG or LPS lethal shock (128). In contrast, the involvement of IRF5 in type I IFN production from these initial studies was not clearly addressed and therefore remained somewhat controversial.

In chapter IV of this thesis, I describe my work, which dissects in detail the involvement of IRF5 in the innate response to viral infection, particularly clarifying its role in the regulation of type I IFN responses.

3) IRF1 and IRF2

IRF1 and IRF2 were the first identified IRFs that could bind the IFNα and β enhancesome regions (129, 130). However in IRF1 deficient MEFs, type I IFN was normally induced (131, 132). IRF2 can bind to the same recognition site as IRF-1, and antagonize transcription of IRF1-dependent promoters (119). IRF2 was known as a negative regulator of type I IFN. IRF2 deficient mice developed inflammatory skin disease, and some IFN inducible genes, such as oligo-adenylate synthetase (OAS) and IRF7 are upregulated (133). Moreover, it is reported that IRF2 can bind to the same IFN-stimulated regulatory element (ISRE) as Interferon-stimulated gene factor 3 (ISGF3) (134). These results imply that the function of IRF2 is not to control the expression of IFNα/β, but rather an excessive IFNα/β signaling by attenuating ISGF3 mediated IFN inducible gene expression (133).

4) IRF9

IRF9 is a central component of type I IFN signaling. IRF9 forms a complex with STAT1 and STAT2, named ISGF3, which is critical for induction of ISGs. In IRF9 deficient mice, type I IFN signaling pathways are impaired (135).

5) IRF4,6,8

IRF4 and 8 share high homology, and are primarily expressed in immune cells, such as macrophages and DCs. IRF4 mRNA is induced by various TLR ligands, and IRF4 shares the same binding site as IRF5 on MyD88. TLR induced IRF4 can compete with IRF5, for MyD88 binding, thus inhibiting IRF5 activity (127). Consistent with these observations the level of proinflammatory cytokines induced by TLR ligands in IRF4 deficient peritoneal macrophages is elevated, which is the opposite of what is seen in IRF5 deficient mice. IRF4 deficient mice are more sensitive to CpG-B induced shock. All these data present IRF4 as a negative regulator of TLR-dependent IRF5 responses (136).

IRF8 also plays a role in TLR9 signaling in DCs. In IRF8 deficient DCs, CpG induction of proinflammatory cytokines such as TNFα and IL-6 are totally abrogated and NFκB was not activated, while the response to LPS remains intact (137). IRF8 is also involved in the induction as well as the feedback phase of type I IFN induction by virus and TLR in DCs (138). Little is known about the function of IRF6 in response to viral infection, while it has been reported that

polyIC treatment changes the cytosolic IRF6 phosphorylation pattern and can induce a small portion of IRF6 to translocate into nucleus (139).

1.3.2 Type I IFN signaling and ISGs

The classic signaling pathway for IFNα and IFNβ to induce cellular responses employs Janus kinase (JAK) and signal transducer and activator of transcription (STAT) factors. In steady state condition, the IFNAR1 is associated with tyrosine kinase 2 (TYK2), and IFNAR2 is associated with JAK1. IFNα/β binding to the receptor promotes the IFNAR1/IFNAR2 dimerization on the cell membrane to initiate a tyrosine phosphorylation cascade. IFNAR1/IFNAR2 dimerization facilitates the cross-phosphorylation of TYK2 and JAK1 for activation, which in turn phosphorylate STAT1 and STAT2. After being activated, STAT1 and STAT2 heterodimers recruit IRF9 (p48) to form the transcriptional complex called ISGF3, which translocates into the nucleus and binds to the ISREs of many type I IFN stimulated genes to regulate their expression (140, 141) (Figure 1.2). Besides this classic STAT1/STAT2/IRF9 complex pathway, type I IFNs also activate other types of STAT complexes, such as STAT3 homodimers, STAT5 homodimers as well as STAT1-STAT3 heterodimers, all of which translocate into nucleus and bind different promoter elements in response to type I IFN (142).

ISGs

ISGs contain ISRE elements in their promoters and are inducible by type I IFN signaling. They are the effectors of type I IFN for the antiviral, antiproliferative and immunomodulatory functions. Over 400 ISGs have been identified by microarray analysis including the well known dsRNA-dependent protein kinase (PKR), Mx1, ISG15, OAS and RNAseL for their antiviral activities. Despite the robust induction of hundreds of ISGs, the biological function of most of these genes is poorly understood (143). I will detail below the function of some ISGs involved in the type I IFN antiviral activity.

1) PKR

PKR is a serine-threonine kinase with a kinase domain at the C-terminus, and two dsRNA binding motifs at the N-terminus, which are well conserved among the dsRNA-binding proteins. There is no specificity described of dsRNA sequence for PKR binding. dsRNA that bind to the RNA binding motif are intermediates of replication generated in infected cells by various viruses. For example, RNA viruses, such as influenza virus, produce dsRNA intermediates when synthesizing new genomic RNA copies. In contrast DNA viruses as VV, adenovirus or HSV, transcribe partially complementary mRNA to form dsRNA due to the bidirectional open reading frames in their DNA genomes. When binding to viral dsRNA, PKR is activated by autophosphorylation and then phosphorylates the eukaryotic initiation factor 2a (eIF2a), which in turn inhibits viral gene translation (140, 144). Besides, in response to a specific group of RNA viruses including EMCV, Theiler's murine encephalomyelitis virus and Semliki

Forest virus, PKR is required for IFNα/β production by maintaining the integrity of newly synthesized IFNα/β mRNA to promote their translation (145).

2) OAS and RNase L

OAS protein is located in the cytosol and can be activated by specific dsRNA motifs, which usually originated from viral RNA. Activated OAS can convert ATP into 2'-5' linked AMP-oligomers (2-5A), which in turn binds to RNAse L monomers and promote the formation of active RNAse L homodimers. The N-terminus of RNAse L involves the 2-5A binding and C-terminus contains the enzymatic domain involved in viral ssRNA cleavage, thereby inhibiting viral protein synthesis. Active RNAse L also cleaves some cellular self mRNA. Cleaved cellular RNA can then act as RIG-I ligands and amplify type I IFN induction (146). The OAS-RNAse L system is important in response to SV, EMCV, WNV and Coxackie virus B4 since cells deficient in RNAse L are more permissive to these viruses (140, 144, 147).

3) Mx proteins

Mx proteins are GTPases, which are strictly controlled by type I IFNs. Mx proteins are expressed abundantly in the cytosol and can interfere with viral replication by binding to viral nucleocapsid proteins in the cytoplasm and inhibiting their trafficking into the nucleus. Mx proteins play a critical role against influenza virus, VSV and Measles virus (140, 144, 147).

4) Other ISGs

ISG15 is an IFN inducible gene that conjugates to its target proteins in a reversible reaction called ISGylation. ISG15 has more than 150 target proteins identified, such as RIG-I, JAK1, STAT1, MxA, PKR, RNaseL, which are important in the innate immune response. Although the function of ISGylation is not yet clear, ISG15 has been reported to prevent the degradation of IRF3 thus enhancing type I IFN production. Besides its intracellular function, the large amount of secreted ISG15 in response to type I IFN may also act as a cytokine like molecule to further modulate immune response, while the mechanism is not yet clear (148). ISG15 deficient mice are more sensitive to HSV-1, Sindbis virus, and influenza virus infection (144, 149). ISG20 is an IFN inducible 3'-5' exonuclease that can cleave viral ssRNA to inhibit viral protein production. It was shown that ISG20 had antiviral activity against VSV infection (150).

1.4 Innate immune response to viral infection

Host cells recognize viral pathogens through PRRs, which in turn activate intracellular signaling cascades, leading to the production of type I IFN and other cytokines and chemokines. Inflammatory cytokines and chemokines recruit and activate effector cells and are often the leading contributor to the morbidity and mortality associated with viral pathogenesis. Type I IFN is the major antiviral

cytokine, which acts to restrict viral replication and activate other immune cells important in viral clearance.

1.4.1 Viral recognition and type I IFN activation

Viral proteins, viral genomes, or nucleic acids produced during viral reproduction are all sensed by PRRs. Three major types of PRRs are currently recognized as the cellular sensors involved in the induction of type I IFN by viruses: TLRs, RNA helicases and cytosolic DNA sensors. As detailed above in "1.2 Pattern recognition receptors",

Of particular relevance to this thesis are the mechanisms underlying the sensing of RNA. Endosomal TLRs and cytosolic RLRs can recognize the viral RNA to induce type I IFN production. TLR3 recognizes dsRNA, including the viral replication intermediates. TLR7/8 recognize single ssRNA, and TLR9 recognizes unmethylated CpG DNA motifs. RIG-I senses cytosolic 5'-triphosphate-ssRNA as well as short dsRNA, while MDA5 senses long dsRNA. Cell surface TLRs, TLR2 and TLR4, can recognize viral proteins to induce proinflammatory cytokine production.

The detection of viral pathogens for type I IFN production involves differential PRRs and signaling components in different cell types. PDCs are known to produce large amount of type I IFN and express very high level of TLR7 and TLR9 and very low level of TLR3, RIG-I and MDA5 (15, 38). Thus PDC are mainly dependent on TLR7/9 for their recognition of viral nucleic acids. For

example, PDCs induced IFNα in response to NDV is normal in cells lacking RIG-I, but severely decreased in the absence of MyD88 or TLR7 (151). In PDCs, binding of nucleic acids to TLR7 and TLR9 activates signaling leading to the recruitment of the adaptor protein MyD88, which forms a complex with IRAK1 (152), IRAK4 (153), TRAF6 (154) and TRAF3 (155). The complex activates IRF7 through IKKα (156) for IFNα production, as well as NFκB and MAP Kinase pathways for inflammatory cytokine production.

In contrast, RLRs play critical roles in the production of type I IFN in a variety of other cell types, including conventional DCs. TLR3 has been suggested to recognize dsRNA from engulfed apoptotic bodies of viral infected cells (157). When TLR3 gets activated, it recruits TRIF, which in turn forms a complex with RIP1, TRAF6 and TRAF3 (155, 158-160). RIG-I and MDA5 share the same adaptor protein MAVS, which forms a complex with TRAF6 (85) and TRAF3 (161). Both of these complexes activate IRF3 and IRF7 through TBK1/IKKε (162, 163) (Figure 1.3).

Viruses use the endocytic pathway to facilitate their entry into cells as well as their exit from cells (164). It is in this compartment where they encounter TLRs. PDCs take up viruses as well as inactive viral particles into the endosomal compartment, where they get degraded exposing the viral nucleic acid to TLR7 or TLR9 (165). This process does not require viral replication in the cytosol. In the case of viruses that replicate in the cytosol, it is less clear how the viral genomes are detected via TLRs. One model is that autophagy is activated in

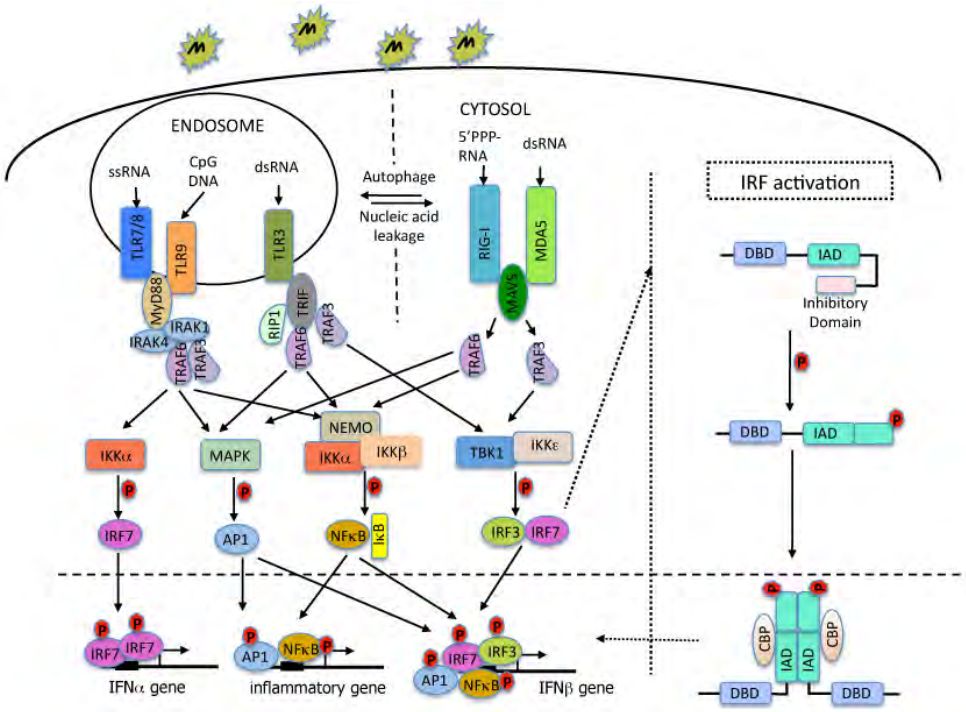


Figure 1.3 IFNB Gene Transcription after Viral Infection. Viral nucleic acids are mainly recognized by endosomal TLR 3 (dsRNA), TLR9 (CpG DNA) and TLR7/8 (ssRNA), and cytosolic RLRs, RIG-I (5'PPP-RNA) and MDA5 (dsRNA). In PDCs, activated TLR7/8 and TLR9 recruit the adaptor protein MyD88, which forms a complex with IRAK1, IRAK4, TRAF6, and TRAF3. This complex activates IRF7 through IKKα for IFNα production, as well as NFκB and MAP Kinase pathways for inflammatory cytokine production. Activated TLR3 recruits TRIF, which in turn forms a complex with RIP1, TRAF6 and TRAF3 to activate NFκB signaling or TBK1 to activate IRF3 and turn on IFN genes. RIG-I and MDA5 share the same adaptor protein MAVS, which forms a complex with TRAF6 and TRAF3 for type I IFN and inflammatory cytokine production, respectively. TRAF3 forms a complex with TBK1/IKKε to phosphorylate IRF3 and IRF7 . Phosphorylation of IRF3 alleviates the auto-inhibitory domain and releases the transactivation domain, which permits IRF3 dimerization and nuclear translocation. IRF7 undergoes a similar activation procedure, and forms heterodimers with IRF3 or homodimers. Dimerized IRFs activate type I IFN transcription together with coactivator, such as CBP.

PDC, which delivers RNA products from viruses in the cytosol to endosomal TLRs (166). Therefore, endosomal TLRs are not restricted to sensing ligands processed through endocytic pathways. Autophagy is a mechanism involved in normal turnover of cellular contents, but is also activated upon infection to target cytosolic pathogens for destruction in the endosome. Genetic studies using mice lacking components of the autophagy machinery (autophagy-related proteins (Atgs)) have revealed a role for autophagy in the sensing of cytosolic viruses (167). In Atg5 deficient PDCs, the production of both type I IFN and the inflammatory cytokine IL-12 p40 induced by VSV and Sendai virus, which replicates in the cytosol was severely impaired. The type I IFN production in PDC response to these viruses required live viral infection in the cytoplasm and endosomal TLR7 (166), which suggested that autophagy is the bridge necessary for the TLR7-dependent production of type I IFNs and cytokines in PDCs after certain viral infections.

1.4.2 Regulation of type I IFN by viral evasion

Viruses are rapidly evolving parasites. They require the host cell protein synthesis for their survival and reproduction. When host receptors detect viral infection, potent cytokines especially type I IFN will be quickly produced to directly kill the invaders and induce antigen-specific responses. Viruses have evolved all kinds of strategies to block their recognition by the immune system and fight against the production of antiviral effectors, so that they can proliferate

and spread in the host. These strategies target different stages of the PRR signaling, including: 1) interfering with the induction of type I IFN—targeting the receptors, the adaptors, the kinases, the transcription factors; 2) interfering with IFN-induced signaling and 3) inhibiting interferon effector proteins. Besides the evasion of host detection, viruses have also been reported to subvert the host signaling for their own benefit. Some examples are shown below.

1) Targeting the induction of type I IFN

Influenza viruses target the type I IFN production pathway at several steps. Its NS1 protein can sequester dsRNA produced during viral replication thereby preventing the RNA from being detected by the cytosolic receptor RIG-I. The NS1 protein also interacts with host Trim25, an E3 ubiquitin ligase essential for RIG-I activation. NS1 blocks Trim25 to inhibit RIG-I ubiquitination and binds to the adaptor MAVS to block the signaling pathway (168). Ebola virus (EBOV) expresses VP35 protein which binds IKKε and TBK1 as a pseudosubstrate to block IRF3/7 activation (169). HSV-8 developed a very unique strategy by expressing viral homologs of cellular IRFs, called vIRFs, to interfere with both the IFN induction and IFN-induced signaling pathway (170).

2) Targeting type I IFN signaling and effector ISGs

Vaccinia virus expresses a soluble IFNα/β receptor, B19, which can sequester IFNα/β produced by the infected cells, thus blocking IFN downstream signaling (171). Ebola virus and Marburg virus, which both belong to the *Filoviridae* family, express VP24 to inhibit the IFNα/β signaling pathway by

preventing STAT1 and STAT2 phosphorylation and STAT1 nuclear accumulation (169). CMV has evolved mechanisms to directly limit ISG function: hCMV proteins pIRS1 and pTRS1, and mCMV proteins pm142 and pm143 can counteract PKR pathway directly and inhibiting OAS/RNase L pathway activation (172).

1.4.3 Paramyxoviruses

The *paramyxoviridae* family includes some of the most prevalent disease-causing human pathogens as well as viruses infecting other animals (measles virus, RSV, parainfluenza virus, metapneumovirus, NDV, SV, Hendra virus, Nipah virus, etc.). The family is classified into two subfamilies: 1) the paramyxovirinae, which contains five genera, *Respirovirus*, *Rubulavirus, Avulavirus, Morbillivirus*, and *Henipavirus*; 2) the Pneumovirinae, which contains two genera, *Pneumovirus* and *Metapneumovirus*. Viruses of *paramyxoviridae* are enveloped, containing non-segmented, single stranded negative sense RNA genomes. Paramyxoviruses replicate in the cytoplasm of infected cells. The viral genome is between 15-19 kb in length and functions as template for two purposes: 1) for transcription of mRNA to produce new viral proteins; 2) for synthesis of anti-genome positive strand RNA to produce new viral genomes. The newly produced genomes and proteins are then packaged with the lipid envelopes derived from the plasma membrane of host cells. Paramyxovirus genome contains 6-10 genes in conserved order, flanked by transcriptional

control sequences. They encode genome-associated proteins (nucleocapsid protein N, phosphoprotein P, and large polymerase L), envelope localizing proteins (fusion protein F, attachment protein HN, or H or G, some viruses also have a small hydrophobic protein SH), and matrix protein (M). Some viruses also encode non-structural proteins (NS). The P gene also extends the coding capacity of some viruses by encoding alternative protein products through “RNA editing”, such as V/W/I/D (173).

N, P, L proteins

The N protein is an RNA binding protein. It coats both the negative viral genome and the positive anti-genome. N protein is a major structural protein. The encapsidation of the RNA not only prevents the RNA from host nuclease digestion, but also turns the RNA into the biologically active form essential for transcription and replication. It also helps for the virion assembly. L protein usually associates with N and P protein to form a complex called RNA polymerase (RNAP) and L is the catalytic subunit of the RNA dependent RNAP. It is believed that L protein is responsible for catalyzing all the synthesis of RNA products, including transcription of mRNA and replication of genomic RNA. The P protein is a part of the RNAP, essential for viral RNA synthesis. Besides encoding the P protein, the P gene from the Paramyxovirinae subfamily has overlapping open reading frames that give multiple distinct protein products, such as “V”, “C’’, “W”, “D”, or “I” proteins by the process called “RNA editing”. These

alternative P gene products are not essential for viral replication but the deletion of these products results in severe attenuation.

F, HN (H or G), SH, and NS proteins

F protein mediates viral entry by fusion between the viral envelope and the host plasma membrane. F protein is synthesized as an inactive form F0, which needs to be cleaved by host protease into the active F1 form. The F protein of paramyxovirus promotes membrane fusion at neutral pH, with the exception that the membrane fusion of certain HMPV strains is induced at low pH (174, 175). The F proteins expressed at the host plasma membrane during viral reproduction also can mediate the fusion with neighboring cells for virus spread.

HN (H or G) protein is the cell attachment protein. It binds to the cellular receptor to facilitate virus binding to target cells and trigger F-protein mediated fusion. Depending on the activity of the attachment protein, hemagglutinin (H) – binding sialic acid, and neuraminidase (N) – cleaving sialic acid, the Respirovirus, Rubulavirus and Avulavirus attachment proteins are denoted HN, Morbillivirus attachment protein is denoted H, Henipavirus, Pneumovirus and Metapneumovirus attachment proteins are denoted G, without H or N activities.

Some of the viruses, such as PIV5, mumps virus and viruses from Pneumovirinae have SH protein. It is a type II integral membrane protein, but the function is not yet clear. Pneumovirinae family members also encode NS1 and NS2 proteins. While the function of NS proteins in viral growth remains unclear,

the NS1 an NS2 proteins from RSV can interfere with host type I IFN production (176).

1.4.4 Innate immune response to paramyxoviruses

Paramyxoviruses, especially SV and NDV, are potent type I IFN inducers. Their nucleic acids, or the RNA products produced during viral reproduction can be detected by PRRs, including the cytosolic RLRs, RIG-I and MDA5 (94, 151, 177-181), and in PDCs are sensed by endosomal TLRs, TLR7, TLR8 (177). It was also reported that the viral F protein from RSV could activate TLR4 for type I IFN production (56, 182).

Sendai virus (SV) and Newcastle disease virus (NDV)

SV and NDV both belong to the Paramyxovirinae subfamily. They are used in the laboratory as experimental tools to dissect the mechanisms underlying the production of type I IFNs. Following the discovery of RIG-I it was shown that, both SV and NDV failed to induce type I IFN production in RIG-I-deficient cells. Similar results were observed in RIG-I deficient bone marrow derived CD11c+CDCs. This was in contrast to the response in MyD88/TRIF double deficient cells, which suggested the importance of RIG-I in sensing these viruses. In contrast to these observations in MEFs and BMDMs, bone marrow derived Flt3L-DCs (PDC) showed that the IFN response to these viruses was dependent on TLR7. Like SV, NDV induced type I IFN was RIG-I dependent in splenic CD11c+CDCs, and was MyD88 dependent in splenic PDCs (151). This

differential recognition was also revealed using MAVS deficient cells. MAVS is required for the type I IFN and IL-6 activation by SV in MEFs, macrophages, and CDCs, but not in PDCs (183).

Respiratory syncytial virus (RSV) and Human metapneumovirus (HMPV)

RSV and HMPV both belong to Pneumovirinae subfamily. Similar to the response to SV and NDV, the type I IFN activation by RSV was also found to be RIG-I/MAVS dependent in murine MEFs, macrophages, and CDCs. After *in vivo* infection, IFNα/β and IL6 levels in bronchial lavage fluid were found to be totally dependent on MAVS. In contrast, TNFa, MCP-1 and IL-1b were found to be dependent on both MAVS and MyD88. In purified bone marrow derived Flt3L-PDCs, RSV failed to activate type I IFN (184), consistent with the observation that the RSV A2 strain can block type I IFN production by human PDCs, probably through inhibiting MyD88 signaling pathway (185). There was also a report showing that RSV NS1 and NS2 proteins could inhibit type I IFN production in A549 cells and human monocyte-derived macrophages (176).

HMPV is a relatively recently identified member of the paramyxovirus family (Figure 1.4). Phylogenetic analysis has revealed two major genetic clusters for HMPV, designated as group A and B, which have been further subdivided into four main subtypes A1, A2, B1 and B2 (186, 187). At the outset of my studies, there was no information available regarding the mechanisms by which the immune systems detect HMPV viruses. Although, a recent study has

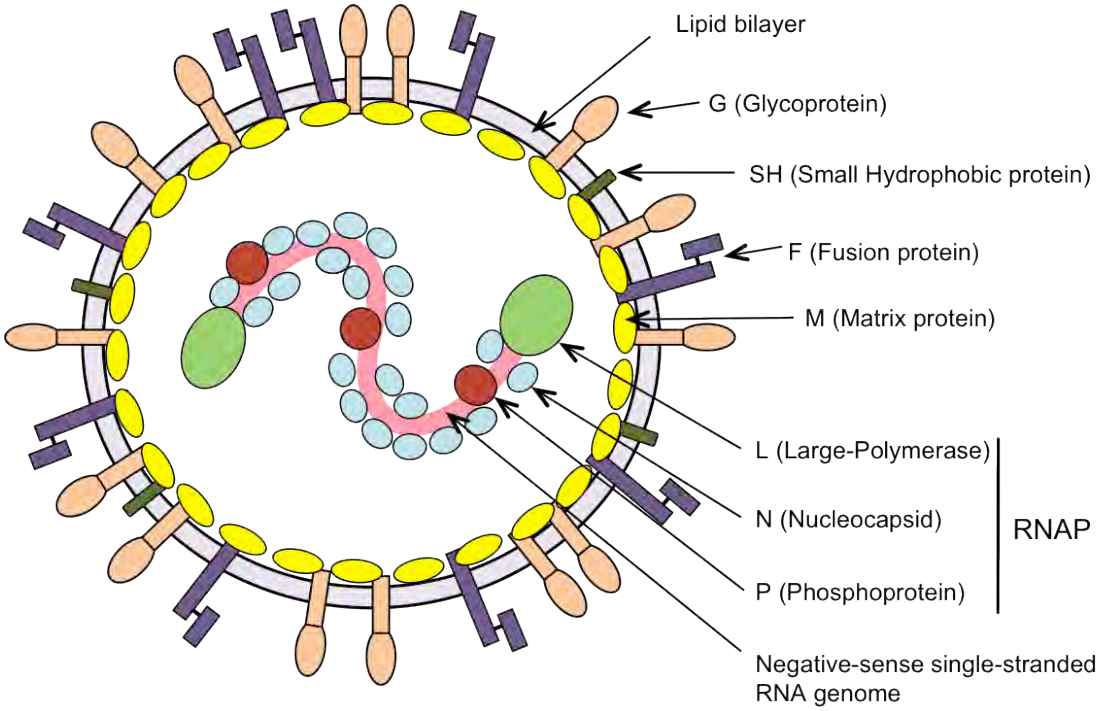


Figure 1.4 Schematic diagram of a HMPV. HMPV belongs to the paramyxovirus family. It contains a negative-sense single-stranded RNA genome, which forms a complex with the L, N and P protein. The virus is enveloped with G, SH and F proteins embedded in the Lipid bilayer, and M protein aligning inside.

implicated the RIG-I pathway in sensing the HMPV strain CAN97-83 (A2 strain) (188). In Chapters II and III, we investigated the innate immune response to HMPV in detail by dissecting the role of TLR and RLR signaling pathways.

1.5 Thesis objective

This thesis study focuses on understanding the molecular mechanisms involved in sensing RNA viruses and the means by which viruses in general turn on protective immune responses.

The objective of this thesis includes:

1) An investigation of the innate immune sensing of HMPV viruses, by examining the receptors and the signaling pathways involved in type I IFN induction by this virus. Using different human cell lines as well as primary human and murine monocytes and PDCs, we demonstrated that type I IFN production during infection with HMPV viruses involves differential sensing mechanisms, which work in a cell-type specific manner.

2) The second objective in this study was to explore the mechanisms underlying the differential induction of type I IFN by two closely related HMPV stains. These mechanistic studies in particular led us to explore the role of the phosphoprotein from HMPVB1 virus and identify this molecule as an immune evasion gene interfering with type I IFN induction.

3) The third objective for my studies was to explore downstream signalling pathways in IFN gene regulation. In particular, these efforts focused on defining

the function of IRF5 in anti-viral innate immune responses. A careful examination of IRF5 deficient cell responses to TLR ligands revealed the involvement of IRF5 not only in the induction of type I IFNs in response to some TLR ligands in specific cell types, but also unveiled the importance of IRF5 in the regulation of inflammatory cytokines. Furthermore, we also uncovered a novel role for IRF5 in the NOD2-RIP2 pathway in response to Mtb infection.

Preface to Chapter II

This Chapter has been published in:

Goutagny N, **Jiang Z**, Tian J, Parroche P, Schickli J, Monks BG, Ulbrandt N, Ji H, Kiener PA, Coyle AJ, Fitzgerald KA. 2010. Cell type-specific recognition of human metapneumoviruses (HMPVs) by retinoic acid-inducible gene I (RIG-I) and TLR7 and viral interference of RIG-I ligand recognition by HMPV-B1 phosphoprotein. *J Immunol.* 184(3):1168-79.

* **Goutagny N and Jiang Z** contributed equally.

In this Chapter, **Zhaozhao Jiang** contributed to Figure 2.1 b, c, e; Figure 2.3 a, b, c; Figure 2.4; and Figure 2.5 a, c.

Chapter II innate immune sensing of HMPV

Abstract

HMPV are recently identified *Paramyxoviridae* that contribute to respiratory tract infections in children. No effective treatments or vaccines are available. Successful defense against virus infection relies on early detection by germline encoded pattern recognition receptors and activation of cytokine and type I interferon genes. In this study, we investigated the ability of two prototype strains of HMPV (A1 [NL\1\00] and B1 [NL\1\99]) to induce type I IFN in different cell types. Recently, RIG-I has been shown to sense HMPV. Sensing of HMPVA1 virus occurs *via* RIG-I in most cell types tested including human cell lines and purified human monocytes. We have also identified 5'- triphosphate RNA as the HMPV viral ligand triggering the RIG-I/IFN-I response. In these cell types, HMPVB1 failed to elicit type I IFN production. In contrast, PDC displayed a unique ability to sense both the A1 and B1 strains and in this case sensing was *via* TLR-7 rather than RIG-I. Collectively, these data reveal differential mechanisms of sensing for two closely related viruses, which operate in cell-type specific manners.

Introduction

HMPV is a newly described virus responsible for lower respiratory tract infections in children (189). The virus was first isolated in the Netherlands in 2001. Compared to its closest human relative, RSV, HMPV has a worldwide prevalence and causes a broad spectrum of illnesses that range from asymptomatic infection to severe bronchiolitis. Serological studies have revealed that virtually every child has been exposed to HMPV by the age of 5 (189). Depending on the population analyzed, 5 to 15% of respiratory infections and 12 to 55% of otitis media may be attributed to HMPV infection (186). Retrospective studies have revealed that HMPV is not a new virus, but rather one that has been circulating for about 50 years (189). There is currently no effective treatment or vaccine available for HMPV infection. Recent studies in mice have revealed an important role for T cells in anti-viral immunity and pathogenesis (190), however our current understanding of the innate immune response to HMPV is limited.

HMPV is an enveloped virus, containing a single-stranded negative-sense RNA genome, encoding 8 open reading frames. Based on its sequence homology to the avian pneumovirus, it was assigned to the *Metapneumovirus* genus within the *Paramyxoviridae* family, which includes some of the most prevalent viruses known (Measles virus, RSV) (173). Phylogenetic analysis has revealed two major genetic clusters for HMPV, designated as group A and B, which have been further subdivided into four main subtypes A1, A2, B1 and B2 (186, 187). Unlike most viruses which enter cells by receptor-mediated

endocytosis, most paramyxoviruses deliver their genome into the cytoplasm directly by fusion with the plasma membrane (164).The attachment (G, H or HN) and fusion (F) proteins are critical for mediating these events (191). Most other viruses enter cells by receptor-mediated endocytosis and are delivered to the endosomal compartment where the acidic environment is critical for viral fusion and the release of viral genomes into the cytosol. Several classes of germline encoded pattern recognition receptors, which recognize different components of viruses, have been identified. In most cases, viruses are sensed via their genomes or their replicative or transcriptional activities (192). The recognition of RNA and DNA viruses has been shown to involve endosome-localized TLRs, including TLR7 and TLR9, which are predominantly expressed on PDC, the major producers of IFNα *in vivo* (90). Both Influenza virus and VSV are sensed by TLR7 in PDC (42), whereby recognition of the genomes of these ssRNA viruses is tightly linked to viral fusion and uncoating (41). DNA viruses such as HSV are sensed in PDC via TLR9. Induction of IFNα by the TLR7/9 pathway is mediated by the TLR adapter MyD88 and the transcription factor IRF7 (122, 193).

Sensing of RNA and DNA viruses also occurs in the cytosol and in the case of RNA viruses, is mediated by a second class of immune sensors, the RNA helicases, RIG-I and MDA-5. Genetic evidence has revealed that RIG-I and MDA-5 discriminate between different classes of RNA viruses (90, 151). RIG-I is required for triggering anti-viral responses against several *Flaviviridae,*

Paramyxoviridae, Orthomyxoviridae and Rhabdoviridae, whereas MDA-5 is required for the response against picornaviruses like EMCV (90, 194). RIG-I senses the nascent 5' triphosphate moiety of viral genomes or virus derived transcripts of negative-sense ssRNA viruses, whereas MDA5 is activated by long dsRNA, a typical intermediate of replication of plus-sense ssRNA viruses. RIG-I and MDA-5 induce type I IFN responses by recruiting a CARD domain containing adapter molecule, MAVS (83) and triggering IRF3 activation to regulate type I IFN gene transcription. Some DNA viruses are also sensed by a pathway involving RIG-I, however in this case viral DNA is transcribed by RNA polymerase III into an RNA intermediate which is then recognized by RIG-I (101, 102).

A recent study has implicated the RIG-I pathway in sensing of the HMPV strain CAN97-83 (A2 strain). HMPV CAN97-83 induces IFNβ and chemokine gene expression in a RIG-I dependent manner (188). Here, we have examined the role of RIG-I in the detection of HMPV viruses by comparing 2 prototype strains NL\1\00 (A1) and NL\1\99 (B1) in human primary cells and cell lines. Although closely related, only the HMPV-A1 strain activated type I IFN gene transcription in most cell types examined. In both human cell lines as well as highly purified human monocytes the A1 but not the B1 strain induced type I IFN. In these cell types, induction of IFNα/β by HMPV-A1 was mediated by the recognition of 5'-triphosphate viral RNA through RIG-I and its downstream adaptor MAVS. In contrast to human cell lines and monocytes, PDC produced

type I IFN upon infection with both A1 and B1 strains. In PDC, treatment of cells with lysosomotropic agents that prevented endosomal acidification blocked IFNα induction by both viruses, which was mediated by TLR7. Taken together these data emphasize the unique ability of PDC to sense and induce type I IFN in response to viruses that appeared “invisible” to most other cell types. These data also indicated that the possibility of two closely related viruses to differentially antagonize innate immune sensing mechanisms.

Results

HMPV-A1 can activate IFNB gene in human cell lines and primary cells.

In an effort to understand the underlying mechanisms regulating the innate immune response to HMPV viruses, we tested the ability of 4 major strains (A1, B1, A2 and B2) to induce type I IFN by examining IFNβ reporter gene activity in infected cells. 293T cells were transfected with a reporter gene under the control of the IFNB gene enhancer, and then were infected with the 4 strains of HMPV viruses separately. We measured the luciferase value as the readout of IFNβ reporter activation. The HMPV-A1 strain induced the IFNβ reporter expression at a high level, while the other strains failed to drive this reporter (Figure 2.1a).

We also observed HMPV-A1 stain activated IFNB gene in the human hepatoma cell line, Huh7 (Figure 2.1b) and the human alveolar epithelial cell line, A549 (Figure 2.1c). Similar observations were made when the endogenous IFNβ transcript levels were measured. HMPV-A1 induced IFNB gene transcription. (Figure 2.1d). We also tested IFNα4 reporter and observed that HMPV-A1 strain activated this reporter. (Figure 2.1e). In the same experimental conditions, HMPV-B1 strains failed to activate type I IFN reporter or transcription in these cell lines.

We also tested the induction of type I response by HMPV viruses in primary cells. Human peripheral blood mononuclear cells (PBMC) were purified

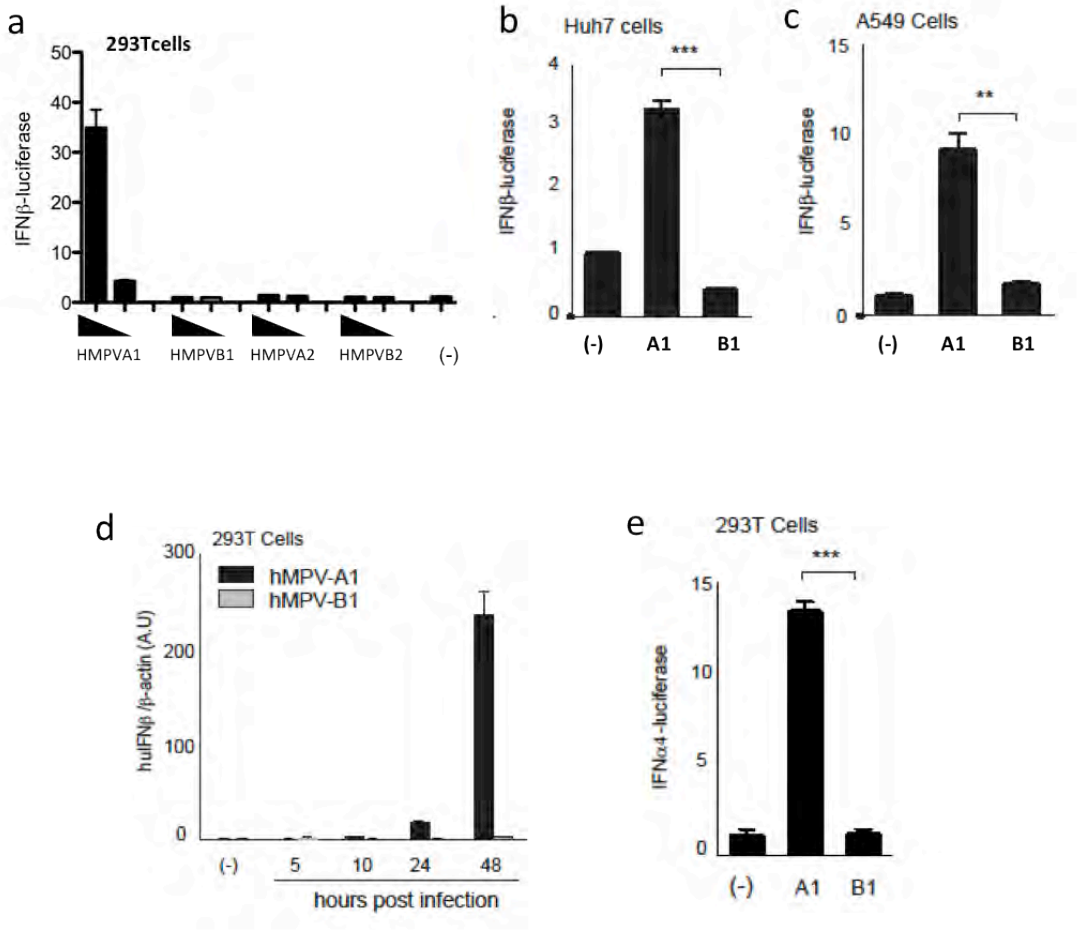


Figure 2.1 HMPV A1 and B1 strains differentially induce type I IFN gene expression (a-c, e) 293T, Huh7 or A549 cells were transfected with the full length IFNβ promoter or IFNα4 promoter. Cells were infected with HMPV-A1, HMPV-B1, HMPV-A2, or HMPV-B2 (from $5x10^4$ to $5x10^2$pfu/ml for 293T, $5x10^4$pfu/ml for other lines) for an additional 24h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (d) 293T cells were stimulated with HMPV-A1 or HMPV-B1 (5.10^4pfu/ml) for the indicated time. Levels of human IFNβ and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFNβ over 100 copies of β-actin.

from whole blood and infected with HMPV viruses and the production of the type I IFN (IFNα) was measured by Enzyme-linked immunosorbent assay (ELISA). Contrary to previous observations in human cell lines, both the HMPV-A1 and HMPV-B1 strains induced type I IFN in PBMC (Figure 2.2a). Both IFNα and IFNβ mRNA levels were induced as measured by quantitative PCR (Figure 2.2b). Monocytes and PDC are the major producers of type I IFN in PBMC. We therefore purified monocytes and PDC from total PBMC. Monocytes responded to HMPV-A1 but not B1 (Figure 2.2c) while PDC responded to both viruses (Figure 2.2d). These results revealed the unique ability of PDC to induce type I IFN in response to HMPV-B1, which may signal through a different pathway than in cells lines and monocytes. We first focused on HMPV-A1 stain in order to investigate the mechanism of type I IFN induction by HMPV in cell lines and then used both A1 and B1 strains to study the response in PDC.

HMPV-A1 triggers type I IFN gene transcription via RIG-I/MAVS pathway

Since RIG-I is a sensor of paramyxoviruses and HMPV has been shown to trigger RIG-I signaling (83, 90, 188, 195), we investigated the contribution of the RIG-I pathway to IFNB gene activation upon HMPV-A1 stimulation. We first examined the role of MAVS, which is the adaptor protein for RIG-I and MDA5 for signaling to downstream kinases and transcription factors. The involvement of MAVS was assessed using the NS3/4A protease from Hepatitis C Virus (HCV).

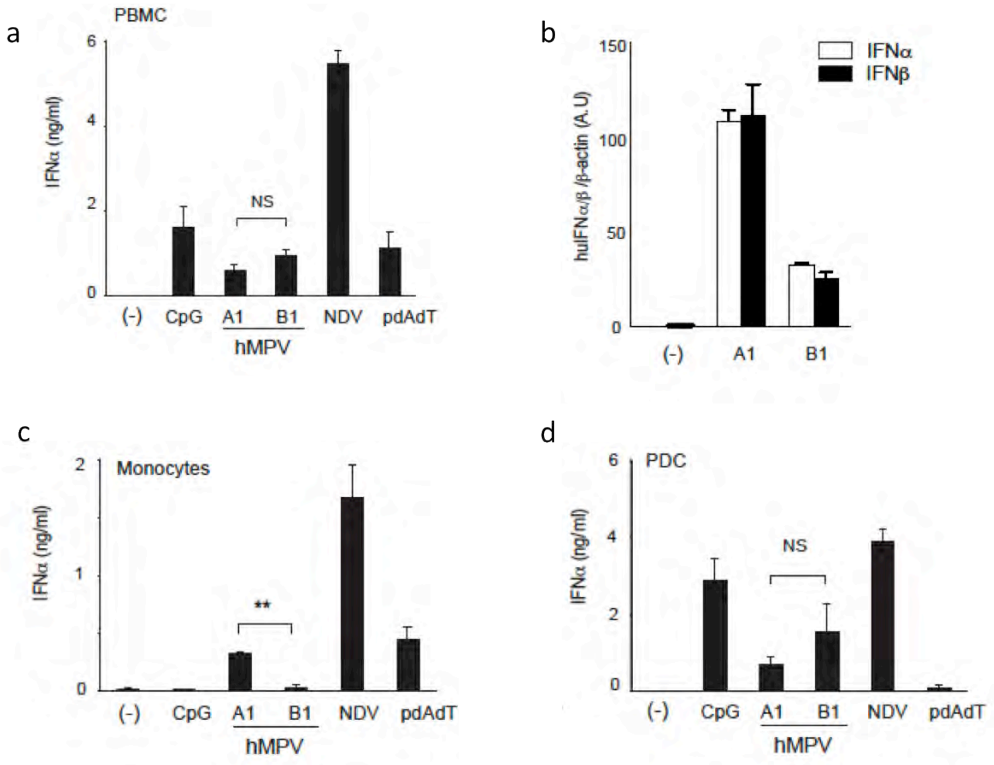


Figure 2.2 Induction of type I IFN in primary cells in response to HMPV-A1 and B1 is cell type dependent. (a, c, d) Total PBMC, monocytes or PDC were stimulated with HMPV-A1, HMPV-B1 ($2x10^5$pfu/ml), CpG-A 2216 (3mM), NDV (8HAU/ml), or poly(dAdT)•poly(dAdT) (5mg/ml) for 24h. Protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (b) PBMC cells were stimulated with HMPV-A1 or HMPV-B1 (2.10^5pfu/ml) for 18h. Levels of human IFNα, IFNβ and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFNα or IFNβ over 100 copies of β-actin.

NS3/4A cleaves MAVS off the mitochondria and inactivates MAVS, thereby disrupting RIG-I signaling (196). NS3/4A can therefore be used as a tool to implicate MAVS signaling in a particular response. The effect of NS3/4A on the HMPV-A1-induced IFN response was tested in 293T cells. Increasing concentrations of wild-type (WT) NS3/4A protease dose-dependently blocked the induction of IFNβ by HMPV-A1 as well as that induced by SV (Figure 2.3a). Importantly, the protease inactive mutant (NS3/4A-S139A) had no effect. The results indicated that MAVS is involved in the IFNβ activation pathway in response to HMPVA1.

Then we tested the involvement of RIG-I using the hepatoma cell line (Huh7) and the Huh7.5 sub-line that bears a natural mutation in RIG-I (T55I), which renders it inactive (197). The transcriptional enhancer of the IFNB gene contains four positive regulatory domains (PRDI–IV), which bind distinct transcriptional regulators that act cooperatively to activate IFNB gene expression. The transcription factors that bind to these elements include NFκB, which binds to PRDII; IRF-3 and -7, which bind to adjacent PRDIII and PRDI sites, collectively referred to as PRDIII-I, and the heterodimeric transcription factor ATF-2/c-Jun, which binds to PRDIV. Huh7 and Huh7.5 cells were transfected with the IFNB-PRDIII-I (IRF) reporter gene and then infected with HMPV-A1 or NDV. Activation of the IFNB-PRDIII-I element was completely abrogated in the Huh7.5 cell line in response to HMPV-A1 (Figure 2.3b). The IFNβ response following NDV infection (which is known to be RIGI-I-dependent) was also inhibited in the Huh7.5 cells,

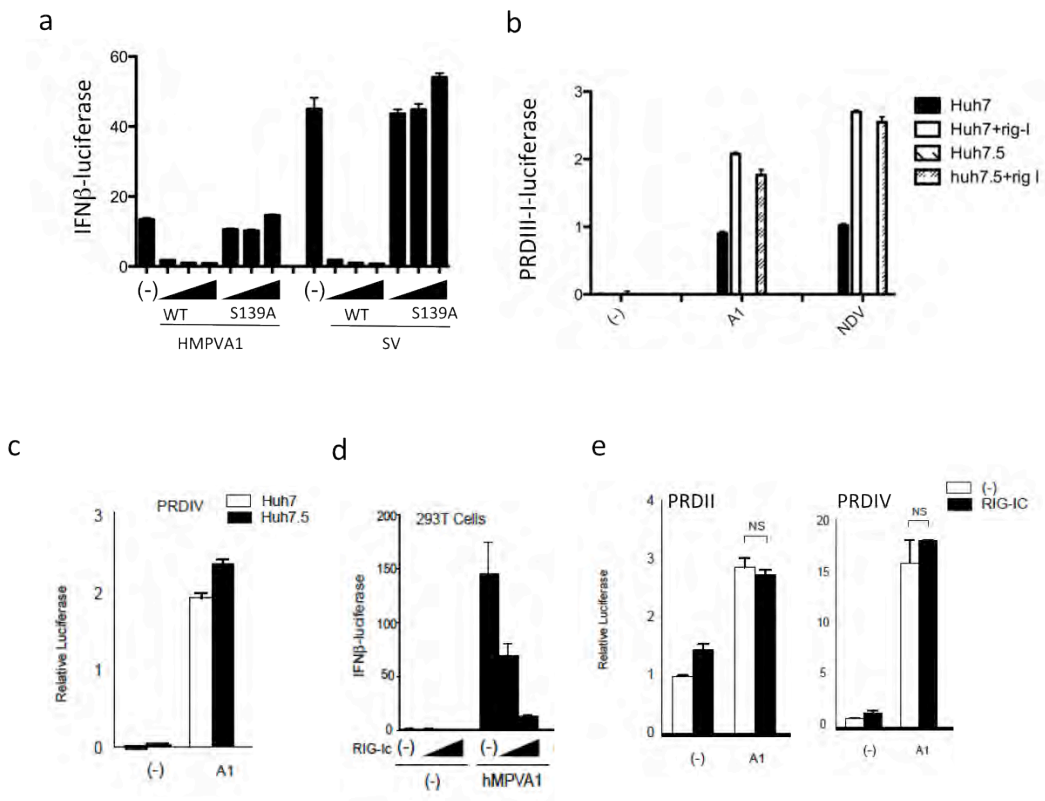


Figure 2.3 HMPV-A1 triggers type I IFN gene transcription via RIG-I/MAVS pathway (a) 293T cells were transfected with the IFNβ reporter gene and increasing concentrations (5-80ng/well) of the WT or S139A inactive NS3/4A protease from HCV. Cells were stimulated for an additional 24h with HMPV-A1 ($5x10^4$pfu/ml) or SV (400HAU/ml). Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) Parental human hepatoma cell line Huh7 and Huh7.5 were transfected with the IFNβ reporter gene in the absence or presence of pEFBos-huRIG-I Flag (40ng/well). Cells were stimulated with HMPV-A1 ($5x10^4$pfu/ml) or NDV (64HAU/ml) for an additional 24h. Results are normalized by renilla luciferase and presented as arbitrary units after correction between the 2 cell lines using the pGL3-control. (c) Parental human hepatoma cell line Huh7 and Huh7.5 were transfected with the PRDIV reporter gene and stimulated with HMPV-A1 ($5x10^4$pfu/ml) for an additional 24h. Results are normalized by renilla luciferase and presented as arbitrary units after correction between the 2 cell lines using the pGL3-control. (d) 293T cells were transfected with increasing amounts of RIG-IC (0-2-20ng/well) along with the IFNB reporter and stimulated with HMPV-A1 ($5x10^4$pfu/ml). Luciferase activity was measured 24h post-transfection and data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (e) 293T cells were transfected with or without RIG-Ic (2ng/well) along with the PRDII or PRDIV element reporter and stimulated with HMPV-A1 ($5x10^4$pfu/ml) for an additional 24h. Luciferase data are expressed as fold induction relative to the reporter-only control and are the mean ± SD.

consistent with published results. Moreover, reconstitution of Huh7.5 cells with WT RIG-I fully restored the response to both HMPV-A1 and NDV. Interestingly, IFNB-PRDIV (MAP kinase) reporter gene activation by HMPV-A1 was not inhibited in Huh7.5 cells (Figure 2.3c).

We also found that a dominant negative version of RIG-I, consisting of the helicase domain only (RIG-IC), dose-dependently inhibited the IFNβ response in 293T cells elicited by HMPV-A1 virus (Figure3d). Consistent with the results in Huh7.5 cells, IFNB-PRDII (NFκB) and IFNB-PRDIV (MAP kinase) reporter gene activation by HMPV-A1 were not inhibited by RIG-IC overexpression in 293T cells (Figure 2.3e). Collectively these observations suggest that HMPV viruses can trigger RIG-I to turn on type I IFN production and also trigger RIG-I-independent signaling events leading to NFκB and AP-1 activation. Thus, RIG-I as well as additional yet unidentified PRR driven pathways contribute to the host response to this virus.

Although MDA5 also uses MAVS as adaptor protein for downstream signaling, MDA5 did not appear to be involved in the recognition of HMPV-A1. A dominant negative mutant form of MDA5 did not inhibit the induction of the IFNβ reporter by HMPV-A1 (Figure 2.4a left panel), but blocked the IFN response to WT MDA5 (Figure 2.4a right panel). A recent paper reported that NOD2 was a very early receptor for RSV (belongs to paramyxovirus family, the closest relative of HMPV) to trigger IFNβ response in human cell line 293T (198). In our hands, overexpression of RIG-I but not NOD2 in 293T cells enhanced the induction of

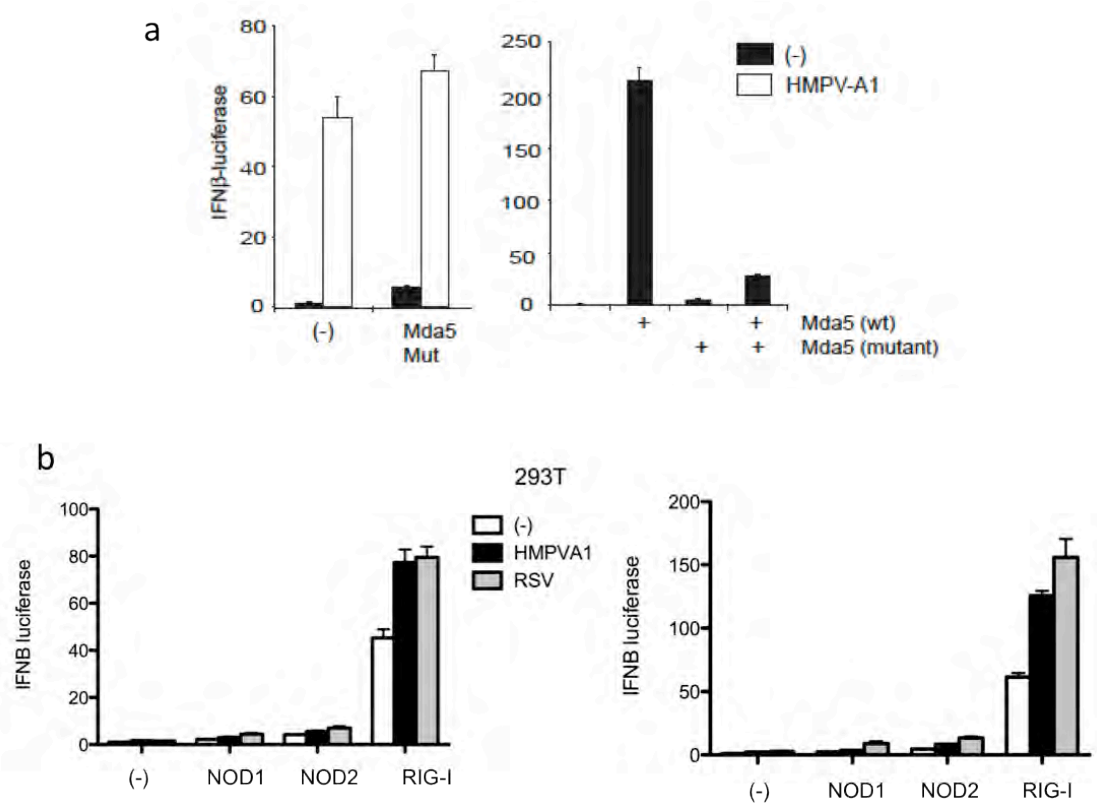


Figure 2.4 Type I IFN activation by HMPVA1 does not involve MDA5 or NOD2 (a) 293T cells were transfected with the IFNβ reporter gene and WT or a dominant negative mutant of MDA-5 (80 ng/well). Cells were stimulated with HMPV-A1 ($5x10^4$pfu/ml) for additional 24hrs. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) 293T cells were transfected with IFNB reporter gene and indicated NOD1, NOD2, or RIG-I expression plasmids (40ng/well). Cells were stimulated with HMPVA1 ($5x10^4$pfu/ml) or RSV A2 strain ($5x10^4$pfu/ml) for additional 6hrs (left panel) or 10hrs (right panel). Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD.

IFNβ luciferase activity by HMPV-A1 and RSV even at early time points. The results suggested a major role for RIG-I in the sensing of those viruses and NOD2 did not seem to be involved in this response (Figure 2.4b).

We next examined the requirement for viral replication in the HMPV-A1 induced type I IFN response. We compared live virus to either heat or UV-inactivated HMPV-A1 on IFNβ reporter gene activity in 293T cells. UV- and heat-inactivation of HMPV-A1 and NDV completely blocked their ability to induce IFNB reporter gene activity (Figure 2.5a). Similar results were observed in purified human monocytes, the activation of IFNα was totally lost (Figure 2.5b). The 5'-triphosphate moiety of the viral RNA is the major ligand recognized by RIG-I (178, 199). To further investigate the nature of the ligand in HMPV viruses that triggers RIG-I, viral RNA was purified from HMPV-A1 and transfected into the cytoplasm of 293T cells using lipofectamine. Transfection of the viral RNA induced the IFNβ reporter gene and removal of the phosphate groups by calf intestinal alkaline phosphatase (CIAP) or digestion of the viral RNA by RNAse A abrogated this response (Figure 2.5c). IFN induction by the double stranded DNA analog, poly(dA-dT)·poly(dA-dT), was unaffected by either CIAP or RNAse A treatment. Moreover, RIG-IC also dose-dependently inhibited the IFNβ response in 293T cells elicited by either HMPV-A1 virus or by HMPV-A1 viral RNA (Figure 2.5d). These data indicate that 5'-triphosphate RNA is the ligand for RIG-I.

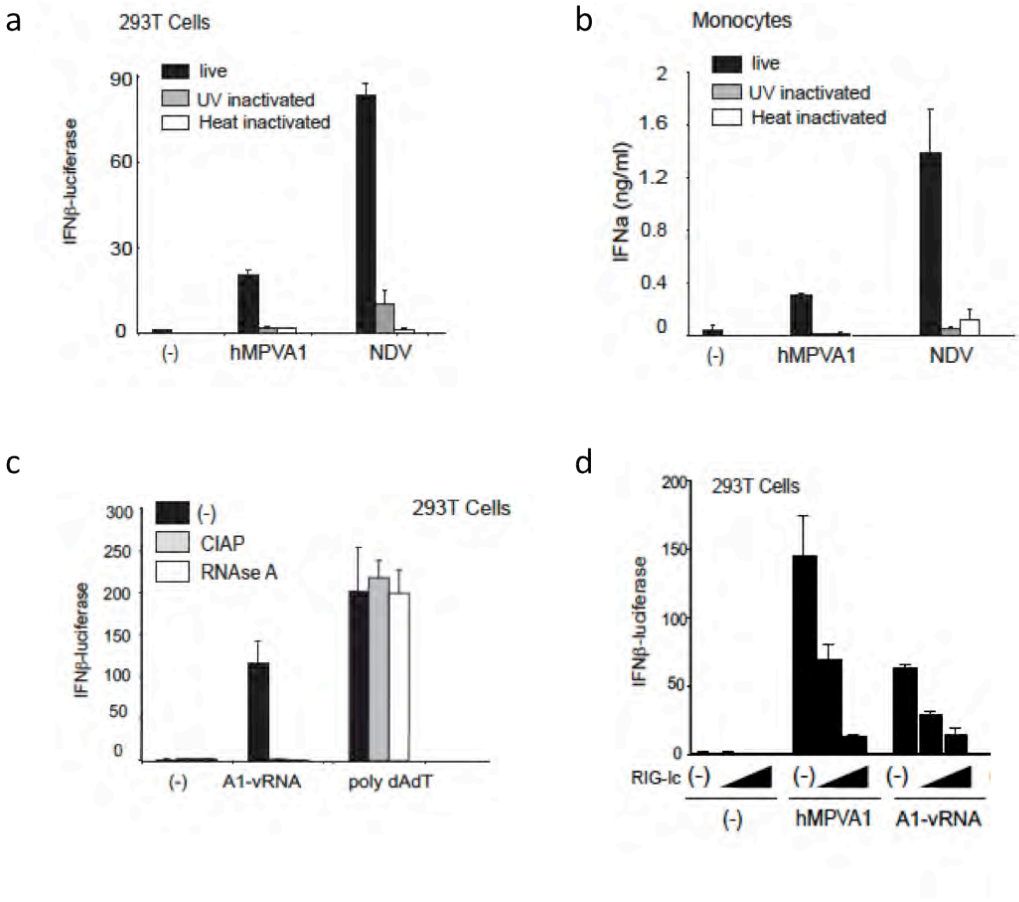


Figure 2.5 Induction of IFNβ by HMPV-A1 requires viral replication. (a) 293T cells were transfected with the full length IFNβ promoter. Cells were stimulated 24h later with live, UV- or heat-inactivated HMPV-A1 ($2x10^5$pfu/ml) or NDV (8HAU/ml) for an additional 24h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) Freshly isolated monocytes were stimulated for 24h with live, UV- or heat-inactivated HMPV-A1 ($2x10^5$pfu/ml) or NDV (8HAU/ml). Human IFNα protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (c) 293T cells were transfected with viral RNA purified from HMPV-A1 (80ng) or poly(dAdT)•poly(dAdT) (20ng) along with the IFNβ and TK-renilla reporter genes. vRNAs and poly(dAdT)•poly(dAdT) were treated with calf intestinal alkaline phosphatase (CIAP) or RNAse A prior to stimulation as indicated. (d) 293T cells were transfected with increasing amounts of RIG-IC (0-2-20ng/well) along with the IFNB reporter and vRNAs (80ng), or stimulate with HMPV-A1 ($5x10^4$pfu/ml). In all cases, luciferase activity was measured 24h post-transfection and data are expressed as fold induction relative to the reporter-only control and are the mean ± SD.

Detection of HMPV viruses in PDC does not involve RIG-I but occurs via an endosomal sensing pathway

As shown in Figure 1, both A1 and B1 strains induce type I IFN production in PDC. The studies outlined above indicate that it is possible that besides RIG-I others receptors mediated these events. Quantitative PCR analysis revealed that PDC express RIG-I and MAVS albeit at much lower levels than that found in monocytes (200). To examine the contribution of the RIG-I pathway in PDC responses to HMPV viruses, we monitored PDC from mice lacking MAVS. PDC from MAVS-deficient mice responded normally to both HMPV viruses (as well as to NDV, HSV and Influenza viruses) (Figure 2.6a). These latter three viruses have been shown to signal in PDC via TLRs (see below). R848 and CpG DNA, ligands for TLR7 and TLR9 respectively, also induced IFNβ normally in MAVS-deficient PDC. Of note MAVS-deficient myeloid DCs or macrophages are severely compromised in NDV induced IFN responses (Figure 2.6b).

In order to define the mechanisms sensing these viruses in human PDC, we investigated the requirement for viral replication in mediating these responses. In contrast to our observations in 293T and monocytes (Figure 2.5a,b), UV- and heat-inactivation of HMPV-A1, B1 and NDV only partially affected their ability to induce IFNα production (Figure 2.6c). Altogether, these studies confirmed that sensing of HMPV viruses in PDC is not mediated *via* the RIG-I cytosolic pathway.

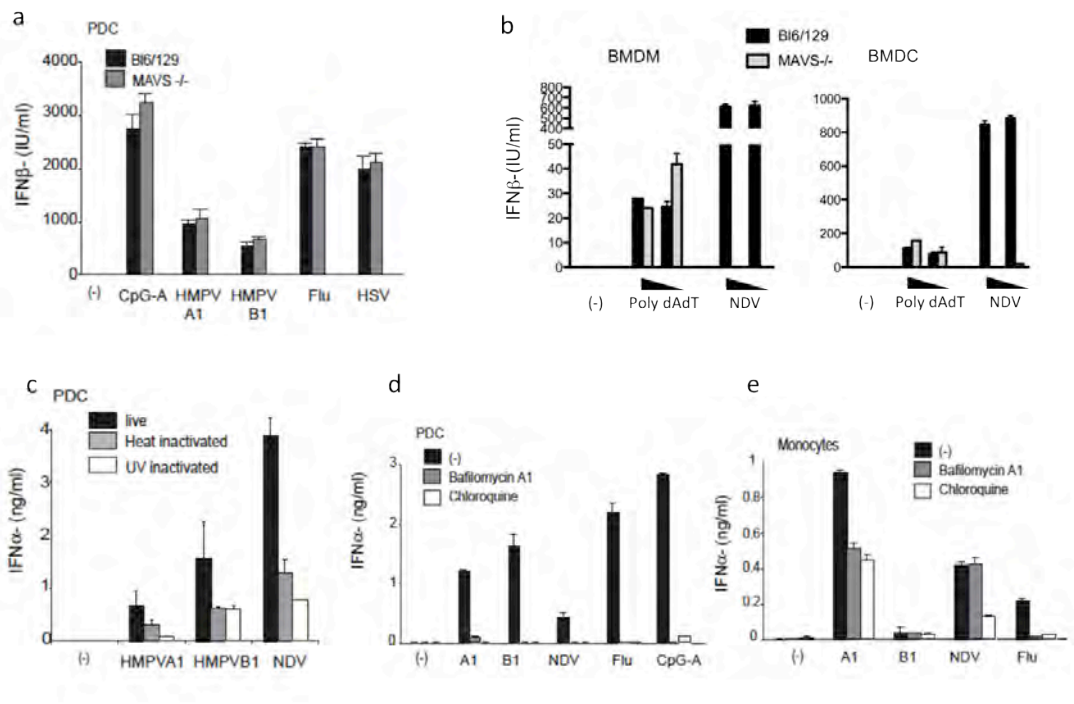


Figure 2.6 Induction of type I IFN in PDC in response to both HMPV-A1 and B1 is through endosomal pathway. (a). PDC from C57Bl6/129 and MAVS-/- mice were stimulated for 24h with CpG-A (2mM), Heat-inactivated Influenza (MOI=0.1), HSV (MOI=100) HMPV-A1, HMPV-B1 ($2x10^5$pfu/ml). Mouse IFNβ protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (b). BMDM and BMDCs from C57Bl6/129 and MAVS-/- mice were stimulated 24h with PolydAdT (5-2.5ug/ml) or NDV (32-3.2HAU). Mouse IFNβ protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (c). Freshly isolated PDC were stimulated for 24h with live, UV- or heat-inactivated HMPV-A1, HMPV-B1 ($2x10^5$pfu/ml) or NDV (8HAU/ml). Human IFNα protein levels were measured in the supernatant by ELISA and presented as the mean ± SD. (d-e). Freshly isolated PDC and monocytes were stimulated with HMPV-A1, HMPV-B1 ($2x10^5$pfu/ml), NDV (8HAU/ml), Heat-inactivated Influenza (MOI=0.1), CpG-A (2mM) for 24 hours. Cells were pre-incubated with chloroquine (1mM) or bafilomycin A1 (4mM for PDC, 10mM for monocytes) for 1 hour were indicated. Human IFNα protein levels were measured in the supernatant by ELISA and presented as the mean ± SD.

Since PDC express high levels of TLR7 and TLR9 and sense viruses *via* endosomally localized TLRs, we next examined the role of this system in the detection of HMPV-A1 and B1 viruses. PDC were treated with chloroquine or bafilomycin A1, two lysosomotropic agents that act by raising the intra-endosomal pH or by specific inhibition of the vacuolar ATPase, respectively (201) (41). Induction of IFNα by HMPV-A1 and B1 in PDC was totally abrogated when cells were pretreated with either chloroquine or bafilomycin A1 (Figure 2.6d). Responses to CpG-A were also completely blocked by chloroquine and bafilomycin A1, consistent with published results (201). In contrast to PDC responses, induction of IFNα by HMPV-A1 in monocytes was less affected (Figure 2.6e). IFNα Induction in response to Influenza is presumably down in chloroquine or bafilomycin A1 treated conditions because of a failure of the virus to infect.

For viruses that enter cells by receptor-mediated endocytosis, viral fusion and uncoating events are tightly coupled to recognition of viral ligands by endosomally localized TLRs. Therefore, inhibition by chloroquine and bafilomycin could implicate TLRs in the sensing of these viruses. To examine the precise role of endosomal TLRs in the recognition of A1 and B1 viruses, PDC were isolated from WT, TLR7 and TLR9-deficient mice and examined IFNβ secretion post-virus infection. As expected, induction of IFNβ by CpG-A and HSV1 was entirely dependent on TLR9 (Figure 2.7a). In contrast, IFNα induced in response to Influenza was unaffected in TLR9-deficient PDC but was completely defective in

TLR7-deficient PDC. HMPV-A1 and -B1 IFNβ production in PDC was induced normally in TLR9-deficient PDC and fully impaired in TLR7-deficient PDC. We also confirmed the TLR7 dependency in the human system using ISS661, a previously characterized oligonucleotide-based inhibitors for TLR7 signaling (48). Pre-treatment with ISS661 blocked the induction of IFNα by HMPV-A1 and B1 (Figure 2.7b). Pretreatment with CpG2088, an inhibitor of TLR9 signaling (202, 203) had no effect. These results reveal that the unique ability of PDC to induce type I IFN in response to HMPV-B1 correlates with the sensing of HMPV viruses through TLR7, and not the cytosolic RNA helicase pathway.

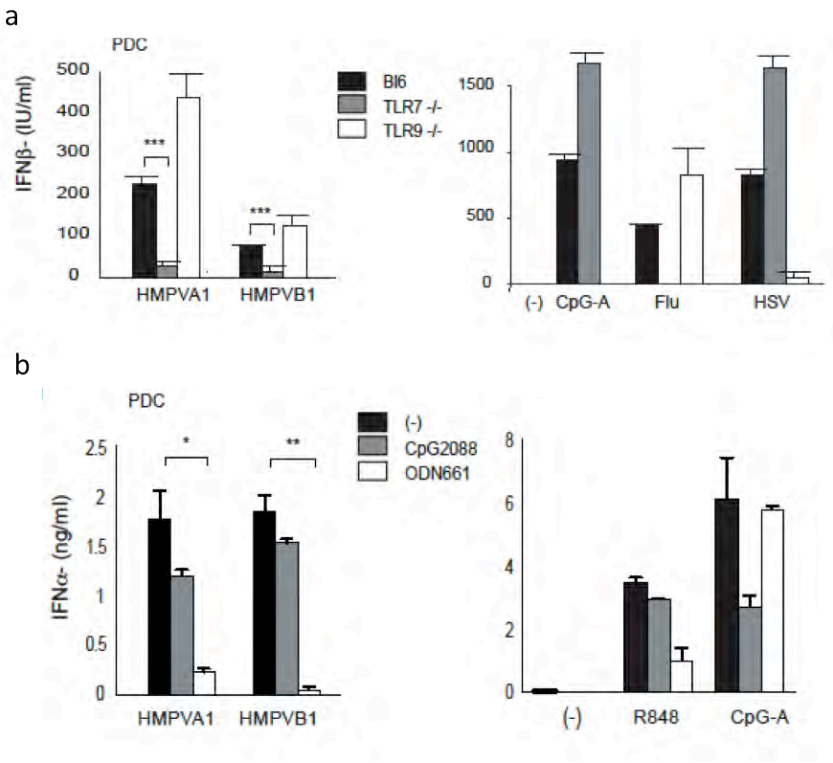


Figure 2.7 Induction of type I IFN in PDC in response to both HMPV-A1 and B1 is TLR7-mediated. (a). Mouse PDC from C57Bl6, TLR7-/- and TLR9-/-mice were stimulated for 24h with CpG-A (2mM), Heat-inactivated Influenza (MOI=0.1), HSV (MOI=100) HMPV-A1, HMPV-B1 ($2x10^5$pfu/ml). Mouse IFNβ protein levels were measured by ELISA and presented as the mean ± SD. (b) Freshly isolated PDC were stimulated with HMPV-A1, HMPV-B1 ($2x10^5$pfu/ml), R848 (10nM), CpG-A (2mM) for 24 hours. Cells were pre-incubated with CpG2088 (0.1mM) or ISS661 (2mM) for 1 hour were indicated. Human IFNα protein levels were measured in the supernatant by ELISA and presented as the mean ± SD.

Discussion

HMPV and RSV viruses are major contributors to respiratory tract infections in infants and young children. In most infants, these viruses cause symptoms resembling those of the common cold. However, in infants born prematurely, children with chronic lung disease, or children with congenital heart disease, these viruses can result in a severe or even life threatening disease. As many as 125,000 hospitalizations occur annually in children less than one year old due to lower respiratory infection or bronchiolitis (204). Developing new therapeutics to prevent and treat these infections is therefore of considerable importance.

Limiting virus infection requires rapidly mounted defences, which include in large part the release of type I IFN (IFNα/β). Interferon limits viral replication directly and enhances viral clearance by activating adaptive immunity. Understanding how viruses are sensed and how type I IFN is regulated may facilitate the rational design of novel anti-viral therapeutics and/or better vaccine candidates useful in the prevention or treatment of lower respiratory tract infections in children. In this study, we demonstrate that type I IFN production during infection with HMPV viruses involves differential sensing mechanisms, which work in a cell-type specific manner. Sensing of HMPV-A1 virus occurs *via* the cytosolic RNA helicase RIG-I in most cell types, with the exception of PDC. A recent study by Casola and colleagues also implicated RIG-I in the sensing of HMPV in airway epithelial cells (188). We have confirmed these observations

using mice with targeted deletions in the RIG-I pathway and have extended these studies to include an analysis of additional cell types. We have identified 5'-triphosphate RNA as the HMPV viral ligand triggering the RIG-I-IFNβ response. Importantly, we also identified a RIG-I-independent pathway for sensing HMPV-A1 and B1 viruses in epithelial cell lines. HMPV-A1 and B1 viruses activated NFκB and AP-1 dependent reporter genes in a RIG-I independent manner. These data suggest additional mechanism of HMPV sensing. The NLR family member, NOD2 was recently shown to act as a cytosolic sensor for RSV infection (198), while our preliminary results suggested that NOD2 is not a receptor sensing HMPV for type I IFN production. However, further studies are required to properly delineate if NOD2 or other receptors also senses HMPV and especially towards NFκB and AP-1 signalling described herein.

In this study we also compared the innate response to two closely related clinical viral isolates. While only HMPV A1 strain elicit a type I IFN response in monocytes and all the cell lines tested and the B1 strain failed to do that, both strains induced type I IFN responses in PDC. IFN production induced by both HMPV strains in PDC was sensitive to bafilomycin A1 and chloroquine and was dependent on TLR7. The current model of anti-viral sensing in PDC suggests that TLR-mediated recognition of viruses occurs without direct infection and that the presence of viral genomic nucleic acids within the endosomal/lysosomal compartment is sufficient to trigger TLRs. Iwasaki and colleagues demonstrated recently that RNA viruses such as VSV which do not enter cells via the

endosomal compartment but replicate in the cytosolic compartment where cytosolic viral replication intermediates are then delivered into the lysosomal compartment by the process of autophagy to trigger TLRs (166). Our preliminary data assessing the role of autophagy in the recognition of HMPV viruses in PDCs showed that induction of IFNα by PDC in response to both HMPV strains, as well as VSV as previously published was abrogated by 3-methyladenine and wortmannin pretreatment. These data suggest that viral RNA may be delivered to TLR7 in the endosome *via* autophagy.

Generally cell entry of paramyxoviruses requires two glycoproteins: the attachment (G, H or HN) and fusion (F) proteins. In the case of HMPV viruses, however, analysis of recombinant viruses lacking the G protein has suggested that attachment and fusion is mainly dependent on the F protein (205). The F protein is a type I glycoprotein, synthesized as an inactive precursor, F0 and subsequently converted into its biologically active form, the heterodimer F1/F2. The majority of *Paramyxoviridae* F proteins are cleaved intracellularly by host cellular proteases, most notably furin. Cleavage of the F-protein from HMPV however, requires secretory proteases, which restrict HMPV viruses to the lumen of the respiratory and enteric tract for replication *in vivo. In vitro* the addition of trypsin to process the F0 protein into its mature form allows efficient propagation of the virus (206). In contrast to most other *Paramyxoviridae* F proteins that require neutral pH for membrane fusion, cleavage of the HMPV F protein might therefore require low pH conditions (175). These findings might indicate a

requirement for low-pH compartments such as the endosome for entry of HMPV viruses in PDC. Receptor-mediated endocytosis at low pH was indeed recently shown in Vero cells for HMPV-A2 strain (207). Since PDC do not need to be infected to induce type I IFN, the ability of PDC to respond may be a result of uptake of viral particles to the endosome directly.

Altogether, our data unveil 2 different mechanisms for sensing of HMPV viruses in the host. Such cell type specific involvement of the RIG-I versus TLR pathways in induction of antiviral responses is not unique to HMPV viruses. This differential sensing has previously been reported in the case of sensing of NDV (151). One can imagine how important it is that more than one mechanism exists to turn on IFN gene regulation. This is particularly important in situations where pathogenic viruses inactivate the IFN arm of the immune response. By employing more than on strategy to turn on IFN, the host allows a back up plan should one arm be deactivated. Defining the role of the RLRs and TLRs in sensing HMPV provides a framework for understanding the evolutionary pressures on the host to constantly evolve mechanisms that are able to overcome viruses' ability to dampen protective responses. Such an understanding of how viruses are detected is essential for the development of vaccines to harness the power of the innate immune system for the benefit of the host.

Material and methods

Cells and mice.

HEK 293, 293T, alveolar epithelial cells (A549) and Vero cells were from ATCC (Manassas, VA). The human hepatocellular carcinoma cell lines Huh7 and Huh7.5 were from C. Rice (Rockefeller University, New York, NY). B16-FLT3L producing cells were from G. Dranoff (HMS, Boston, MA) (208). All of the above cell lines were maintained in DMEM (Mediatech Inc, Herndon VA) supplemented with 5% FBS (Hyclone, Logan UT) and 10mg/ml Ciprofloxacin (Mediatech Inc). C57/Bl6 and C57/BL6-129 F1 mice were from Jackson Laboratories (Bar Harbor, ME). MAVS$^{-/-}$ mice on a mixed C57/BL6x129 background were from Z.J. Chen (UT Southwestern, Dallas, TX). TLR7$^{-/-}$ and TLR9$^{-/-}$ mice were from S. Akira (Osaka University, Osaka, Japan). Animal studies have been reviewed and approved by the University of Massachusetts Medical School institutional animal care and use committee.

Reagents

The HMPV viral isolates A1 (NL\1\001), B1 (NL\1\99), A2 (N\L\00\17) and B2 (N\L\94\01) were provided by Medimmune Inc. (Gaithersburg, MD) and were propagated as previously described in IMDM 4% BSA trypsin (206, 209). Influenza virus (strain A/PR/8/34) was from Charles River Laboratories (Boston, MA). NDV (LaSota strain) was from P. Pitha (Johns Hopkins, Baltimore, MD). HSV-1 (KOS strain) was from D. Knipe (HMS, Boston, MA). HMPV and NDV

were inactivated by heating at 56°C for 30 min or by UV cross-linking at a dose of 2 Joules/cm^2. RSV A2 strains was from Medimmune Inc. (Gaithersburg, MD). CpG-A (CpG 2216), CpG 2088 (48) and ISS661 (203) were from IDT (Coralville, IA). Poly (dA-dT)•Poly (dA-dT) was from GE Healthcare (Piscataway NJ). Chloroquine, and Bafilomycin A1 were from Sigma-Aldrich (St. Louis, MI).

Plasmids

The IFNβ luciferase, pGL3-PRDII, -PRDIII-I and -PRDIV luciferase reporter genes were from T. Maniatis (Harvard, Cambridge, MA). The IFNα4 luciferase reporter gene was from S. Akira (Osaka, Japan). Human RIG-I flag and RIG-IC (helicase domain only) were from T. Fujita (Tokyo, Japan). A dominant negative mutant form of MDA5 (MDA5-T789M) was from D. Conte (C. Mello Lab, UMASS Medical School, Worcester, MA). pME18-NS3/4A-myc and pME18-NS3/4A-S139A-myc were from Z.J. Chen (UT Southwestern, Dallas, Texas). NOD1 and NOD2 plasmids are from Dr. Nunez (U of Michigan Medical School, Ann Arbor, MI) pGL4-TK renilla luciferase and pGL3-control luciferase were from Promega (Madison, WI).

Mouse and human primary cell isolation

Human PBMC were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (GE Healthcare). Monocytes and PDC were purified from PBMC using CD14 microbeads and the diamond PDC isolation kit respectively as recommended by the manufacturer (Miltenyi Biotec). Purity of these

populations was assessed by staining for CD14 and BDCA2 expression by flow cytometry, and was greater than 95%. Mouse PDC were purified from spleens of mice injected with BL6-FLT3L producing cells. Briefly, mice were injected subcutaneously with 5 x 10^6 cells in 300µl PBS. Spleens were harvested within 2 weeks after injection and PDC were isolated using the PDC isolation kit II (Miltenyi Biotec). Purity was assessed by staining for CD11c, Ly6C and mPDCA1 expression by flow cytometry, and was greater than 85%.

Reporter assays

Luciferase reporter assays were conducted as previously described (95). Briefly, 293T cells or A549 cells (2.10^4 cells/well in 96-well plates) were transfected with the indicated luciferase reporter genes together with TK Renilla-luciferase reporter gene and with of the indicated expression plasmids using Genejuice (Novagen, Madison, WI). Luciferase activity was measured as previously described (210). Huh7 and Huh7.5 cells (4.10^4 cells/well in 96-well plates) were transfected as above except that these cells were also transfected with 40ng of a pGL3 control reporter, which drives constitutive luciferase activity in order to normalize the data between the two cell lines. Data were normalized according to manufacturer recommendations (Promega).

Viral RNA Purification

Viral RNA from HMPV-A1 and B1 was extracted using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Viruses were concentrated by

ultracentrifugation for 2h at 4 °C at a speed of 27,000 RPM using a SW28 rotor (Beckman, CA). RNA (1.5mg) was treated with or without 10U of calf intestinal alkaline phosphatase (CIAP, Fermentas) for 3h at 37°C or RNase A (Promega) for 1h at 37°C. All samples were then treated for 15min at 85°C to inactivate the enzymes. Viral RNA was transfected into 293T cells using lipofectamine 2000 at a ratio 1:1 (weight/volume) along with the IFNβ luciferase and the TK-renilla reporter genes according to the manufacturer instructions (Invitrogen).

ELISA

For ELISA analysis, human PBMC (2.10^5/well), monocytes (1.10^5/well) and PDC (4.10^3/well), or mouse PDC (5.10^4/well) were plated in 96 well plates in 100 ml and stimulated for 24h. Poly (dA-dT)•poly (dA-dT) was transfected as previously described (211) at 5ug/ml using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio 2:1 (weight/volume). Human IFNα was measured according to the manufacturer's recommendations (Bender Medsystems, Burlingame CA). A murine IFNβ sandwich ELISA was used as previously described (212).

RNA extraction and real time PCR

293T cells (2.10^6cells per 10 cm plate) were stimulated with HMPV-A1 or HMPV-B1 ($3.2.10^5$pfu/ml) for 5 to 48h and RNA isolated using RNeasy (Qiagen, Valencia CA). cDNA were synthesized as previously described (95) using the SuperScript III enzyme (Invitrogen). Quantitative real-time PCR analysis was

performed using SYBR green reagent (Invitrogen) on a DNA engine Opticon 2 cycler (Bio-Rad, Hercules CA) using the following primers: IFNβ-F CAGCAATTTTCAGTGTCAGAAGC; IFNβ-R CATCCTGTCCTTGAGGCAGT; IFNα-F GTGAGGAAATACTTCCAAAGAATCAC; IFNα-R TCTCATGATTTTCTGCTCTGACAA; β–actin-F CCTGGCACCCAGCACAAT; β–actin-R GCCGATCCACACGGAGTA. The specificity of amplification was assessed for each sample by melting curve analysis, and the size of the amplicon checked by electrophoresis. PCR efficiency was calculated for both vRNA with ten-fold dilutions of the cDNA with the formula $E = 10^{[-1/slope]}-1$ as previously described (213). Relative quantification was performed using standard curve analysis. All gene expression data were normalized with β-actin and are presented as a ratio of gene copy number per 100 copies of β -actin +/-SD.

Statistical analysis

Differences between groups were analyzed for statistical significance by using a t-test in Prism software. $P<0.05$ was considered statistically significant. ***, ** and * represent p values of <0.001, <0.01 and <0.05, respectively.

Preface to Chapter III

This Chapter has been published in:

Goutagny N, **Jiang Z**, Tian J, Parroche P, Schickli J, Monks BG, Ulbrandt N, Ji H, Kiener PA, Coyle AJ, Fitzgerald KA. 2010. Cell type-specific recognition of human metapneumoviruses (HMPVs) by retinoic acid-inducible gene I (RIG-I) and TLR7 and viral interference of RIG-I ligand recognition by HMPV-B1 phosphoprotein. *J Immunol.* 184(3):1168-79.

* **Goutagny N and Jiang Z** contributed equally.

In this chapter, **Zhaozhao Jiang** contributed to Figure3.1 b, d, e; Figure3.2; Figure3.3 c, d, and Figure3.4.

Chapter III Interference with type I IFN production by HMPV

Abstract

Acute viral infection activates a type I IFN antiviral response that is essential for curbing viral replication until adaptive immunity clears the virus. Usurping IFN induction pathways is a common tactic employed by viruses to enable their replication within host cells. Our previous study showed the differential induction of type I IFN by two closely related HMPV strains. Both viruses were able to infect and replicate and the naked viral RNA of both could trigger RIG-I if delivered by lipofection into the cytoplasm, however in contrast to HMPVA1 virus, the live HMPVB1 virus failed to activate RIG-I. The data suggests that the B1 virus interferes with the RIG-I dependent type I IFN signalling pathway. Although many reports have shown that non-structural proteins from paramyxoviruses block type I IFN signalling, it is still not clear how HMPV evades IFN inducing pathways. The major focus of this study was to understand the mechanism underlying the inability of HMPVB1 virus to trigger type I IFN production. The failure of the HMPV-B1 strain to elicit type I IFN production was dependent on the B1 phosphoprotein, which specifically prevented RIG-I-mediated sensing of HMPV viral 5’ triphosphate RNA. Our data unveil a new strategy of HMPV virus to interfere with host innate defence.

Introduction

Innate responses against virus infection is initiated by the recognition of viral pathogens by PRRs such as TLRs and RLRs, which drive signalling and turn on anti-viral gene expression to limit virus infection by rapidly mounted defences including in large part the release of type I IFN (IFNα/β). Interferon limits viral replication directly and enhances viral clearance by activating adaptive immunity. In the previous chapter, we demonstrated that type I IFN production during infection with HMPV viruses involves differential sensing mechanisms, which work in a cell-type specific manner. While both HMPV-A1 and HMPV-B1 strains can trigger type I IFN production in PDCs, only the HMPV-A1 strain activated type I IFN gene transcription in most cell types examined although these two strains are closely related. In PDCs, HMPVA1 and B1 strains are sensed by TLR7 to trigger type I IFN production. In contrast in various human cell lines as well as primary human monocytes, the induction of type I IFN by HMPV-A1 was mediated by the recognition of 5'triphosphate viral RNA through RIG-I and its downstream adaptor MAVS. In these cell types, type I IFN was not induced by HMPVB1 strain, which implies that the RIG-I/MAVS pathway is not activated or is inhibited by this strain.

HMPV belongs to the family of paramyxoviruses, which contains single-negative-strand RNA viral genome that is considered as good type I IFN inducers. Some of them such as SV and NDV are usually used in the laboratory as a tool for experimental type I IFN induction. In order to survive,

paramyxoviruses developed a variety of strategies to escape the detection and antiviral response of the host, especially to counteract the strong and direct antiviral activity of type I IFN. By using the viral non-structural proteins encoded by their small genome, paramyxoviruses can inhibit type I IFN signaling. Besides coding the phosphoprotein P protein that is the essential component of viral polymerase complex, P gene also has overlapping open reading frames that give multiple distinct protein products, such as “V”, “C’’, “W”, “D”, or “I” proteins by the process called “RNA editing”. These alternative P gene products are not essential for viral replication but the deletion of these products results in severe attenuation associated with the failure to control type I IFN of the host (214). For example, the V protein targets STAT protein for the inhibition of IFN signaling. Parainfluenza virus 5 V protein was shown to mediating STAT1 degradation (215). However, in the *Pneumovirinae* subfamily that HMPV belongs to, the P gene only encodes one protein. The closest relative to HMPV, RSV, has been reported to use non-structural (NS)-1 and NS2 proteins to inhibit type I IFN signaling pathway by targeting STAT2 protein (216). HMPV stain CAN97-83 was shown to interfere with IFN signaling in lung epithelial cells by inhibiting STAT1 phosphorylation (217). But this strain has also been shown to phosphorylate STAT1 (218). Thus the interference with type I IFN by HMPV is still not clear.

In this chapter, we explored the mechanisms underlying the failure of the HMPVB1 viral strain to trigger type I IFN in human cell lines in order to understand the evasion of type I IFN system by this virus. We found that the B1

strain antagonizes IFN production through the P phosphoprotein. In the context of the virus, the HMPV-B1 phosphoprotein prevented RIG-I from sensing the viral genome during infection, which is a newly described strategy that HMPV employs to subvert viral clearance.

Results

The HMPV-B1 virus fails to activate type I IFN in human cells lines as well as human monocytes.

As shown in data above, both HMPV-A1 and B1 strains induce type I IFN production in PDC through TLR7 (Figure 2.2d and 2.6e), while in human embryonic kidney (HEK) 293T cells and monocytes only A1 activates the IFNB reporter (Figure 2.1a, 2.2c). We then closely investigated the mechanism underlying the difference between these two closely related strains A1 (NL\1\00) and B1 (NL\1\99). We found that besides 293T cells, HMPV-B1 strain failed to induce IFNβ reporter gene activity in the other cell lines we tested such as Huh7 and A549 (Figure 2.1b,c). The HMPV-B1 strain also failed to induce IFNα4 reporter gene (Figure 2.1e). Similar observations were made when the endogenous IFNβ transcript levels were measured in infected 293T cells. HMPV-A1 induced IFNB gene transcription while HMPV-B1 failed to do so (Figure 2.1d).

To exclude the possibility that the failure of the B1 virus to induce type I IFN induction was due to a failure to infect or replicate in these cells, we first compared viral infection and replication in both monocytes and PDC. Upon infection with paramyxoviruses, the viral F protein is first synthesized as an inactive precursor F0, which is subsequently converted into the fusogenic F1

form by cellular proteases (219). A HMPV-specific anti-F antibody has been characterized previously and shown to detect both F0 and F1 forms of the F protein from both A1 and B1 strains (209). The level of F-protein expression was monitored in infected cells by flow cytometry. Similar percentages of purified monocytes (about 20% of CD14+ cells) and PDC (about 45% of BDCA-2+ cells) expressing the F protein were observed for both HMPV strains (Figure 3.1a). This indicated that both strains can infect these cell types.

In 293T cells, comparable levels of expression of the F protein (both F0 and F1 forms) were detected in both HMPV-A1 and B1 infected 293T cells when examined by western blotting (Figure 3.1b). We excluded that the staining detected might originate from the initial inoculums since no signal was detected at 2h post-infection. The amount of F proteins from both A1- and B1-infected cells were increasing at comparable pace with longer incubation of viruses with infected cells, which indicated the A1 and B1 viruses replicated inside 293T cells at a similar rate. Replication of HMPV-A1 and B1 was also examined by quantifying the level of viral transcripts and measuring viral titers in infected cells. To ensure similar PCR efficiency for both strains, the primer-binding sites were designed in a region of the gene encoding the L protein, which was identical in both strains. Equivalent levels of A1 and B1 transcripts were detected in infected cells throughout the course of infection (Figure 3.1c). Viral titers were also equivalent for both strains (Figure 3.1d). This was also true in 293T, A549 and Huh7 cells (Figure 3.1e). Indeed, the levels of B1 virus exceeded that of A1 strain

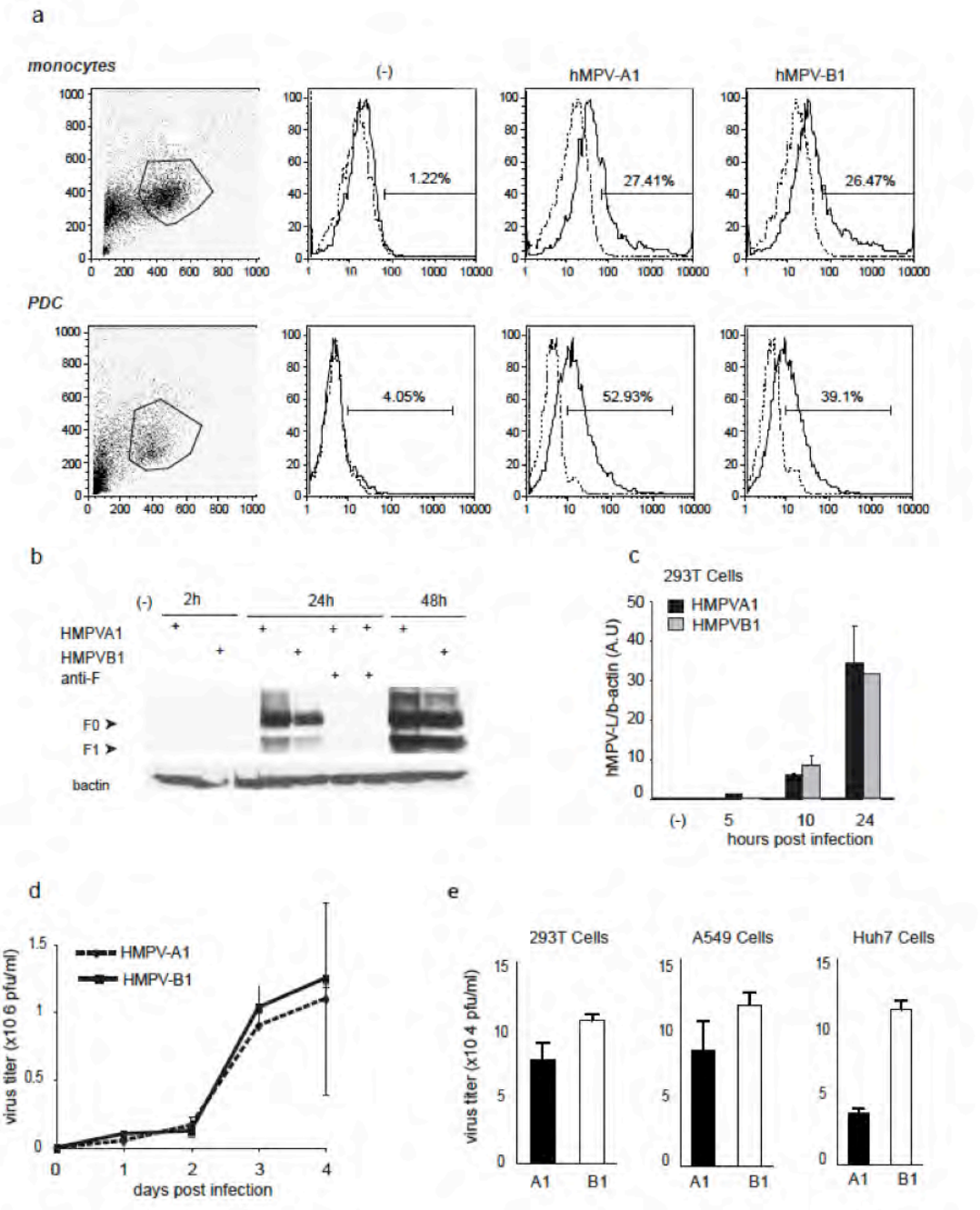

a
monocytes
(-)
hMPV-A1
hMPV-B1
1.22%
27.41%
26.47%
PDC
4.05%
52.93%
39.1%
b
(-)
2h
24h
48h
HMPVA1
HMPVB1
anti-F
F0
F1
bactin
c
293T Cells
HMPVA1
HMPVB1
hMPV-L/b-actin (A.U)
(-)
5
10
24
hours post infection
d
HMPV-A1
HMPV-B1
virus titer (x10 6 pfu/ml)
days post infection
e
293T Cells
A549 Cells
Huh7 Cells
virus titer (x10 4 pfu/ml)
A1
B1

Figure 3.1 HMPV-A1 and HMPV-B1 infect and replicate in primary cells and cell lines. (a) Purified monocytes ($6x10^5$ cells/well) and PDC (2.10^5 cells/well) were cultured for 24h in presence of HMPV-A1 and B1 ($3.2x10^5$pfu/ml). Cells were harvested and stained for specific cell surface antigen (CD14 or BDCA2) and F protein expression using an anti-F biotinylated antibody and PE or APC-conjugated steptavidin. Results are presented as histogram overlays of anti-F antibody staining versus isotype control. Displayed percentages were substracted from the isotype control. (b). Infection of 293T cells by HMPV was assessed by immunoblot analysis. Cells ($6x10^5$cells/well) were infected with HMPV-A1 or HMPV-B1 ($5x10^4$pfu/ml) for 2 to 48h. A specific anti-F biotinylated antibody and HRP-conjugated steptavidin were used to detect F protein in cell lysates. F0 and F1 forms of the F protein are indicated by an arrow. Anti-F #338 neutralizing antibody was used to show the specificity. The lower panel shows β-actin levels in the samples. (c) Replication of HMPV in 293T was quantified by real-time PCR. Cells were infected with HMPV-A1 or HMPV-B1 ($5x10^4$pfu/ml) for the indicated time points. Levels of HMPV-L gene and human β-actin were quantified in RNA samples using specific primers. Results are presented in arbitrary unit as the ratio of HMPV-L gene over 100 copies of β-actin. (d-e) Virus titers were determined after infection of several cell types ($3x10^5$/well) with HMVPA1 or B1 strain (10^5pfu/ml). Virus titers were measured for the indicated time points after infection in Vero cells (d) or after 24h post infection in 293T, A549 and Huh7 cells (e) and presented as pfu/ml.

at the time point, probably because A1 virus activated the expression of antiviral type I IFN. We conclude therefore from these studies, that both viruses infect and replicate in all cells tested, however PDC were unique in their ability to induce type I IFN in response to HMPV-B1.

The HMPV-B1 virus fails to activate Interferon Regulatory Factor-3.

Then we test all the positive regulatory domains of the IFNB gene transcriptional enhancer using PRDIII-I (IRF), PRDII (NFκB) and PRDIV (MAP Kinase) reporters. Only A1 strain activated the PRDIII-I reporter gene, which responds to IRF3 and 7 (Figure 3.2a). We also monitored activation of a reporter gene containing multimerized ISRE elements from the ISG-54 promoter. Consistent with a failure to induce the PRDIII-I element from the IFNB promoter, HMPV-B1 virus also failed to drive the ISG54-ISRE (Figure 3.2b). In contrast, both HMPV-A1 and B1 induced the PRDII/NFκB and PRDIV/ATF2/c-jun reporter genes (Figure 3.2c and d).

We next monitored endogenous IRF3 activation by examining the formation of IRF3 dimers in virus-infected cells. IRF3 is normally present in the cytoplasm of resting cells as a monomer. Virus infection triggers the phosphorylation and dimerization of IRF3 followed by its nuclear translocation. Infection of 293T cells with HMPV-A1 induced IRF3 dimerization in a manner similar to that observed with NDV, our positive control (Figure 3.2e). In contrast,

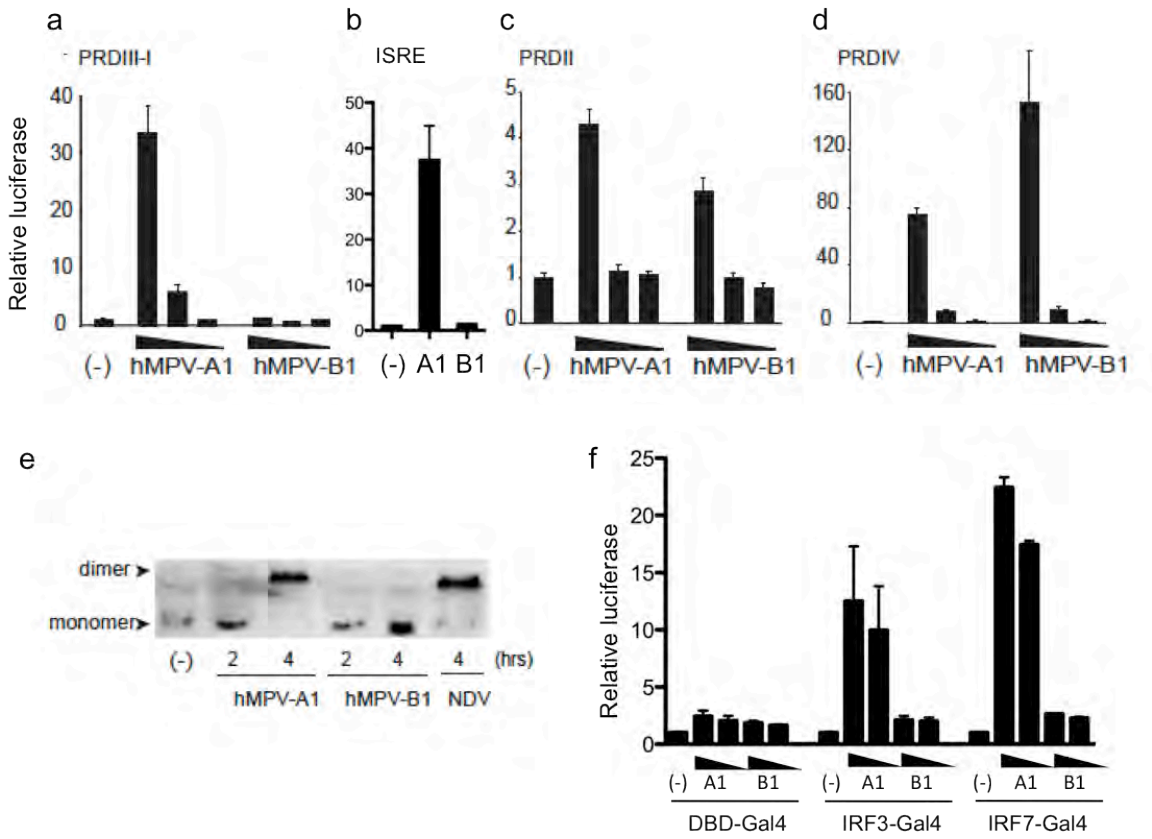
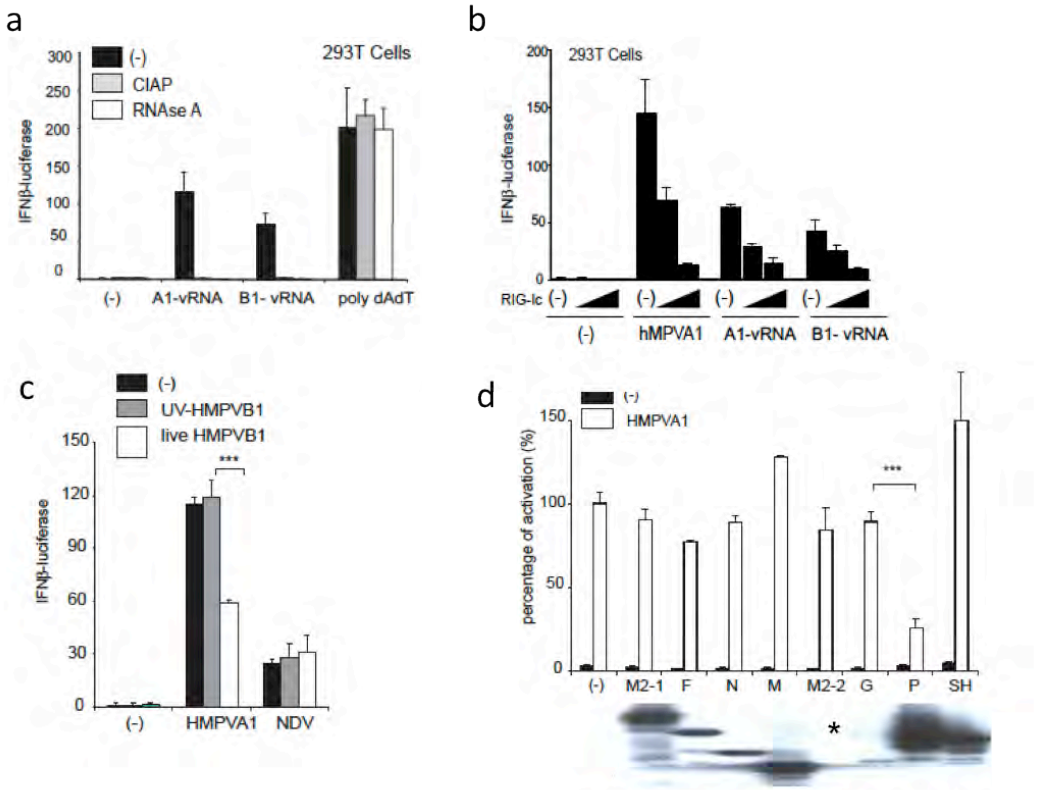


Figure 3.2 Differential activation of IRF3 by the HMPV A1 and B1 strains. (a-d, f) 293T cells were transfected with the IFNB PRDIII-I, ISG54-ISRE, PRDII, PRDIV or Gal4 reporter genes as detailed in Material and Methods. Cells were infected with HMPV-A1 or HMPV-B1 (range from $5x10^4$ to $5x10^2$pfu/ml, $5x10^4$ pfu/ml for ISRE reporter, $5x10^4$ to $5x10^3$pfu/ml for Gal4 reporter) for an additional 24h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (e) A549 cells were stimulated with viruses for the indicated period of time. Nuclear protein were extracted and analyzed by native PAGE. The monomer and dimeric forms of IRF3 are indicated by arrows.

cells infected with the HMPV-B1 strain failed to lead to dimerization of endogenous IRF3. These observations were confirmed using an *in vitro* assay for IRF3 and IRF7, which utilizes a hybrid protein consisting of the yeast Gal4-DBD fused to IRF-3 or IRF-7 lacking its own DBD (220). Reporter gene expression from the Gal4 upstream activation sequence in this assay requires IRF activation (220). HMPV-A1 but not B1 activated both the Gal4-IRF3 and IRF7 reporters (Figure 3.2f). Taken together these data provide clear evidence that the B1 strain fails to trigger IRF3/7 activation and as a result fails to trigger IFNβ production.

The HMPV-B1 P protein prevents sensing by RIG-I.

We next traced back upstream the RIG-I/MAVS pathway to understand the differential responses of the human cells lines to HMPVA1 and B1 strains. As shown in the data above, the 5'-triphosphate viral RNA of HMPVA1 is the ligand recognized by RIG-I. We wanted to know if the vRNA of HMPVB1 was also recognized by RIG-I. The viral RNA was purified from HMPV-B1 and transfected into the cytoplasm of 293T cells *via* lipofection. Similar results were observed as using HMPVA1 viral RNA, transfection of the viral B1-RNA induced the IFNB reporter gene and removal of the phosphate groups by CIAP or digestion of the viral RNA by RNAse A abrogated this response (Figure 3.3a). Moreover, RIG-IC also dose-dependently inhibited the IFNβ response in 293T cells elicited B1 viral RNAs (Figure 3.3b). These data revealed that the naked



* M22 expression can be detected in repeatable WB assays

Figure 3.3 IFNB gene can be activated by vRNA from both HMPV-A1 and B1 viruses via RIG-I. (a) 293T cells were transfected with viral RNA purified from HMPV-A1 or B1 (80ng), or poly(dAdT)•poly(dAdT) (20ng) along with the IFNβ and TK-renilla reporter genes. vRNAs and poly(dAdT)•poly(dAdT) were treated with calf intestinal alkaline phosphatase (CIAP) or RNAse A prior to stimulation as indicated. luciferase activity was measured 24h post-transfection and data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) 293T cells were transfected with increasing amounts of RIG-IC (0-2-20ng/well) along with the IFNB reporter and vRNAs (80ng), or stimulate with HMPV-A1 ($5x10^4$pfu/ml). Luciferase activity was measured 24h post-transfection and data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (c) 293T cells were transfected with the IFNB reporter gene one day prior to stimulation. Cells were pre-incubated with live or UV ($2J/cm^2$) inactivated HMPV-B1 (10^5pfu/ml) for 24 hours. Cells were then stimulated with HMPV-A1 ($5x10^4$pfu/ml) or NDV (8HAU) for an additional 24hours. Results are normalized by renilla luciferase and presented as fold induction relative to the reporter-only control and are the mean ± SD. (d) 293T cells were transfected with the IFNβ PRDIII-I reporter gene and 40ng of plasmids encoding HMVB1 proteins as indicated. Cells were stimulated for an additional 24h with HMPV-A1 ($5x10^4$pfu/ml). Data were normalized by renilla luciferase and are expressed as a percentage of activation relative to conditions with the virus in the absence of exogenous proteins and are the mean ± SD. Protein expression levels were analyzed by WB.

viral genome of HMPV-B1 virus can be sensed by RIG-I, if delivered to the cytosol. However, the inability of the B1 strain to be sensed suggested that in the context of the virus, the HMPV-B1 viral RNA is not detected. There might be some proteins prevent the access of RIG-I.

To test this hypothesis, we first tested if preincubation with the HMPV-B1 strain could interfere with signaling initiated by the HMPV-A1 strain or other viruses that are sensed by RIG-I. Cells were transfected with the IFNB reporter gene, pre-incubated for 24 hours with live or UV-inactivated HMPV-B1 strain before infection with the A1 strain or NDV. Luciferase activity was then monitored 24 hours post infection. Preincubation with live HMPV-B1 reduced IFNβ induction upon HMPV-A1 infection (Figure 3.3c). In contrast, preincubation with UV-inactivated virus did not block the induction of IFNβ. The activation of the IFNβ reporter gene in response to NDV was not affected. These data suggest that the B1 virus interferes with the induction of IFNβ reporter by A1 virus and that the inhibitory effect was not due to antagonism downstream of RIG-I itself. Therefore, the B1 strain likely blocks sensing of HMPV viral RNA and not RIG-I function *per se*.

Each individual cDNA from the B1 strain (with the exception of the L polymerase) were cloned into mammalian expression vectors and their effects on type I IFN induction upon infection with the A1 strain examined by reporter assay. Plasmids encoding B1 cDNAs were transfected into 293T cells together with the PRDIII-I reporter gene, whose activity was monitored after infection of cells with

HMPV-A1 virus. Table 3.1 shows the percentage identity between A1 and B1 proteins. While the M, SH, M2-1, N, M2-2, F and G proteins had little or no effect, the P protein blocked the induction of the reporter (Figure 3.3d). Importantly, western blotting revealed good expression levels of all of these proteins in transfected cells.

We next generated HEK293 cell lines stably expressing the B1-virus P protein in order to test if the B1 protein could prevent induction of IFNβ upon delivery of viral RNA into the cytoplasm. Viral RNA from both A1 and B1 strains triggered a strong IFNβ response in parental HEK293 cells but the response was largely impaired in HEK293 cells expressing B1-P protein. Importantly, IFNβ induced by vRNA from NDV or VSV was unaffected (Figure 3.4a). These data are consistent with the possibility that the HMVP-B1 P protein functions in a specific manner as an inhibitor, blocking IFN production in response to HMPV viral RNA.

To further examine the inhibitory effect of the B1 virus P protein in a more physiologically relevant manner and in the context of the virus, we generated chimeric viruses in which the P protein from the A1-strain was replaced with that of the B1 strain. Both WT and chimeric HMPV-A1 virus were recovered from cDNA and their ability to induce type I IFN examined by reporter assays. Consistent with our data with the A1 clinical isolate, WT HMPV-A1 virus recovered from cDNA (referred to as A1R) induced IFNβ reporter in HEK293 cells, however when A1 virus expressing the P protein from B1 virus (APB) was

Table 3.1 Percent identity between HMPV-A1 and HMPV-B1 proteins

Protein	Identity (%)
	86.1
N	94.9
M	97.3
F	93.7
M2-2	88.9
M2-1	8.6
SH	8.9
G	11.3
L	91.7

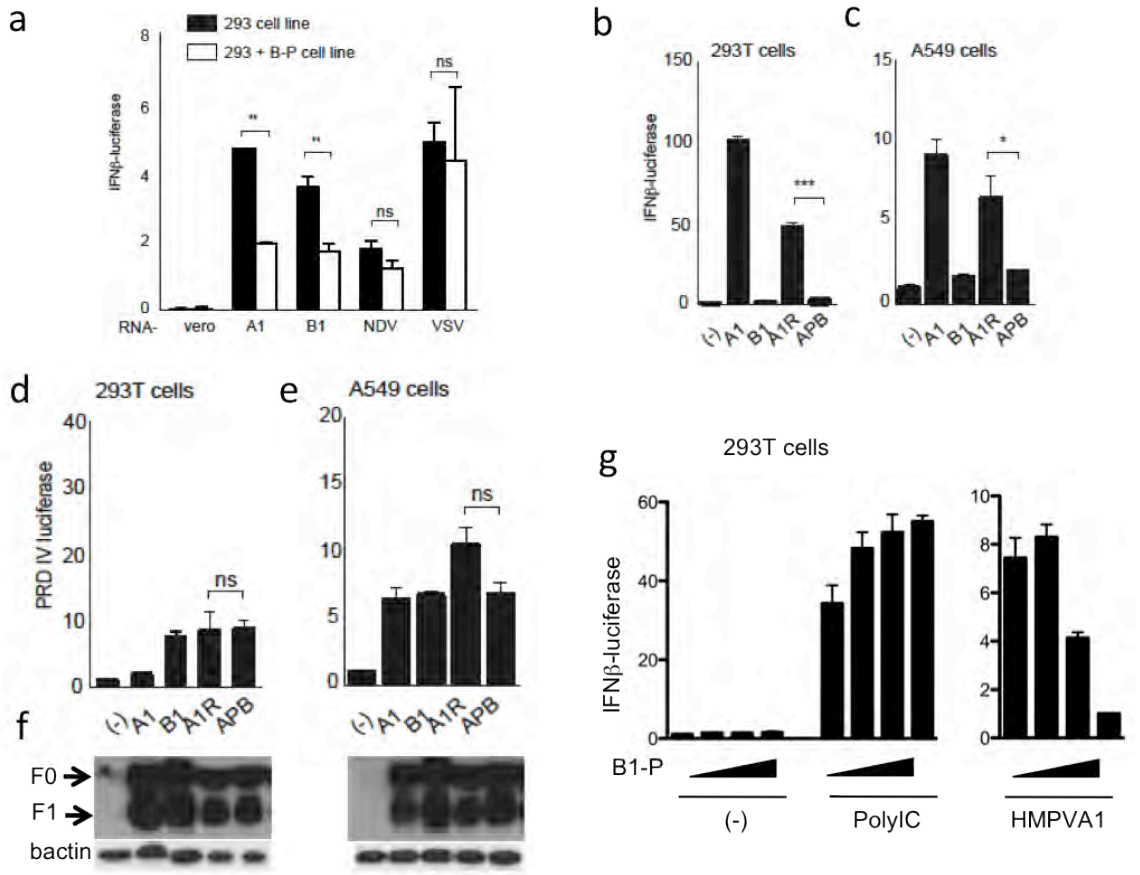


Figure 3.4 HMPV-B1 P protein prevents RIG-I from sensing HMPV vRNA. (a) HEK293 cells and HEK293 cells stably expressing the P protein from HMPV-B1 were transfected with the full length IFNβ reporter gene along with the viral RNA (80ng) purified from HMPV-A1, B1, NDV, or VSV, or with total RNA (80ng) purified from Vero cells as control. Results are normalized by renilla and pGL3 control luciferase, and presented as mean ± SD. (b-f) 293T and A549 cells were transfected with the full length IFNB or the PRDIV reporter along with the TK-renilla reporter gene, one day prior to stimulation. Cells were infected for 24 hours with the following viruses: HMPV-A1, HMPV-B1, A1R, or the chimeric virus APB ($5x10^4$pfu/ml). Results are normalized by renilla luciferase and are presented as fold induction relative to the reporter-only control and are the mean ± SD. A western blot of F protein levels is shown (f). (g) 293T cells were transfected with the IFNβ reporter gene and plasmids encoding HMVB1 P protein(5-40ng). Cells were stimulated for an additional 24h with PolyIC (200ug/ml) or HMPV-A1 ($5x10^4$pfu/ml). Results are normalized by renilla luciferase, and presented as mean ± SD.

examined, no reporter gene activity was detected (Figure 3.4b). Similar data were obtained when A549 cells were examined (Figure 3.4c). Since both the A1 and B1 strains could induce PRDII and PRDIV-luciferase reporters, we also compared the effect of A1R and APB viruses on induction of these reporters in both HEK293 and A549 cells. In contrast to the IFNβ reporter, both A1R and APB induced both of these reporter genes in 293T cells (Figure 3.4d) and A549 cells (Figure 3.4e). Moreover, analysis of F-protein levels in virus infected cells revealed similar levels in cells infected with all viral strains (Figure 3.4f). These results provide compelling evidence that the HMPV B1-P protein prevents RIG-I mediated sensing and signaling resulting in IRF3–dependent type I IFN gene transcription.

Discussion

HMPV is a major contributor to lower and upper respiratory tract infections in infants and young children. Acute viral infections activate type I IFN antiviral responses that are essential for viral clearance. HMPV belongs to the paramyxovirus family that employs a variety of non-structural proteins including the alternative products of P gene and proteins encoded by NS genes to circumvent the IFN response thus to make the better environment for viral replication and expansion. Although many reports have shown the function of these proteins to inhibit type I IFN signalling in different genus (221), it is still not clear how HMPV counteracts the production of type I IFN. In the previous chapter, we demonstrated that type I IFN was induced during infection with HMPV viruses. HMPVA1 and B1 strains have the abilities to induce similar level of type I IFN in PDCs through endosomal TLR7. Sensing of HMPVA1 virus occurs *via* the cytosolic RNA helicase RIG-I in most cell types tested including human cell lines and purified human monocytes. We have also identified 5'-triphosphate RNA as the HMPV viral ligand triggering the RIG-I/type I IFN response. In these cell types, HMPVB1 failed to elicit type I IFN production.

The major focus of this chapter was to understand the mechanism underlying the inability of HMPVB1 virus to trigger type I IFN production by comparing the innate response to two closely related strains. Despite its ability to infect and replicate as efficiently as the A1 virus and despite the ability of naked viral RNA to trigger RIG-I if delivered by lipofection to the cytoplasm, the live

HMPVB1 virus failed to activate RIG-I as HMPVA1 can do. The fact that the B1 virus could prevent type I IFN induction by the A1 virus, but not that induced by NDV indicates that RIG-I signalling *per se* is not blocked by the B1 virus. Like the A1 virus, purified B1 viral RNA could trigger IFN and pretreatment of the B1 viral RNA with RNAse or removal of phosphate groups with alkaline phosphatase ablated sensing of the viral RNA by RIG-I. These studies suggest that RNA of both strains are ligands for RIG-I, however, in the context of the virus, the B1 viral RNA is prevented from being sensed by RIG-I. During viral infection, RNA viruses like HMPV fuse with the cell membrane and deliver their ribonucleoprotein (RNP) complex into cells. The RNP complex consists of the viral RNA associated with the viral polymerase L, the nucleoprotein N and the phosphoprotein P. Upon fusion, the viral RNA is protected from free cellular RNases by this protein complex. Proteins within the RNP therefore could prevent the recognition of the vRNA by the RIG-I pathway. In fact, our studies using overexpressed B1 proteins indicated that the B1 virus P protein but not other HMPV proteins could block IFN production by live A1 virus or viral RNA. Although this approach indicated that the B1 P protein was the most likely candidate, we found that if we overexpressed the A1 P protein we could also observe an inhibitory effect. To determine if the B1 P protein was responsible for the inhibitory effect in the context of the virus, we generated recombinant viruses, where we replaced the P protein in the A1 virus with that from the B1 virus. This is a more physiologically relevant system to assess its contribution where

associations of the B-1 P with other viral proteins could also be accounted for. A recombinant A1 virus encoding the P protein from the B1 strain was generated and its ability to induce RIG-I signalling examined. Unlike the WT virus recovered from cDNA, the recombinant A1 virus containing the B virus P protein had a substantially reduced ability to induce IFN, suggesting that in the context of the entire virus, B1-P may indeed prevent RIG-I from sensing HMPV virus. Specific inhibition of RIG-I sensing by the B1-P protein could be because of higher levels of expression of the P protein in the B1 virus rather than what is found in the A1 virus. Another possibility is that the B-1 P protein could have higher affinity for the RNA or for other components of the RNP complex (HMPV RNA or N and L), which would preclude the RNA from being sensed. The two P proteins share 86% identity (see table 3.1) and therefore unique residues in B1-P could account for these effects. The inhibitory effect of the B1 virus P protein was restricted to the RIG-I pathway, since the B1 virus did not prevent induction of IFNB reporter gene activity by PolyIC/TLR3 signaling (Figure 3.4g).

Usurping IFN induction pathways is a common tactic employed by viruses to enable their replication within host cells. Viral IFN antagonists strike at just about every level of the IFN regulatory network, but by far the best-studied strategies relate to the ability of viral proteins to counteract RNA sensing and signaling events. Examples include Influenza virus NS1, which inactivates RIG-I (199), HCV, NS3/4A, which cleaves and inactivates MAVS (86) and the phosphoprotein of Borna disease virus and Rabies virus, which target the IRF3

kinase, TBK1 (222, 223). In the case of RSV, the genome encodes two NS1 and NS2 proteins known to inactivate the IFN response (176). A recent study from Casola and colleagues implicated the G protein from another strain of HMPV in evasion of the RIG-I signaling pathway (224). A recombinant hMPV lacking the G protein (rhMPV-ΔG) was developed as a potential vaccine candidate and shown to be attenuated in the respiratory tract of a rodent model of infection. Casola and colleagues found that rhMPV-ΔG-infected airway epithelial cells produced higher levels of chemokines and type I interferon compared to cells infected with rhMPV-WT. They showed that RIG-I was the target of G protein inhibitory activity. Indeed the G protein associated with RIG-I and inhibited RIG-I-dependent gene transcription. Our overexpression data with the HMPV-B1 virus however do not support a role for the B1-virus G protein but rather indicate that the B1 phosphoprotein can prevent RIG-I from sensing the viral RNA. However, we had problems to recover the recombinant B1 virus encoding P protein from A1 strain, it is possible that there's other protein in the B1 virus contributing to the antagonism.

Altogether, our data unveil a new strategy of HMPV virus to interfere with host innate defence. We proposed a model showing how these two HMPV strains are differentially sensed and how HMPV-B1 strain counteracts the recognition by RIG-I (Figure 3.5). A better understanding of the interactions between viral proteins and host innate immune defences is critical to improving

the knowledge about the pathogenesis of viruses, which will contribute to the development of better approaches for treatment and vaccines.

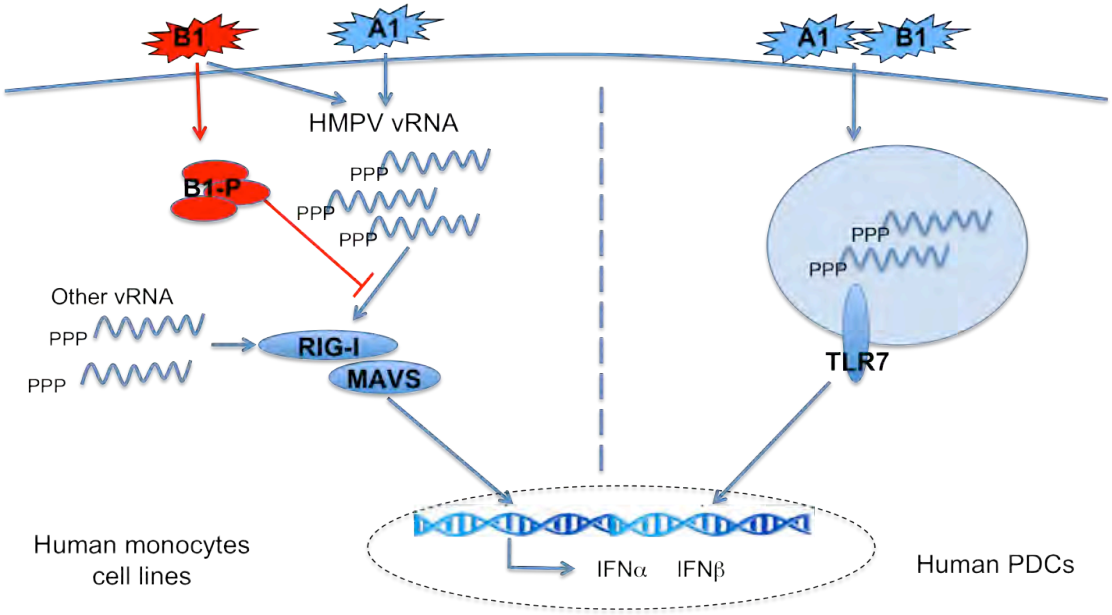


Figure 3.5 Schematic model of differential recognition of HMPV-A1 and B1 strains. In most cell types tested including human cell lines and isolated human monocytes, only HMPVA1 virus, but not B1 virus induces type I IFN production. The response is mediated by the cytosolic receptor RIG-I and its adaptor molecule MAVS. Purified viral RNA from both A1 and B1 viruses can be recognized by RIG-I. The HMPVB1 virus encodes an antagonist, P protein, which can prevent the recognition of B1 viral RNA by RIG-I. This ability to block vRNA recognition is unique to HMPV viruses as the B1 P protein does not appear to prevent RIG-I from sensing other viral RNAs. In contrast, we noted that in PDCs, there is no blocking of viral recognition, since TLRs control the induction of type I IFNs in response to both HMPVA1 and B1 viruses. These responses in PDCs do not involve RIG-I/MAVS pathway but instead involves sensing by endosomal Toll-like receptor 7.

Material and methods

Cell lines.

HEK 293, 293T, alveolar epithelial cells (A549) and Vero cells were from ATCC (Manassas, VA). The human hepatocellular carcinoma cell lines Huh7 was from C. Rice (Rockefeller University, New York, NY). HEK293 cells stably expressing the P protein from HMPV-A1 and B1 were generated by transfection with cDNAs encoding P proteins, which were generated by PCR. All of the above cell lines were maintained in DMEM (Mediatech Inc, Herndon VA) supplemented with 5% FBS (Hyclone, Logan UT) and 10mg/ml Ciprofloxacin (Mediatech Inc). BSR-T7/5 cells were from K. Conzelmann (Munich, Germany) and were cultured in DMEM 10%FCS/ciprofloxacin with G418 (0,5mg/ml).

Reagents

The trypsin-independent HMPV viral isolates A1 (NL\1\001) and B1 (NL\1\99) were provided by Medimmune Inc. (Gaithersburg, MD) and were propagated as previously described in IMDM 4% BSA trypsin (206, 209). Newcastle disease virus (NDV, LaSota strain) was from P. Pitha (Johns Hopkins, Baltimore, MD). HMPV B1 was inactivated by UV cross-linking at a dose of 2 Joules/cm^2. Poly (dA-dT)•Poly (dA-dT) was from GE Healthcare (Piscataway NJ). The monoclonal antibodies specific for the HMPV F protein were generated and characterized by Medimmune Inc. (Gaithersburg, MD) (209). Biotinylated hamster monoclonal antibody #1017 was used at 1mg/ml for staining of the F

protein by immunoblotting and flow cytometry. Anti β-actin was from Sigma-Aldrich (St. Louis, MI).

Plasmids

The IFNβ luciferase, pGL3-PRDII, -PRDIII-I and -PRDIV luciferase reporter genes were from T. Maniatis (Harvard, Cambridge, MA). ISG54-IFN-stimulated regulatory element (ISRE) was from Stratagene (La Jolla, CA). Human RIG-IC (helicase domain only) were from T. Fujita (Tokyo, Japan). pGL4-TK renilla luciferase and pGL3-control luciferase were from Promega (Madison, WI). All viral proteins (with the exception of L) from HMPV-B1 were cloned individually into pEF-Bos-Flag by PCR using full-length cDNA as template (225). Clone sequences were verified by sequencing and protein expression in 293T cells by western blotting with anti-Flag M2 (Sigma-Aldrich, St. Louis, MI).

Human primary cell isolation

Peripheral blood mononuclear cells (PBMC) were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (GE Healthcare). Monocytes and PDC were purified from PBMC using CD14 microbeads and the diamond PDC isolation kit respectively as recommended by the manufacturer (Miltenyi Biotec). Purity of these populations was assessed by staining for CD14 and BDCA2 expression by flow cytometry, and was greater than 95%.

Reporter assays

Luciferase reporter assays were conducted as previously described (95). Briefly, 293T cells or A549 cells (2.10^4 cells/well in 96-well plates) or Huh7 cells (4.10^4 cells/well in 96-well plates) were transfected with the indicated luciferase reporter genes together with thymidine kinase Renilla-luciferase reporter gene and with of the indicated expression plasmids using Genejuice (Novagen, Madison, WI). Luciferase activity was measured as previously described (210).

Viral RNA Purification

Viral RNA from HMPV-A1 and B1 was extracted using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Viruses were concentrated by ultracentrifugation for 2h at 4 °C at a speed of 27,000 RPM using a SW28 rotor (Beckman, CA). RNA (1.5mg) was treated with or without 10U of calf intestinal alkaline phosphatase (CIAP, Fermentas) for 3h at 37°C or RNase A (Promega) for 1h at 37°C. All samples were then treated for 15min at 85°C to inactivate the enzymes. Viral RNA was transfected into 293T cells using lipofectamine 2000 at a ratio 1:1 (weight/volume) along with the IFNβ luciferase and the TK-renilla reporter genes according to the manufacturer instructions (Invitrogen). Viral RNA from NDV and VSV were from T. Morrison and S. Zhou, respectively (UMASS Medical School, Worcester, MA).

RNA extraction and real time PCR

293T cells (2.10^6cells per 10 cm plate) were stimulated with HMPV-A1 or HMPV-B1 ($3.2.10^5$pfu/ml) for 5 to 48h and RNA isolated using RNeasy (Qiagen,

Valencia CA). cDNA were synthesized as previously described (95) using the SuperScript III enzyme (Invitrogen). Quantitative real-time PCR analysis was performed using SYBR green reagent (Invitrogen) on a DNA engine Opticon 2 cycler (Bio-Rad, Hercules CA) using the following primers: β–actin-F CCTGGCACCCAGCACAAT; β–actin-R GCCGATCCACACGGAGTA; L1-F TTGCATGAGGTACCTTGGATTG; L1-R AGAGTGCATTATCACACATCA. The specificity of amplification was assessed for each sample by melting curve analysis, and the size of the amplicon checked by electrophoresis. PCR efficiency was calculated for both vRNA with ten-fold dilutions of the cDNA with the formula $E = 10^{[-1/slope]}-1$ as previously described (213). Relative quantification was performed using standard curve analysis. All gene expression data were normalized with β-actin and are presented as a ratio of gene copy number per 100 copies of β -actin +/-SD.

Western-Blotting

Whole cell extracts were prepared using lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, freshly supplemented with protease and phosphatase inhibitors. Cells were placed on ice for 15min and centrifuged for 15min at 14,000rpm. 30mg of protein were analyzed by 10% SDS-PAGE and subjected to immunoblot analysis using anti-F and anti-β-actin antibodies as indicated. The IRF3 dimerization assay was performed as follows. A549 cells (10.10^6cells /10ml in 10cm plate) were stimulated with HMPV-A1, B1 (10^5 pfu/ml) or NDV (40 HAU) as indicated.

Nuclear proteins were extracted using Nuclear Extraction Kit (Active Motif, CA). 25 µg of protein was analyzed by 8.5% PAGE and subjected to immunoblot analysis using anti-IRF3.

Generation of chimeric viruses

Chimeric viruses where the P proteins both HMPV strains were exchanged, were generated by PCR using naturally occurring MluI and PacI enzymes and Esp31 enzyme to generate overlapping PCR fragments. PCR fragments were generated using full length cDNA as previously described (225). For each virus strain, 3 PCR products were generated and cloned in topo cloning vector (Invitrogen). From the MluI site to the ATG of the P protein, the P protein and to the end of P up to PacI site. Ligations of MluI to P and P to PacI from each strain were ligated with the P protein from the opposite virus strain into pCDNA3. HMPV-A1 and B1 full length was generated by partial digestion using MluI and PacI and used to clone the 3 pieces by PCR from pCDNA3. Colonies were screened by digestion and sequenced by PCR.

Virus recovery and titration

Recovery of recombinant HMPV was performed as described previously (225). Briefly, BSR-T7 cells were transfected with 5 µg full-length HMPV cDNA plasmid, 2 µg pCITE-N, 2 µg pCITE-P, 1 µg pCITE-L and 1 µg pCITE-M2.1 using Genejuice (Novagen). Two days later, the BSR-T7 cells were scraped and co-cultured with Vero cells in IMDM with 4% BSA for 7 days. Viruses were titered

as followed. Twenty-four-well plates containing 95% confluent monolayers of Vero cells were inoculated with 200µl of 10-fold serial virus dilutions. After 2 h at 37 °C, 0.8ml of 0.5% methylcellulose/DMEM with 3% FCS was added. Cells were incubated for an additional 5 days. Methylcellulose overlays were removed and cells were fixed with 80% acetone. Cells were incubated with HMPV-specific 1017 biotinylated antibody (Medimmune) for 1 h at 37 °C, followed by incubation with horseradish peroxidase-labelled streptavidin (BD bioscience, CA). Plaques were quantified after incubation with a freshly prepared solution of 3,3' Diaminobenzidine (DAB) (Vector Laboratories, CA) to determine viral titers.

Statistical analysis

Differences between groups were analyzed for statistical significance by using a t-test in Prism software. $P<0.05$ was considered statistically significant. ***, ** and * represent p values of <0.001, <0.01 and <0.05, respectively.

Preface to Chapter IV

Portions of his Chapter have been published separately in:

1. Paun A, Reinert JT, **Jiang Z**, Medin C, Balkhi MY, Fitzgerald KA, Pitha PM. 2008. Functional characterization of murine interferon regulatory factor 5 (IRF-5) and its role in the innate antiviral response. *J Biol Chem*. 23;283(21):14295-308.

This was a collaborative study between the Pitha and Fitzgerald laboratories. **Zhaozhao Jiang** performed experiments examining the role of IRF5 in inflammatory cytokine and type I IFN responses in different immune cell subsets.

2. Pandey AK, Yang Y, **Jiang Z**, Fortune SM, Coulombe F, Behr MA, Fitzgerald KA, Sassetti CM, Kelliher MA. 2009. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. *PLoS Pathog.* 5(7):e1000500.

This was a collaborative study between the Kelliher, Sassetti and Fitzgerald laboratories. **Zhaozhao Jiang** performed reporter assay testing the activation of IRF5 by RIP2 for type I IFN induction in 293T cells.

In this Chapter, **Zhaozhao Jiang** contributed to Figure4.1, Figure4.2, and Figure4.3.

Chapter IV IRF5 function in innate immune response

Abstract

IRFs are key regulators of innate immune signaling and were first implicated as critical transducers of type I IFN responses. IRF3 and IRF7 have been best studied and their role in IFN gene regulation in TLR, RLR and cytosolic DNA sensing pathways is now clear. In contrast, although IRF5 has also been implicated in anti-viral defenses, its precise role is much less clear. In particular very little was known about murine IRF-5. Murine IRF-5, unlike the heavily spliced human gene, is expressed as a full-length transcript, with only a single splice variant. The murine IRF-5 protein can be activated downstream of MyD88 to form homodimers and bind to and activate transcription of type I interferon and inflammatory cytokine genes. In this chapter, by examining immune responses in IRF5-deficient mice, we found that IRF5 contributes to various aspects of TLR signaling. These studies revealed that IRF-5 was critical for TLR3-, TLR4-, and TLR9-dependent induction of TNFα in CD11c+CDCs from mouse spleen. In addition, TLR9, but not TLR3/4-mediated induction of type I IFN transcription, was dependent on IRF-5 in these cells. In addition, these studies also found that the role of IRF5 in these responses was restricted to splenic cells, no role for IRF5 was found in macrophages or DCs generated from bone marrow.

We also contributed to the discovery of a new mechanism that triggered IRF5. Stimulation of NOD2-RIP2-IRF5 signaling pathway by MDP components from Mtb induced type I IFN production. This study identified a new role for IRF5

besides its known function in MyD88 dependent TLR7/9 signaling pathways. These data reveal the cell type-specific importance of IRF-5 in MyD88-mediated antiviral pathways as well as RIP2-dependent anti-bacterial responses and the widespread role of IRF-5 in the regulation of inflammatory cytokines.

Introduction

The IRF family has been reported to have multiple functions in the immune system. These include regulation of immune cell development and transcription of type I Interferon genes. The search for transcription factors activating the promoters of IFNA and IFNB genes led to the identification of IRF-3 and IRF-7. Another member of the IRF family, IRF-5, was then identified and also implicated in innate immunity. HuIRF-5 is expressed primarily in DCs and B cells and shows some properties that are different from IRF-3 and IRF-7. Unlike IRF-3 and -7, IRF5 contains two nuclear localization signals, and consequently low levels of nuclear IRF-5 can be detected in the nucleus of uninfected cells (226). Human IRF-5 is expressed in multiple spliced variants, and some of these are transcriptionally inactive and may function as dominant negative mutants (227). HuIRF-5 was shown to regulate IFN gene expression in response to some viruses and TLR ligands. A role for IRF-5 in IFNα synthesis has also come from genetic studies of SLE, which is characterized by a constitutive expression of IFNα/β. Variants of IRF-5 have now been linked to SLE (228), as well as inflammatory bowel disease (229).

In vitro experiments have shown that in infected cells, HuIRF-5, like IRF-3 and IRF-7, is activated by phosphorylation, resulting in nuclear translocation and stimulation of IFNA gene expression (230). Studies from the Pitha lab have shown that IRF-5 specifically up-regulates early inflammatory cytokines and chemokines in addition to IFNα (226). While the TLR3-TRIF pathway activates

both IRF-3 and IRF-7 via TBK-1, these mediators do not appear to regulate IRF5. In contrast, IRF-5 is activated by MyD88-dependent pathways through a TRAF6- and IRAK1-dependent mechanism (125). Initial analysis of the role of IRF-5 in the innate antiviral response in Irf5$^{-/-}$ mice showed impairment in the TLR9-mediated induction of IL-6 and TNFα in CDC. However, unlike PDCs from Irf7$^{-/-}$ mice, which are severely compromised in CpG-DNA-induced IFN responses, PDCs from Irf5$^{-/-}$ mice did not show any defect in the induction of IFNα (128). The impairment of TNFα, IL-6, and IL-12p40 was not limited to TLR9-mediated induction but was also observed in the TLR4 and TLR3 responses of splenic macrophages; however, the induction of type I IFN in Irf5$^{-/-}$ cells in response to these inducers was not examined (128). Recent observations have shown that IRF-5 plays a role in IFNα induction by RNA-containing immune complexes present in the sera of SLE patients (231). Yasuda et al. stimulated a mixture of CDC and PDC derived from the bone marrow of C57BL/6J and Irf5$^{-/-}$ mice with IgG from lupus sera, the TLR9 ligand CpG-A, or the TLR7 ligand R848 and showed that production of IFNα and IL-6 was largely abolished in Irf5$^{-/-}$ cells, a result that is in contrast to the report by Takaoka et al. (128).

To clarify the apparent contradictions between the roles of HuIRF-5 in the antiviral response (in particular the type I IFN response) *in vitro* and MuIRF-5 *in vivo*, the work described in this chapter determined the functional properties of MuIRF-5 *in vitro*. These studies revealed that IRF-5 plays an important role for

IFNα induction in CD11c+CDCs from mouse spleen and also revealed the important role of IRF5 in regulation of TNFα downstream of multiple TLRs.

In addition to examining the role of IRF5 in TLR signaling, we also examined the role of IRF5 in additional pathways important in innate immunity. These studies led to the finding that RIP2, which functions in NOD signaling can also act as an upstream mediator of IRF5 signaling. RIP2 plays a critical role in activating the signaling pathways downstream of the NOD1/2, which recognize bacterial PGN fragments (232). NOD1 and NOD2 belong to the NLR family. In contrast to TLRs which recognize microbial components at the cell surface or inside endosomes, NLRs are cytosolic receptors specialized in bacterial recognition. In response to Lm infection, activated NOD1 and NOD2 recruit RIP2 for downstream signaling and proinflammatory cytokine production (232). RIP2 is a serine-threonine kinase that belongs to the same family as RIP1, a mediator of TLR signaling. Overexpression of RIP2 stimulates NFκB activity and induces apoptosis (233). In RIP2 deficient MEFs, NOD1 and NOD2 cannot activate NFκB signaling, suggesting a critical role of RIP2 in NOD1/2 pathway (234). NOD2 has also been shown to be involved in type I IFN production upon intracellular bacteria infection since the induction of IFNβ was reduced in NOD2 deficient macrophages during infection with either Lm or Mtb (235). In this chapter, we investigated the function of IRF5 in the NOD2-RIP2 pathway. Our data revealed that IRF5 could be activated by RIP2. Furthermore, this led to studies in

collaboration with the Kelliher and Sasetti laboratories, which showed that IRF5 was involved in Mtb induced IFNβ production. Mtb infection activates more than one signaling pathway for type I IFN expression. Live Mtb induced IFNβ is TBK1 dependent and dependent on both IRF3 and 5. *N*-glycolyl MDP could also turn on IFNB gene expression via NOD2-RIP2-IRF5 and did not involve IRF3. This is the first work to implicate IRFs in signaling downstream of NLRs and the first to reveal the important role of IRF5 in response to bacterial infection.

Results

IRF5 plays important role in antiviral and anti-inflammatory function *in vivo.*

Previous data in our lab had shown that IRF5 was involved in TLR7/9 signaling for induction of type I IFN in a MyD88 dependent manner (125). Initial analysis of the role of IRF-5 in the innate antiviral response in Irf5$^{-/-}$ mice showed that induction of IL-6 and TNFα in splenic DC following CpG DNA treatment was severely compromised in IRF5-deficient mice. Surprisingly, PDCs from Irf5$^{-/-}$ mice produced normal levels of IFNα (128). TNFα, IL-6, and IL-12p40 were also decreased in response to ligands for TLR4 and TLR3; however, the induction of type I IFN in Irf5$^{-/-}$ cells in response to these inducers was not examined. We therefore wanted to perform a more detailed study to examine the role of IRF5 in antiviral and anti-inflammatory response and especially in type I IFN production.

We generated mouse BMDC and stimulated them with ligands for TLR2 (Pam2Cysk4), TLR3 (polyIC), TLR4 (LPS), TLR7/8 (R848), TLR9 (CpG-B) and viruses. Mouse TNFα and IFNβ levels were quantified in the supernatant by ELISA. No difference in TNFα level was observed between WT and IRF5 deficient BMDC (Figure 4.1a). While Pam2Cysk4, R848 and CpG-B didn't induce detectable IFNβ, LPS, PolyIC and viruses (SV, NDV) induced comparable amounts of IFNβ in WT and IRF5 BMDCs (Figure 4.1b). We also generated mouse BMDM, stimulated them with TLR3, TLR4, and TLR9 ligands, and monitored IFNβ RNA level by real-time PCR. We observed similar IFNβ levels in

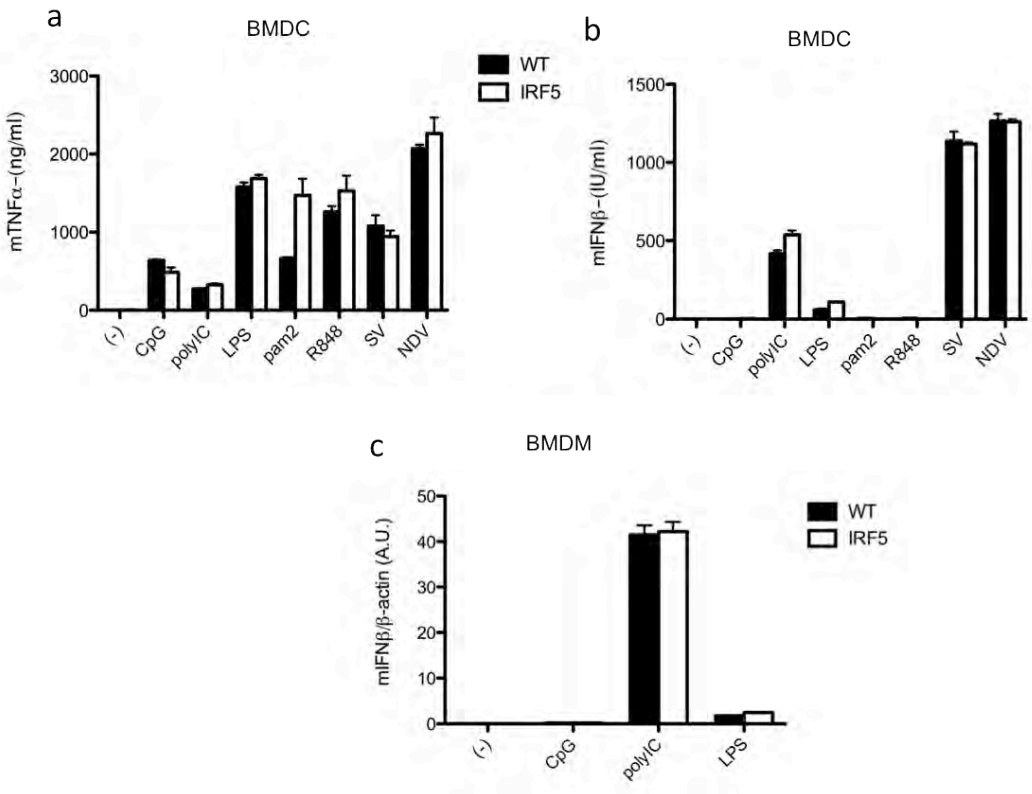


Figure 4.1 IRF5 is not involved in the IFNβ production in BMDC or BMDM (a-b) mouse BMDCs (2.10^5/well) were stimulated with CpG-B (2uM), polyIC (20ug/ml), LPS (20ng/ml), pam2 (10nM), R848 (10nM), SV (40HAU), or NDV (16HAU) for 24hrs. Protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (c) mouse BMDMs (2.10^5/well) were stimulated with CpG-B (2uM), polyIC (20ug/ml), or LPS (20ng/ml) for 6hrs. Levels of murine IFNβ and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFNβ over 100 copies of β-actin.

WT and IRF5 deficient BMDM upon polyIC and LPS stimulation (Figure 4.1c). These data indicated that IRF5 dose not play a role in type I IFN production in response to most TLR ligands in BMDM and BMDC. Moreover, type I IFN production in response to SV and NDV, that mainly signal through RIG-I and MDA-5 in these cell types, was also not dependent on IRF5.

To further examine the role of IRF5 in other cell types, total splenic DCs (panDC) were purified using a mix of CD11c+ and mPDCA1+ beads (to assure isolation of all DC types from spleen including CDC and PDC) and CD11c+CDC were purified using CD11c+ beads from WT and IRF5 deficient mouse spleen, and stimulated with TLR3, TLR4, TLR9 ligands and NDV. Similarly to previously published data, IRF5 deficient splenic panDC and CD11c+CDCs displayed impaired TNFα levels in response to TLR3, TLR4 and TLR9 ligands (Figure 4.2a, b). In contrast to the BMDC and BMDM where no effect of IRF5 deficiency was observed, the splenic panDC population from IRF5 deficient mouse showed a significant decrease in the induction of IFNβ in response to CpG-B DNA. A partial defect in response to TLR3 and NDV was also revealed, while the TLR4 ligand only induced very low levels of IFNβ even in WT cells (Fig 4.2c). CD11c+CDC isolated from mouse spleens contain low numbers of PDC, since mouse PDC express very low levels of the CD11c surface marker. We found that IFNβ RNA levels were comparable between WT and IRF5 deficient CD11c+CDC in response to TLR3 and TLR4 ligands, while they still displayed significantly impaired IFNβ RNA level in response to the TLR9 ligand, CpG-B (Figure 4.2d).

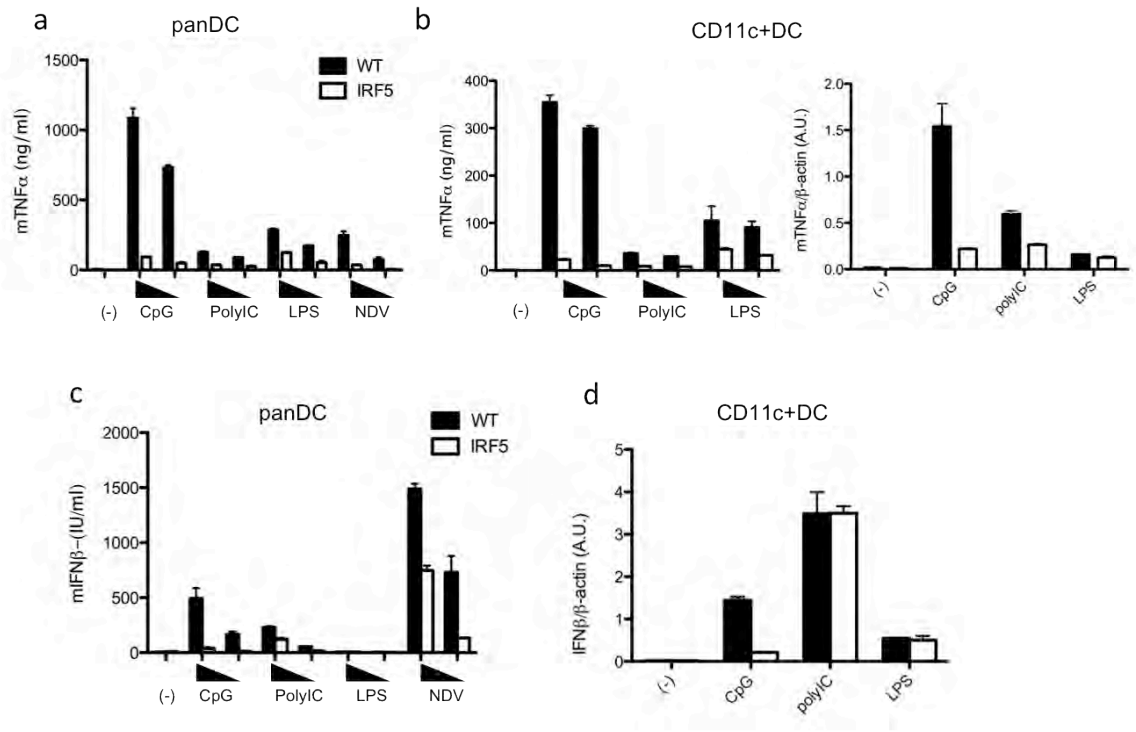


Figure 4.2 IRF5 plays an important role in type I IFN production in CD11c+CDCs (a, c) splenic panDCs (2.10^5/well) were stimulated with CpG (2-0.2uM), polyIC (20-2ug/ml), LPS (20-2ng/ml), or NDV (16-1.6 HAU) for 24hrs. Protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (b) Left panel, CD11c+CDCs (2.10^5/well) were stimulated with CpG (2-0.2uM), polyIC (20-2ug/ml), or LPS (20-2ng/ml) for 24hrs. Protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (b) Right panel and (d), CD11c+CDCs (2.10^5/well) were stimulated with CpG-B (2uM), polyIC (20ug/ml), or LPS (20ng/ml) for 6hrs. Levels of murine TNFα (b) or murine IFNβ (d) and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFNβ over 100 copies of β-actin.

Comparing this results with that of panDC, this data implied that TLR9 ligands triggers IFNβ via IRF5 in contrast to other ligands in splenic CD11c+CDCs which most likely require IRF3 and/or IRF7. IRF5 did not appear to contribute to IFNβ production in response to LPS or polyIC in splenic CD11c+CDCs. These observations support the role of IRF5 as a downstream effector of MyD88 signaling. A similar defect in IFNβ induction was also found when the splenic CD11c+CDC from IRF5 deficient mice were stimulated with the TLR7 ligand R848 (Dr. P. Pitha, unpublished observation). Collectively, these data support an important role for IRF5 in TLR7 and 9 signaling in some DC cell subsets.

IRF5 can be activated by RIP2 and involved in Mycobacterium Tuberculosis (Mtb) induced IFNβ production.

The data presented above support the involvement of IRF5 as a mediator of MyD88 dependent pathways that control IFNβ production. In an effort to search for other pathways in which IRF5 may play a role, we wanted to examine the role of IRF5 in signaling by other receptors. These studies initiated with an examination of the role of RIP1 and RIP2 kinases in IRF5 signaling. RIP1 is a mediator of TNFR signaling (236-239) as well as signaling by TLR3 and 4 ligands (159, 240). RIP2 is a mediator of NOD 1 and NOD2 signaling (232). We thereby tested the activation of IRF5 by RIP2 using a reporter assay. To test the ability of these kinases to regulate IRF5 activation, we took advantage of a sensitive reporter assay, which we had previously used to examine the ability of MyD88 to

turn on IRF5 signaling. HEK293 cells were transfected with an IFNB reporter gene, either in the presence or absence of low concentrations of IRF5. These cells were then co-transfected with increasing concentrations of candidate activators including MyD88. At low concentrations, neither IRF5 nor MyD88 alone can induce IFNB reporter gene activity. However, in the presence of IRF5, MyD88 activates the IFNB reporter gene in a dose dependent manner. Using a similar approach we compared the effect of RIP2 to MyD88 in this assay. As seen in Figure 4.3a, similarly to MyD88, which can activate IRF5 to induce the IFNβ reporter, RIP2 was also a potent activator of IRF5 to induce IFNβ reporter. In contrast, IRF3, another IRF family member that is very critical for early type I IFN production downstream of multiple PRRs, did not appear to function downstream of RIP2 (Figure 4.3b). These results indicated that RIP2 can target the IRF5 pathway to trigger type I IFN production.

As a member of the IRF family like IRF3 and IRF7, IRF-5 can be phosphorylated *in vitro* by TBK1 kinase as shown in *in vi*tro kinase assay (125). Phosphorylated IRF5 translocates to the nucleus and promotes type I IFN expression (230). Like TBK1, RIP2 also contains an N-terminal Serine/Threonine kinase domain (233), however the role of its kinase activity in mediating the signaling in NOD1/2 pathway remains unclear. We thus wanted to test whether IRF5 can be phosphorylated by RIP2 *via* its kinase activity. We performed *in vitro* kinase assay using purified IRF5 protein C-terminal transactivation domain (residues 222-467) (241) and myelin basic protein (MBP) as substrates, and

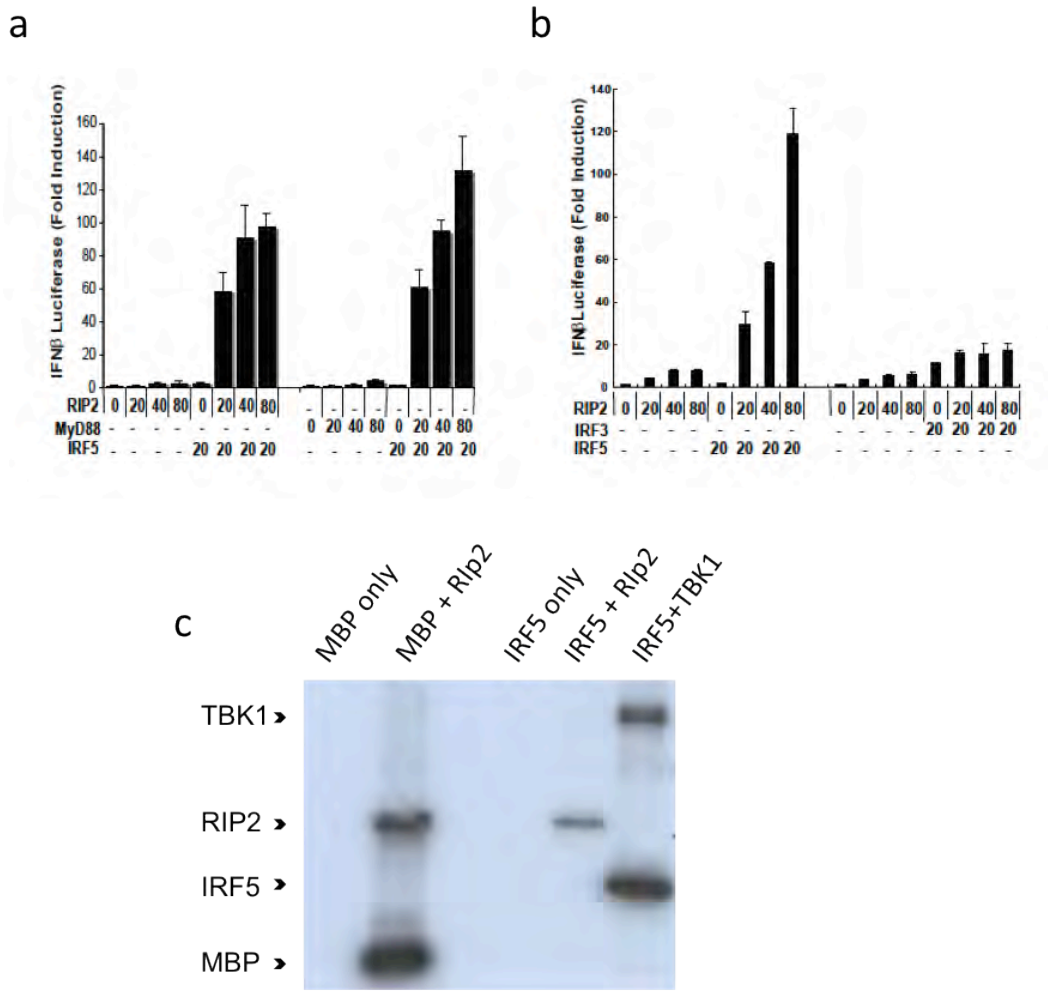


Figure 4.3 IRF5 can be activated by RIP2 for type I IFN production (a) HEK293T cells were co-transfected with IFNβ-luciferase reporter plasmid (40 ng) together with the indicated concentrations of MyD88, IRF5 and RIP2 expression plasmids. (b) HEK293T cells were co-transfected with IFNβ-luciferase reporter plasmid (40 ng) together with the indicated concentrations of IRF5, IRF3 and RIP2 expression plasmids. In both cases, Luciferase activity was measured 24 h later using Dual Luciferase reporter assay system (Promega). The Renilla luciferase gene (40 ng) was co-transfected and used as an internal control. Each experiment was repeated three times. Data are expressed as mean±s.d. of three replicates. (c) the reaction was prepared in 10µl of buffer A (20mM Hepes, pH7.6/20mM β -glycerophosphate/0.1mM sodium orthovanadate/10mM $MgCl_2$/50mM NaCl/1mM DTT) containing 1µg of IRF5 or MBP protein substrate and 10ng recombinant TBK1 protein or RIP2 protein, 50µM ATP, and 5µCi of [γ-^{32}P]ATP (1Ci = 37GBq), and conducted at 30°C for 30 min. Reaction products were subjected to analysis in 10% SDS- PAGE gels.

purified TBK1 and RIP2 proteins as kinases. Unlike TBK1, which successfully phosphorylated IRF5, RIP2 failed to do so (Figure 4.3c). In contrast, RIP2 phosphorylated MBP strongly. These results revealed that RIP2 could not directly phosphorylate IRF5 and instead must activate IRF5 via other mechanisms. One possibility is that downstream of RIP2, other kinases, such as TBK1, might be recruited to activate IRF5 for type I IFN production.

Mtb infection can induce rapid and robust type I IFN production, and our collaborators recently showed that NOD2 and RIP2 played a critical role in this signaling pathway (242). IRF3, but not IRF5, has been shown to be critical for type I infection in early response to bacteria infection, like Lm (243-246). Given the data above that Rip2 and IRF5 can synergistically induce high level of IFNβ in reporter assay, we tested whether IRF5 could be involved in the Mtb induced type I IFN production. We observed that Mtb-induced type I IFN expression was severely impaired in IRF5 deficient macrophages (Figure 4.4a) suggesting that IRF5 played an important role in type I IFN induction. As control, the response to Lm, which is totally dependent on IRF3 (244-246), was unaffected in the absence of IRF5 (Figure 4.4a). While the related RIP1 adaptor protein has been implicated in IRF7 activity in innate anti-viral signaling (247), IRF7 was not involved in this RIP2 pathway since IRF7 deficient macrophages did not display any difference in type I IFN production in response to Mtb infection compare to WT macrophages (Dr. M. Kelliher unpublished data). IRF3 seems to also play a partial role in type I IFN production in response to whole Mtb, since type I IFN

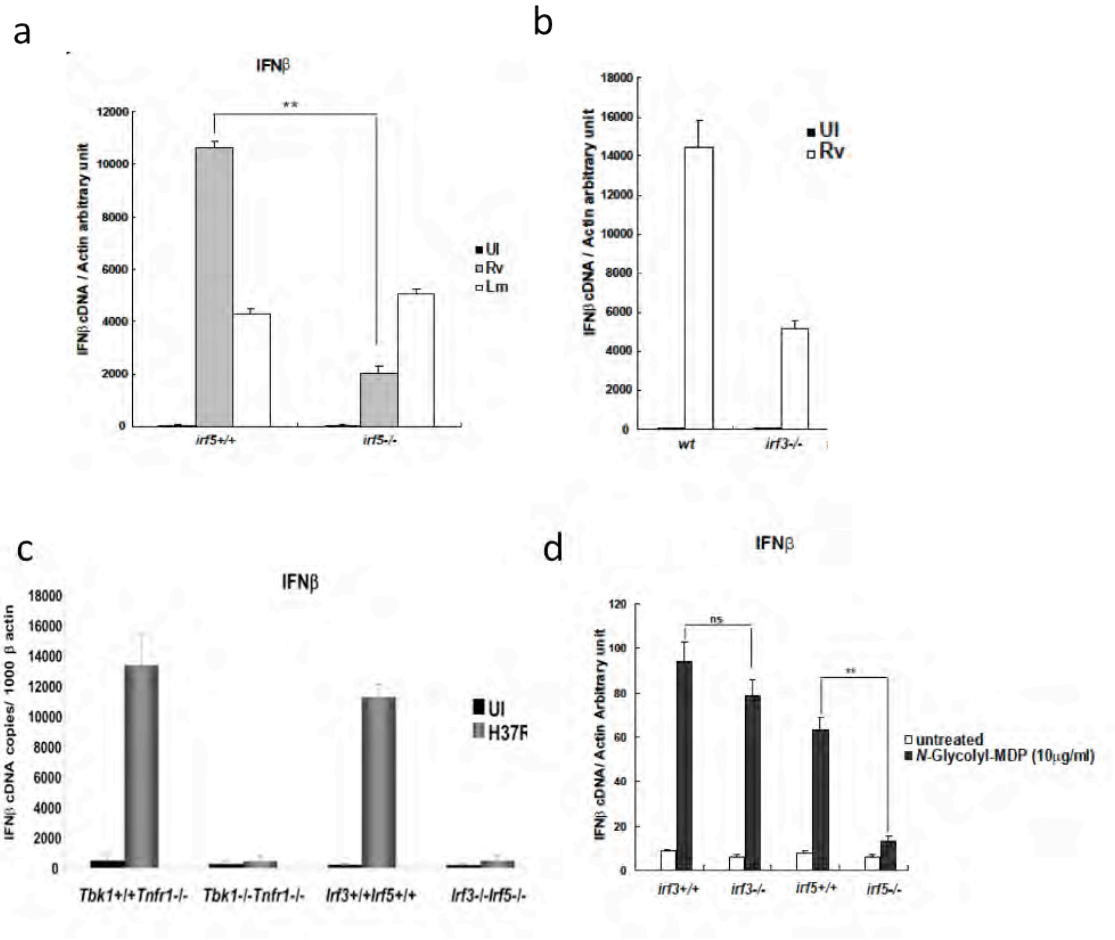


Figure 4.4 IRF5 is involved in IFNβ production induced by MTB (a) BMDM from *irf5−/−* or control littermates were infected with virulent Mtb H37Rv (Rv) at an MOI of 10, or with *Listeria monocytogenes* (Lm) strain 10403S (MOI 10) for 4 hours. (b) BMDM derived from *wt* and *irf3−/−* mice were infected with virulent Mtb H37Rv (Rv) at an MOI of 10 for 4 h. (c) BMDM derived from *Irf3+/+Irf5+/+, Irf3-/-Irf5-/-, Tbk1+/+Tnfr1-/-* and *Tbk1-/-Tnfr1-/-* mice were left uninfected (UI) or infected with virulent Mtb H37Rv (Rv) at an MOI of 10 for 4h. (d) BMDM derived from *irf3−/−* and *irf5−/−* mice and their littermate controls were left untreated or treated for 6 hours with 10 μg/ml of *N*-Glycolyl-MDP. In all cases, RNA was harvested, and IFNβ mRNA level was quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β-actin. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three.

levels were reduced in IRF3 deficient macrophages compared to WT (Figure 4.4b). Furthermore, IFNβ activation is entirely dependant upon IRF3 and IRF5 since IFNβ production was totally abrogated in IRF3/IRF5 double KO cells (Figure 4.4c). Since TBK1 can phosphorylate both IRF3 and IRF5, we also test its role in IFNβ induction upon Mtb infection. These results showed that IFNβ production was totally TBK1 dependent (Figure 4.4c).

The data above showed that the IFNβ induction by the whole TB is TBK1 and IRF3/IRF5 dependent. Since NOD2/RIP2 pathway was triggered by *N*-glycolyl MDP produced by Mtb (242), we assessed the involvement of IRF5 and IRF3 in this MDP/NOD2/RIP2 pathway. Consistent with the luciferase reporter assay above showing that RIP2 can activate IRF5 but not IRF3 for type I IFN induction, the IFNβ production induced by *N*-glycolyl MDP was only dependent on IRF5, and was entirely independent of IRF3 (Figure 4.4d). These data indicated that Mtb activates more than one signaling pathway for type I IFN expression. On one hand the whole bacterium activates both IRF3 and IRF5 pathways while the purified *N*-glycolyl form of MDP produced by Mtb rather triggers NOD2-RIP2-IRF5 pathway for type I IFN production.

Discussion

In recent years, considerable progress has been made in our understanding of the mechanisms underlying the detection of viruses and other pathogens and the signaling pathways leading to the production of type I IFN genes, the key workhorses of the anti-viral response. IRF3 and IRF7 have been known for some time to play a major role in these responses and their role in host-defense to viruses is very clear. The importance of these IRFs is supported by the fact that mice lacking one or other of these IRFs are susceptible to virus induction. Much less was known about the related protein IRF5, which early studies implicated in the antiviral response. A key question that arose from the early studies on IRF5 was whether this factor really participated in the production of type I IFNs.

In this chapter of this thesis, we attempted to clarify this issue. We took advantage of the recently generated mouse lacking IRF5. Four different cell types were isolated and used to investigate the role of IRF5 in IFNβ as well as proinflammatory cytokine production in response to TLR ligands and viral stimulations. Our data revealed the unique involvement of IRF5 in the activation of IFNβ in response to TLR7 and TLR9 signaling (our own data and that of our collaborator, Dr. P. Pitha). This role for IRF5 was apparent in splenic CD11c+CDCs, but not in macrophages or DCs isolated from mouse bone marrow or in PDCs isolated from spleen. TLR7 and TLR9 signal through the adaptor protein MyD88 and recruit a protein complex containing IRAK4, IRFAK1,

TRAF6 and TRAF3. In PDCs, this pathway leads to phosphorylation and activation of IRF7, while in the cells above, this same pathway converges on IRF5. In support of this model, IKKα has been shown to regulate IFNB responses via IRF5 and IRF7. Although IRF7 has clearly been shown to be phosphorylated by IKKα, it is less clear if IKKα is involved in IRF5 phosphorylation. In addition to phosphorylation, K63-linked ubiquitination has also been implicated in IRF5 activation (248, 249). The activation of type I IFN by TLR3 and TLR4 is mediated through the adaptor protein TRIF, which recruits RIP1, TRAF6 and TRAF3 to activate TBK1/IKKε for IRF3 activation. Although IRF5 can be phosphorylated by overexpression of IKKε and TBK1 *in vitro*, the phosphorylation alone did not seem to result in IRF5 nuclear localization or activation (250). These two different pathways may explain the differential involvements of IRF5 in regulating the type I IFN production in CDCs versus other cell types.

Our results with isolated IRF5 deficient splenic panDC also showed similar defects in IFNβ activation in response to TLR9 ligand CpG-B. The panDC population consists of two subtypes: CD11c+CDCs and PDCs. By comparing the results of CDC and panDC, we concluded that the decrease of IFNβ in response to TLR9 ligands in IRF5-deficient cells is due the deficiency of IRF5 in conventional DCs but not PDCs. These observations are consistent with those of Takaoka et. al. which showed that PDC responses to TLR ligands did not depend on IRF5. However, our data expand on these studies by revealing that in some DC subsets, TLR9 and TLR7 activate IRF5 for IFNα production. Thus, we

proposed a model of differential involvements of IRF7 and 5 for type I IFN production in splenic PDCs and CDCs (Figure 4.5).

In addition to the role of IRF5 in IFN production, our data as well as those from Takaoka and colleagues also revealed a role for IRF5 beyond IFN gene regulation. TNFα production was also defective in IRF5 –deficient splenic DCs in response to ligands for several TLRs. This is consistent with the previous report showing the important role of IRF5 in the production of inflammatory cytokine, such as IL-6, TNFα, and IL-12 in splenic DCs and macrophages (128). In this report, the authors analyzed and identified the potential IRF5 binding site, ISRE, in the sequences of these genes, which support a model where IRF5 regulates these inflammatory cytokines via binding to the ISRE sites within the promoters of these genes. In a manner similar to IFNB enhancesome formation, a model could be proposed whereby IRF5 binding to ISRE sites, cooperates with NFκB bound to NFκB sites to coordinate transcription of inflammatory cytokines. Further studies are required however, to definitely prove that IRF5 cooperates with other transcription factors on the promoters of these inflammatory cytokines.

A surprising finding from our studies was the lack of a phenotype in BMDCs and BMDMs from IRF5-KO mice. We found no difference in TLR driven TNFα or IFNβ production between WT and IRF5 deficient cells in response to TLR ligands and viral stimulation. We speculate the reason might be that BMDCs and BMDMs are derived from *in vitro* culture with high doses of cytokines, which could prime these cells during the culture procedure. Thus, the role of IRF5 in

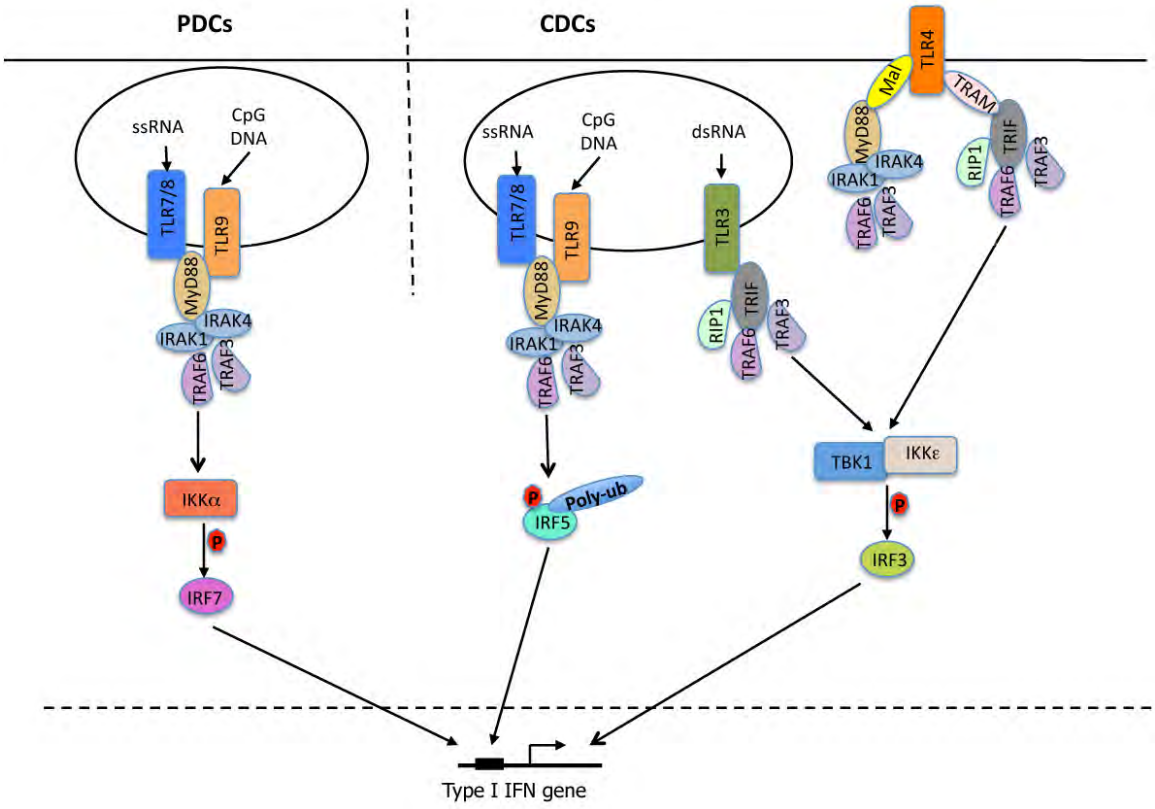


Figure 4.5 Schematic model of differential involvement of IRF5 for type I IFN production in splenic PDCs and CDCs. In PDCs, TLR7/8 and TLR9 signal through the adaptor protein MyD88 and recruit a protein complex containing IRAK4, IRAK1, TRAF6 and TRAF3. In splenic PDCs, this pathway leads to phosphorylation and activation of IRF7 through IKKα for type I IFN production. In the splenic CDCs, these same ligands trigger TLR-MyD88 signaling which does not appear to activate IRF7. Rather, this pathway involves IRF5, which is regulated by both phosphorylation and polyubiquitination. The activation of type I IFN by TLR3 and TLR4 in CDCs is mediated through the adaptor protein TRIF, which recruits TRAF3 to activate TBK1/IKKε and consequently IRF3 driven IFN responses.

these cells may be masked by these priming cytokines, which might elevate expression of IRF7 to compensate for a lack of IRF5.

Besides viruses, bacterial pathogens also trigger type I IFN responses and the mechanisms underlying this response are only beginning to be elucidated. Recent reports indicated that Lm infection or transfection of DNA into the cytosol induce IFNβ response via TBK1 and IRF3. RIP-2 does not play a role in this cytosolic DNA driven IFNB response (244). Our data with RIP2 and IRF5, suggests an additional mechanism by which bacteria can elicit IFNB responses. Moreover, the Mtb studies, reveal that bacterial PAMPs, such as MDP, can synergize with the cytosolic DNA response in order to elicit maximal IFN production (235). In this study, we revealed that MDP generated during Mtb infection can activate NOD2-RIP2-IRF5 signaling and is sufficient to induce type I IFN production. This model therefore expands our understanding of IRF5 function by revealing that in addition to its function in Myd88 dependent TLR7/9 signaling, IRF5 is also a transducer of NOD signaling. When infected with whole Mtb, IFNβ production is impaired but still a significant part is remaining in IRF5 deficient macrophages comparing to WT, indicating that additional pathways exist besides the MDP-NOD2-RIP2-IRF5 pathway. The failure of Mtb to drive IFNβ in IRF3/IRF5 double KO cells supports a model where two distinct pathways cooperate for maximal responses. Understanding the molecular basis for IFN response to pathogens such as Mtb is important as it reveals potential targets to manipulate this response in situations where IRF signaling is beneficial

to host survival. For example, vaccines could be improved by including agents that activate IRF3 as well as IRF5. In contrast, where IRF activation is harmful to the host, such as in autoimmune diseases like SLE, inactivating the IRF5 pathway could have important consequences for the treatment of this debilitating disease.

Material and methods

Cells and mice

HEK 293T, L929 cells were from ATCC (Manassas, VA) and were maintained in DMEM (Mediatech Inc, Herndon VA) supplemented with 5% FBS (Hyclone, Logan UT) and 10mg/ml Ciprofloxacin (Mediatech Inc). C57/Bl6 and C57/BL6-129 F1 mice were from Jackson Laboratories (Bar Harbor, ME). *Irf5*$^{-/-}$ mice was generously provided by Dr. T. Mak (University of Toronto, Canada). *Tbk1-/+* mice were a gift from W.C. Yeh (University of Toronto, Canada) and were bred with *Tnfr1-/-* mice at the University of Massachusetts Medical School until *TBK1*$^{-/-}$*TNFR1*$^{-/-}$ mice were achieved. Animal studies have been reviewed and approved by the University of Massachusetts Medical School institutional animal care and use committee.

Plasmids and Reagents

The IFNβ luciferase reporter gene was from T. Maniatis (Harvard, Cambridge, MA). Full length IRF5-Flag, huIRF3-Flag, and MyD88-HA were from P. Pitha (Johns Hopkins, Baltimore, MD). pCI-RIP2 was from Millennium Pharmaceuticals, Inc (San Diego, CA) . pGL4-TK renilla luciferase and pGL3-control luciferase were from Promega (Madison, WI).

Newcastle disease virus (NDV, LaSota strain) was from P. Pitha (Johns Hopkins, Baltimore, MD). Sendai virus was from Charles River Laboratories (Boston, MA).

The WT *M. tuberculosis* H37Rv strain was cultured in 7H9 medium containing 0.05% Tween 80 and OADC enrichment (Becton Dickinson). Bacterial clumps were removed by passing the washed suspension through a 5 µm syringe filter before infection. Pre-titered stocks of *Listeria monocytogenes* strain 10403 stored at −80°C (kindly provided by Victor Boyartchuk) were recovered for 1 hr at 37°C in 9 ml of Tryptic Soy Broth (BD Biosciences). Bacteria were then washed and resuspended in PBS prior to infection. CpG-B (1826) was from Coley Pharmaceutical Group (Wellesley, MA). poly IC was purchased from Amersham (Piscataway, NJ). LPS was purchased from Sigma-Aldrich (St. Louis, MI) and re-purified with Phenol. Pam_2CSK_4 was purchased from InvivoGen (San Diego, CA).

Luciferase reporter assays and Western-Blotting

Luciferase reporter assays were conducted as previously described (95). Briefly, 293T cells (2.10^4 cells/well in 96-well plates) were transfected with 40ng luciferase reporter genes together with 40ng pGL4-TK Renilla-luciferase reporter gene and with the indicated expression plasmids using Genejuice (Novagen, Madison, WI). Luciferase activity was measured as previously described (210). Whole cell lysate left from reporter assay were analyzed by 10% SDS-PAGE and subjected to immunoblot analysis using anti-Flag antibody (Sigma-Aldrich, St. Louis, MI).

***In vitro* kinase assay**

The kinase assay was performed as previously described (251). Briefly, the reaction was prepared in 10µl of buffer A (20mM Hepes, pH7.6/20mM β-glycerophosphate/0.1mM sodium orthovanadate/10mM $MgCl_2$/50mM NaCl/1mM DTT) containing 1µg of IRF5 or MBP (Upstate, Lake placid, NY) protein substrate and 10ng recombinant TBK1 protein (from Dr. McWhirter, UC Berkeley, CA) or RIP2 protein (Upstate, Lake placid, NY), 50µM ATP, and 5µCi of [γ-^{32}P]ATP (1Ci = 37GBq), and conducted at 30°C for 30 min. Reaction products were subjected to analysis in 10% SDS- PAGE gels.

Real time quantitative PCR analysis

Total RNA was extracted from the macrophage or splenic DCs (2.10^6cells/w in 6w plates) stimulated with TLR ligands or viruses using RNeasy (Qiagen, Valencia CA) or from macrophages (1.10^6cells/w in 6w plates) infected with bacteria or N-Glycolyl-MDP using Trizol reagent (Invitrogen) according to the manufacturer's directions. cDNA were synthesized as previously described (HMPV paper ref 29) using the SuperScript III enzyme (Invitrogen). Quantitative real-time PCR was performed using SYBR green reagent (Bio-Rad, Hercules CA) with the following primers: mIFNβ-F, 5′-CGTCTCCTGGATGAACTCCAC; mIFNβ-R, TGAGGACATCTCCCACGTCA; β -actin-F, 5′-CGAGGCCCAGAGCAAGAGAG; β -actin-R, 5′-CGGTTGGCCTTAGGGTTCAG; mTNFα-F, CAGTTCTATGGCCCAGACCCT; mTNFα-R, CGGACTCCGCAAAGTCTAAG; Results shown are representative of more than three separate infection experiments, with each PCR performed in triplicate. All

values reported were in the linear range of the experiment and were normalized to β-actin values.

Bone Marrow-derived Macrophages, DCs, Splenic DC Preparations

Bone marrow was flushed out of the femurs and differentiated into macrophages for 10 days in DMEM (Mediatech Inc, Herndon VA) supplemented with 20% L929-cell conditioned medium (which produce macrophage colony-stimulating factor), 10% fetal bovine serum (Hyclone, Logan UT), and 10mg/ml Ciprofloxacin (Mediatech Inc). Bone marrow was differentiated into DCs for 10 days in RPMI (Mediatech Inc, Herndon VA) supplemented with 20ng/ml of GM-CSF (PeproTech, Rocky Hill, NJ), 10% fetal bovine serum (Hyclone, Logan UT), and 10mg/ml Ciprofloxacin (Mediatech Inc). Splenic CD11c+CDC or panDC was isolated by positive selection using CD11c+ or panDC MACS beads (Miltenyi Biotec).

ELISA

For ELISA analysis, BMDMs or splenic DCs (2.10^5/well) were plated in 96 well plates in 200ul and stimulated for 24h. mTNFα was measured according to the manufacturer's recommendations (eBioscience, San Diego, CA). A murine IFNβ sandwich ELISA was used as previously described (212).

Chapter V Discussion and Perspectives

5.1 Cell type specific response

Protective innate immune responses begin when PRRs recognize microbial ligands. What has emerged from studies over the last 10-15 years or so is that multiple classes of PRRs recognize microbial components in cell type-specific manners due to the differential expression of receptors in different immune cell types. In chapter II of this thesis, we revealed that the recognition of two closely related viruses, HMPV strains (A1 [NL\1\00] and B1 [NL\1\99]) triggered different classes of PRRs depending on the cell types tested. We began our studies of PRR sensing of viruses with human cell lines. Initially, we focused on HEK293 cells given their wide use in the field of innate immune study. These cell lines have been proven to be very useful model cells to study cytosolic nucleic acid sensing pathways. In fact, RIG-I and most cytosolic DNA sensors have been discovered in HEK293 cells. In addition to the use of HEK293, we also used A549 cells, which are a human airway epithelial cell line commonly used to study immune responses to respiratory pathogens such as HMPV. Although cell lines feature most of the characteristics of primary cells and have the advantage of being able to be expanded in large numbers for biochemical assays and are easily transfectable, they often do not fully represent what is seen in primary cells or in vivo. For these reasons we extended our studies in cell lines to these human viruses in more relevant human primary cells

isolated from human PBMCs. A comprehensive analysis of cell line responses, combined with primary cell analysis is therefore a better approach.

Our studies in cell lines and primary cells allowed us to reveal, that in most cell types tested including human cell lines and purified human monocytes, HMPVA1 was sensed by the cytosolic RNA receptor RIG-I, in which 5'-triphosphate RNA is the viral ligand triggering the response. The exception to this was what we found in PDCs, where both A1 and B1 strains were sensed more or less equivalently to turn on type I IFN responses through a TLR7 dependent mechanism. One explanation for this differential sensing in these cells is that TLR7 expression is restricted to B cells and PDCs (at least in humans) while RIG-I appears to be expressed more broadly. PDCs do not appear to express RIG-I although its expression can be inducible by IFN production. The delayed expression of RIG-I might not cause significant differences in the induction of type I IFN by A1 or B1 viruses since the early robust expression of IFNα in PDC via TLR7 amplifies type I IFN production through IFNR signalling. It is possible however, that after prolonged IFN treatment, RIG-I expression may be turned on and RIG-I could play a role to cooperate with TLRs for later IFN production in these cells. TLR7 is expressed in the endosomal compartment where it samples viral genomes to turn on signalling. The acidic compartment of endosomes unveils the viral RNA for detection in this case. HMPV viruses are paramyxoviruses, which replicate in the cytosol. The lack of RIG-I in these cells means there is no apparent cytosolic detection of this virus. Endosomal sensing

is thus employed. One possibility for these observations is that viruses enter cells via the endocytic pathway in PDCs in contrast to other cell types. Another possibility is that cytosolic viral nucleic acids are delivered to the endosome to TLRs for sensing. Indeed, we have preliminary evidence that suggested a role for autophagy in PDC sensing of HMPV, since 3-MA and wortmannin, which inhibit the autophagy machinery block IFN responses in PDCs. Actually, tracking the viral entry pathways, for example using fluorescently labelled viruses/viral genomes, would be ideal to help explain the differential sensing in different cell types. We did attempt to perform such studies, but failed to clearly visualize virus trafficking patterns using the methods we used. Such cell type specific involvement of the RIG-I versus TLR pathways in induction of antiviral responses is not unique to HMPV viruses, as this differential sensing has previously been reported in the case of sensing of NDV (151). *In vivo*, it is likely that both PDCs and other DC subtypes, as well as macrophages, epithelial cells etc. could contribute to the overall anti-viral response. Indeed in the case of DNA viruses, Rasmussen et. al. also showed that in response to HSV, early production of IFN *in vivo* is mediated through TLR9 in PDCs, while the subsequent type I IFN response comes from several cell types and independent of TLR9. This report is consistent with the idea that the antiviral response is regulated in a cell type specific and temporal manner with different cell types and PRRs contributing at different stages of the infection *in vivo*.

The ability of different cell types to turn on IFN is not only due to the different receptors involved but is also related to the downstream pathways and signaling mediators expressed in those cells. The three major adaptor proteins of TLRs and RLRs, MyD88, TRIF, and MAVS, recruit different combinations of effectors, such as kinases and IRFs depending on the cell types. For example, in most cell types, IRF3 plays the predominant role in IFN induction pathways, however the high level expression of IRF7 in PDCs dictates the preferential induction of IFNα rather than IFNβ in these cells. As suggested by our studies on IRF5, detailed in chapter IV, depending on the cell type, different IRFs may be employed to participate in the IFN response. In the context of the whole animal however, when one monitors total IFN production *in vivo*, this response is a sum of the IFN levels coming from all the different cell types and therefore it might be difficult to clearly reveal the importance of a specific IRF. In this sense, *in vitro* assays with individual cell types would be more relevant than *in vivo* models of whole animals to clarify the specific role of each mediator.

Innate antiviral immune responses *in vivo* involve different subsets of DCs, as well as macrophages, natural killer cells and non-hematopoietic cells. It is important that immune responses to viruses involve tightly controlled and temporally regulated responses. If all cells from different tissues, and locations responded in exactly the same manner at the same time to viral infection, there could be overproduction of interferon and cytokines which could have a damaging effect on the host. For example, overproduction of type I IFNs is

associated with SLE. The cell type specific and temporal manner in which cells regulate IFNs and other cytokines allows effective host-defense while at the same time preventing excessive production of these mediators.

The ability of different cell types to induce the same responses, through different sensors and signalling pathways also adds redundancy to the defence system. Viruses are experts at evading innate defenses, particularly those that turn on IFN. The studies outlined in Chapter III provide a good example of this phenomenon and emphasizes the importance of functional redundancy for the fight between host and invader. Our studies revealed that HMPVB1 strains can prevent the RIG-I pathway from being activated in most cell types. However, the B1 virus is detected normally in PDCs and sensing of B1 in PDCs is not sensitive to B1 P protein mediated antagonism, because in this cell, B1 is not sensed by RIG-I but rather by the TLR system. Thus, HMPV viruses trigger two pathways to generate IFN. The involvement of two pathways means that even when one arm of the response is disabled, a second pathway is still functional to allow an effective immune response. This may be particularly relevant *in vivo* where the PDC are thought to be responsible for most of the IFN produced in the circulation. Even if the virus thwarts sensing by cells at the site of infection, the ability of PDCs to respond normally could still have a significant impact on control of this virus *in vivo*.

Functional redundancy also appears to be important in DNA sensing and in TLR4 signaling. A number of DNA sensing pathways have been identified

including DAI and RNA polymerase III which likely work in redundant manners. Similarly, TLR4 uses four adapter molecules, MyD88, Mal/TIRAP, TRIF and TRAM to coordinate downstream signaling. NFκB can be activated by two different mechanisms in this case. The Mal/MyD88 pathway leads to NFκB activation via IRAK kinases while the TRIF/TRAM arm of this pathway leads to NFκB via RIP1 kinases. One can imagine how pathogens might disarm one of these pathways, but the redundancy built into the system allows the host to still elicit the protective responses required to eliminate the pathogen.

Cell type specific expression of different receptors or responsiveness to a given agent can have practical implications. For example, distinct subtypes of breast tumors that arise from different cell types and these tumors show significant differences in responses to chemotherapeutic agents (252). Drug delivery by targeting drugs to bind to receptors and accumulate inside specific cells is an example of how such an approach could be useful therapeutically. Thus, drugs can act locally in specific tissues or organs, which can increase their efficacy and reduce harmful side effects which might occur if the drug acted systemically.

5.2 Viral antagonism

When potent cytokines especially type I IFNs are quickly produced upon viral infection, viral replication will be restricted. It is clear that viruses have evolved all kinds of strategies to block their recognition by the immune system

and fight against the production of antiviral effectors, so that they can proliferate and spread in the host. Our studies comparing the HMPVA1 and B1 strains led us to identify a novel immune evasion mechanism. We found that the B1 strain failed to induce innate signaling in most cell types and our studies with individual B1 proteins and recombinant viruses in which we swapped P proteins from the B1 virus with those from the A1 virus, allowed us to propose a mechanism whereby in the context of the entire virus, the P protein from the B1 virus is the likely factor that prevents RIG-I from sensing HMPV B1 virus. This conclusion was based on the following observations: 1. that the P protein from the B1 strain could prevent HMPVA1 virus from being recognized, 2. that under conditions when free viral RNA was delivered to the cytosol, the RNA from both A1 and B1 strains could be recognized and turn on RIG-I signaling, 3. that the B1 P protein did not block RIG-I from recognizing NDV, a virus which is sensed by RIG-I and finally, 4). That recombinant A1 virus with the P protein from B1 failed to induce type I IFN production. These combined data suggested to us that in the context of the virus, the B1 P protein was preventing access of RIG-I to the viral RNA. This explanation is particularly strengthened by the fact that free viral RNA is sensed normally. Moreover, the factor that the B1 P protein allows normal sensing of NDV RNA indicates that the block is at the level of HMPV RNA access/sensing. Our failure to recover recombinant B1 virus encoding P protein from the A1 strain prevented us from testing the possibility that this was the only contributing factor. If in the B1 backbone, the P protein from the A1 virus did not

block A1 virus from being sensed, this would have strengthened this argument. We therefore cannot exclude the possibility that other proteins (from the B1 strain) also contribute to this antagonism.

Previously published data has suggested that the G protein could also have an inhibitory effect on the Rig-I pathway (224). These studies however, utilized a different strain of HMPV virus, CAN97-83 strain. In our own studies with A1 and B1 strains, we failed to see any effects of the G protein from the B1 virus on RIG-I sensing. Generating chimeric viruses in the A1 and B1 strains in which the G protein was deleted or exchanged would allow us to clarify this issue. In light of our current data, we propose a model in which the P protein inhibits type I IFN induction through RIG-I by preventing RIG-I from detecting the viral DNA. Importantly, our studies in PDC reveal that the P protein does not prevent TLR7 mediated recognition, since both A1 and B1 viruses are sensed normally via TLR7 in the endosomal. Of note, TLR7 senses ssRNA and a 5' tri-phosphate group is not required for this sensing. Specifically, the B1 P protein masks the RIG-I ligand in the cytosol.

A detailed comparison of *in vivo* responses in mice to HMPVA1 and B1 viruses could be used to further examine the differential innate sensing of these viruses and the impact of the B1 P protein on viral pathogenesis. Based on our findings in this thesis *in vitro*, we would speculate that the failure of the RIG-I pathway to induce IFN in response to the B1 virus might make this strain more virulent allowing greater viral replication. However, the fact that PDC can trigger

IFN in response to B1 could be sufficient to control viral levels *in vivo*. We don't yet have clinical data comparing the effect of these two human viruses in human populations, which would be more relevant than mouse models. One recent study from Vicente & al. described how acute respiratory illness (ARI) due to HMPV-A strains was more severe than that due to HMPV-B viruses (253). One possible interpretation of this finding based on our studies might relate to the fact that the RIG-I pathway also regulates inflammatory cytokines as well as IFN. It is possible that the ability of A viruses but not B1 virus to trigger RIG-I could account for a more aggressive inflammatory response to A1 versus B1 virus. By understanding how viruses trigger and evade innate sensors, one can develop deletion mutants, which are attenuated *in vivo* that could be developed as vaccine candidates.

5.3 Summary

In this thesis, we have studied the innate immune response to RNA viruses and ligands for TLR7 and 9. A clear understanding of how viruses are detected and how viruses subvert these recognition systems and overcome innate defenses is critical for our ability to treat viral pathogenesis and develop new vaccines for viruses.

While most of the work in this thesis focused on RNA sensing of viruses, it is becoming more and more clear that a variety of bacterial pathogens also trigger type I IFN response. It is likely that IFN response to bacteria will also involve other receptor families besides the major viral detecting TLRs and RLRs.

In the second part of chapter IV our data showed that stimulation with MDP isolated from Mtb can activate NOD2-RIP2-IRF5 signaling pathway and is sufficient to induce type I IFN production. While in response to whole Mtb, both IRF3 and IRF5 are required for full activation of type I IFN, again indicating that redundant pathways exist for sensing Mtb infection. The redundancy employed here could also be important in situations where bacterial evasion of one or more of these immune responses comes into play.

Understanding how the activity of IRFs as well as other signaling mediators is regulated can help to understand how disease related versions of these molecules can contribute to disease pathogenesis. For example, some mutations in IRF5 are associated with the pathogenesis of autoimmune disease including inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) (228, 229, 254-256). Our understanding of IRF5 function in TLR and NLR signaling could be of potential importance for developing clinical therapies to treat these important diseases.

In summary, in this thesis, by studying HMPV viruses and IRF5 signaling we have explored new aspects of innate immunity. Our findings help us to understand how important innate sensing can be and in particular reveal that innate sensing and signaling pathways need to be tightly controlled or else there are deleterious consequences for the host: in the case of virus infection, over activation of innate pathways can contribute to viral pathogenesis. While in the

case of IRF5, as suggested by the disease associated SNPs, mutant versions could lead to excessive IFN responses and diseases like SLE. A deeper understanding of the molecular mechanisms involved in interactions between host and microbes will help to develop better preventive and therapeutic strategies against infectious and autoimmune diseases.

Bibliography

1. Passlick B, Flieger D, Ziegler-Heitbrock HW. 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 74: 2527-34
2. Geissmann F, Jung S, Littman DR. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71-82
3. Gordon S, Lawson L, Rabinowitz S, Crocker PR, Morris L, Perry VH. 1992. Antigen markers of macrophage differentiation in murine tissues. *Curr Top Microbiol Immunol* 181: 1-37
4. Martinez-Pomares L, Platt N, McKnight AJ, da Silva RP, Gordon S. 1996. Macrophage membrane molecules: markers of tissue differentiation and heterogeneity. *Immunobiology* 195: 407-16
5. Nimmerjahn F, Ravetch JV. 2006. Fcgamma receptors: old friends and new family members. *Immunity* 24: 19-28
6. Hawlisch H, Kohl J. 2006. Complement and Toll-like receptors: key regulators of adaptive immune responses. *Mol Immunol* 43: 13-21
7. Brown GD. 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol* 6: 33-43
8. Pluddemann A, Mukhopadhyay S, Gordon S. 2006. The interaction of macrophage receptors with bacterial ligands. *Expert Rev Mol Med* 8: 1-25
9. Portnoy DA, Chakraborty T, Goebel W, Cossart P. 1992. Molecular determinants of Listeria monocytogenes pathogenesis. *Infect Immun* 60: 1263-7
10. O'Neill LA. 2006. How Toll-like receptors signal: what we know and what we don't know. *Curr Opin Immunol* 18: 3-9
11. Creagh EM, O'Neill LA. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol* 27: 352-7
12. Langerhans P. 1868. Ueber die Nerven der menschlichen Haut. *Virchows Archiv* 44: 325-37
13. Steinman RM, Cohn ZA. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137: 1142-62
14. Liu K, Nussenzweig MC. 2010. Origin and development of dendritic cells. *Immunol Rev* 234: 45-54
15. Liu YJ. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23: 275-306
16. Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, Gunther G, Johnston I, Lanzavecchia A, Nagasaka T, Okada T, Vermi W, Winkels G, Yamamoto T, Zysk M, Yamaguchi Y, Schmitz J. 2001. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med* 194: 1823-34

17. Blasius A, Vermi W, Krug A, Facchetti F, Cella M, Colonna M. 2004. A cell-surface molecule selectively expressed on murine natural interferon-producing cells that blocks secretion of interferon-alpha. *Blood* 103: 4201-6
18. Trinchieri G, Santoli D. 1978. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J Exp Med* 147: 1314-33
19. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ. 1999. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 284: 1835-7
20. Barchet W, Cella M, Odermatt B, Asselin-Paturel C, Colonna M, Kalinke U. 2002. Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. *J Exp Med* 195: 507-16
21. den Haan JM, Lehar SM, Bevan MJ. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192: 1685-96
22. Fitzgerald-Bocarsly P, Feng D. 2007. The role of type I interferon production by dendritic cells in host defense. *Biochimie* 89: 843-55
23. Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 164: 2978-86
24. Miloud T, Hammerling GJ, Garbi N. 2010. Review of murine dendritic cells: types, location, and development. *Methods Mol Biol* 595: 21-42
25. Vremec D, O'Keeffe M, Hochrein H, Fuchsberger M, Caminschi I, Lahoud M, Shortman K. 2007. Production of interferons by dendritic cells, plasmacytoid cells, natural killer cells, and interferon-producing killer dendritic cells. *Blood* 109: 1165-73
26. Villadangos JA, Young L. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 29: 352-61
27. Robinson SP, Stagg AJ. 2001. *Dendritic cell protocols*. Totowa, N.J.: Humana Press. xvii, 470 p. pp.
28. Proietto AI, Lahoud MH, Wu L. 2008. Distinct functional capacities of mouse thymic and splenic dendritic cell populations. *Immunol Cell Biol* 86: 700-8
29. Segura E, Kapp E, Gupta N, Wong J, Lim J, Ji H, Heath WR, Simpson R, Villadangos JA. 2010. Differential expression of pathogen-recognition molecules between dendritic cell subsets revealed by plasma membrane proteomic analysis. *Mol Immunol* 47: 1765-73
30. Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, Akira S, Wiegand M, Hochrein H, O'Keeffe M, Mann M. 2010. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32: 279-89
31. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SE, Yagita H, Takeda K, van Dommelen SL, Degli-Esposti MA, Hayakawa Y. 2005. Activation of NK cell cytotoxicity. *Mol Immunol* 42: 501-10

32. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 17: 189-220
33. Bryceson YT, March ME, Ljunggren HG, Long EO. 2006. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* 214: 73-91
34. Turvey SE, Broide DH. 2010. Innate immunity. *J Allergy Clin Immunol* 125: S24-32
35. Bowie A, O'Neill LA. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J Leukoc Biol* 67: 508-14
36. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413: 732-8
37. Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M. 2006. Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* 2: e53
38. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, Endres S, Hartmann G. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* 168: 4531-7
39. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 10: 1366-73
40. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529-31
41. Wang JP, Liu P, Latz E, Golenbock DT, Finberg RW, Libraty DH. 2006. Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. *J Immunol* 177: 7114-21
42. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A* 101: 5598-603
43. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303: 1526-9
44. Gorden KK, Qiu XX, Binsfeld CC, Vasilakos JP, Alkan SS. 2006. Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides. *J Immunol* 177: 6584-7
45. Guggemoos S, Hangel D, Hamm S, Heit A, Bauer S, Adler H. 2008. TLR9 contributes to antiviral immunity during gammaherpesvirus infection. *J Immunol* 180: 438-43

46. Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM, Colonna M. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21: 107-19
47. Krug A, Luker GD, Barchet W, Leib DA, Akira S, Colonna M. 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* 103: 1433-7
48. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med* 198: 513-20
49. Takeuchi O, Hoshino K, Akira S. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol* 165: 5392-6
50. Wooten RM, Ma Y, Yoder RA, Brown JP, Weis JH, Zachary JF, Kirschning CJ, Weis JJ. 2002. Toll-like receptor 2 is required for innate, but not acquired, host defense to Borrelia burgdorferi. *J Immunol* 168: 348-55
51. Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783-801
52. Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. *Annu Rev Immunol* 21: 335-76
53. Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, Wagner H, Kirschning CJ, Ter Meulen V, Schneider-Schaulies S. 2002. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J Virol* 76: 8729-36
54. Aravalli RN, Hu S, Rowen TN, Palmquist JM, Lokensgard JR. 2005. Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus. *J Immunol* 175: 4189-93
55. Compton T, Kurt-Jones EA, Boehme KW, Belko J, Latz E, Golenbock DT, Finberg RW. 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J Virol* 77: 4588-96
56. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, Finberg RW. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 1: 398-401
57. Burzyn D, Rassa JC, Kim D, Nepomnaschy I, Ross SR, Piazzon I. 2004. Toll-like receptor 4-dependent activation of dendritic cells by a retrovirus. *J Virol* 78: 576-84
58. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458: 1191-5

59. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 130: 1071-82
60. Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, Davies DR. 2008. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. *Science* 320: 379-81
61. Schroder K, Tschopp J. 2010. The inflammasomes. *Cell* 140: 821-32
62. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411: 599-603
63. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411: 603-6
64. Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Hafner R, Chamaillard M, Zouali H, Thomas G, Hugot JP. 2001. CARD15 mutations in Blau syndrome. *Nat Genet* 29: 19-20
65. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237-41
66. Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V, Tschopp J, Lagente V, Quesniaux VF, Ryffel B, Couillin I. 2009. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. *Am J Respir Crit Care Med* 179: 903-13
67. Griffith JW, Sun T, McIntosh MT, Bucala R. 2009. Pure Hemozoin is inflammatory in vivo and activates the NALP3 inflammasome via release of uric acid. *J Immunol* 183: 5208-20
68. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, Carter AB, Rothman PB, Flavell RA, Sutterwala FS. 2008. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 105: 9035-40
69. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9: 847-56
70. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320: 674-7
71. Gross O, Poeck H, Bscheider M, Dostert C, Hannesschlager N, Endres S, Hartmann G, Tardivel A, Schweighoffer E, Tybulewicz V, Mocsai A, Tschopp J, Ruland J. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459: 433-6

72. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228-32
73. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N, Nunez G. 2006. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* 281: 36560-8
74. Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, Tschopp J. 2008. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452: 103-7
75. Kumagai Y, Akira S. 2010. Identification and functions of pattern-recognition receptors. *J Allergy Clin Immunol* 125: 985-92
76. Amer A, Franchi L, Kanneganti TD, Body-Malapel M, Ozoren N, Brady G, Meshinchi S, Jagirdar R, Gewirtz A, Akira S, Nunez G. 2006. Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. *J Biol Chem* 281: 35217-23
77. Franchi L, Stoolman J, Kanneganti TD, Verma A, Ramphal R, Nunez G. 2007. Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. *Eur J Immunol* 37: 3030-9
78. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430: 213-8
79. Miao EA, Ernst RK, Dors M, Mao DP, Aderem A. 2008. Pseudomonas aeruginosa activates caspase 1 through Ipaf. *Proc Natl Acad Sci U S A* 105: 2562-7
80. Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BI, Flavell RA. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* 204: 3235-45
81. Suzuki T, Franchi L, Toma C, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H, Inohara N, Sasakawa C, Nunez G. 2007. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog* 3: e111
82. Yoneyama M, Fujita T. 2009. RNA recognition and signal transduction by RIG-I-like receptors. *Immunol Rev* 227: 54-65
83. Seth RB, Sun L, Ea CK, Chen ZJ. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122: 669-82
84. Kumar H, Kawai T, Kato H, Sato S, Takahashi K, Coban C, Yamamoto M, Uematsu S, Ishii KJ, Takeuchi O, Akira S. 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. *J Exp Med* 203: 1795-803
85. Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 19: 727-40

86. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437: 1167-72
87. Dixit E, Boulant S, Zhang Y, Lee AS, Odendall C, Shum B, Hacohen N, Chen ZJ, Whelan SP, Fransen M, Nibert ML, Superti-Furga G, Kagan JC. 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* 141: 668-81
88. Sun L, Liu S, Chen ZJ. 2010. SnapShot: pathways of antiviral innate immunity. *Cell* 140: 436- e2
89. Zhong B, Yang Y, Li S, Wang YY, Li Y, Diao F, Lei C, He X, Zhang L, Tien P, Shu HB. 2008. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 29: 538-50
90. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441: 101-5
91. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M, Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol* 82: 335-45
92. McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin HW, Colonna M. 2008. MDA-5 recognition of a murine norovirus. *PLoS Pathog* 4: e1000108
93. Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, Hiiragi A, Dermody TS, Fujita T, Akira S. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 205: 1601-10
94. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M, Jr., Akira S, Yonehara S, Kato A, Fujita T. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175: 2851-8
95. Rothenfusser S, Goutagny N, DiPerna G, Gong M, Monks BG, Schoenemeyer A, Yamamoto M, Akira S, Fitzgerald KA. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 175: 5260-8
96. Venkataraman T, Valdes M, Elsby R, Kakuta S, Caceres G, Saijo S, Iwakura Y, Barber GN. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J Immunol* 178: 6444-55
97. Satoh T, Kato H, Kumagai Y, Yoneyama M, Sato S, Matsushita K, Tsujimura T, Fujita T, Akira S, Takeuchi O. 2010. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A* 107: 1512-7
98. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, Ohba Y, Taniguchi T. 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 448: 501-5

99. Wang Z, Choi MK, Ban T, Yanai H, Negishi H, Lu Y, Tamura T, Takaoka A, Nishikura K, Taniguchi T. 2008. Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules. *Proc Natl Acad Sci U S A* 105: 5477-82
100. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, Uematsu S, Takeuchi O, Takeshita F, Coban C, Akira S. 2008. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 451: 725-9
101. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V. 2009. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol* 10: 1065-72
102. Chiu YH, Macmillan JB, Chen ZJ. 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* 138: 576-91
103. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458: 514-8
104. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 458: 509-13
105. Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, Jahn H, Planyavsky M, Bilban M, Colinge J, Bennett KL, Superti-Furga G. 2009. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol* 10: 266-72
106. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, Hume DA, Stacey KJ. 2009. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* 323: 1057-60
107. Meixenberger K, Pache F, Eitel J, Schmeck B, Hippenstiel S, Slevogt H, N'Guessan P, Witzenrath M, Netea MG, Chakraborty T, Suttorp N, Opitz B. 2010. Listeria monocytogenes-infected human peripheral blood mononuclear cells produce IL-1beta, depending on listeriolysin O and NLRP3. *J Immunol* 184: 922-30
108. Franchi L, Kanneganti TD, Dubyak GR, Nunez G. 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem* 282: 18810-8
109. Warren SE, Mao DP, Rodriguez AE, Miao EA, Aderem A. 2008. Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection. *J Immunol* 180: 7558-64
110. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11: 395-402

111. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264: 1918-21
112. Hwang SY, Hertzog PJ, Holland KA, Sumarsono SH, Tymms MJ, Hamilton JA, Whitty G, Bertoncello I, Kola I. 1995. A null mutation in the gene encoding a type I interferon receptor component eliminates antiproliferative and antiviral responses to interferons alpha and beta and alters macrophage responses. *Proc Natl Acad Sci U S A* 92: 11284-8
113. Randall RE, Goodbourn S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* 89: 1-47
114. Kim TK, Maniatis T. 1997. The mechanism of transcriptional synergy of an in vitro assembled interferon-beta enhanceosome. *Mol Cell* 1: 119-29
115. Maniatis T. 1986. Mechanisms of human beta-interferon gene regulation. *Harvey Lect* 82: 71-104
116. Ryals J, Dierks P, Ragg H, Weissmann C. 1985. A 46-nucleotide promoter segment from an IFN-alpha gene renders an unrelated promoter inducible by virus. *Cell* 41: 497-507
117. Au WC, Moore PA, LaFleur DW, Tombal B, Pitha PM. 1998. Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *J Biol Chem* 273: 29210-7
118. Marie I, Durbin JE, Levy DE. 1998. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO J* 17: 6660-9
119. Paun A, Pitha PM. 2007. The IRF family, revisited. *Biochimie* 89: 744-53
120. Takaoka A, Tamura T, Taniguchi T. 2008. Interferon regulatory factor family of transcription factors and regulation of oncogenesis. *Cancer Sci* 99: 467-78
121. Ishikawa H, Barber GN. 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 455: 674-8
122. Honda K, Taniguchi T. 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6: 644-58
123. Yanai H, Chen HM, Inuzuka T, Kondo S, Mak TW, Takaoka A, Honda K, Taniguchi T. 2007. Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. *Proc Natl Acad Sci U S A* 104: 3402-7
124. Paun A, Reinert JT, Jiang Z, Medin C, Balkhi MY, Fitzgerald KA, Pitha PM. 2008. Functional characterization of murine interferon regulatory factor 5 (IRF-5) and its role in the innate antiviral response. *J Biol Chem* 283: 14295-308
125. Schoenemeyer A, Barnes BJ, Mancl ME, Latz E, Goutagny N, Pitha PM, Fitzgerald KA, Golenbock DT. 2005. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *J Biol Chem* 280: 17005-12

126. Jefferies CA, Fitzgerald KA. 2005. Interferon gene regulation: not all roads lead to Tolls. *Trends Mol Med* 11: 403-11
127. Savitsky D, Tamura T, Yanai H, Taniguchi T. 2010. Regulation of immunity and oncogenesis by the IRF transcription factor family. *Cancer Immunol Immunother* 59: 489-510
128. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S, Honda K, Ohba Y, Mak TW, Taniguchi T. 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434: 243-9
129. Thanos D, Maniatis T. 1995. Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell* 83: 1091-100
130. Au WC, Yeow WS, Pitha PM. 2001. Analysis of functional domains of interferon regulatory factor 7 and its association with IRF-3. *Virology* 280: 273-82
131. Matsuyama T, Kimura T, Kitagawa M, Pfeffer K, Kawakami T, Watanabe N, Kundig TM, Amakawa R, Kishihara K, Wakeham A, et al. 1993. Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell* 75: 83-97
132. Reis LF, Ruffner H, Stark G, Aguet M, Weissmann C. 1994. Mice devoid of interferon regulatory factor 1 (IRF-1) show normal expression of type I interferon genes. *EMBO J* 13: 4798-806
133. Hida S, Ogasawara K, Sato K, Abe M, Takayanagi H, Yokochi T, Sato T, Hirose S, Shirai T, Taki S, Taniguchi T. 2000. CD8(+) T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-alpha/beta signaling. *Immunity* 13: 643-55
134. Yamagata T, Nishida J, Tanaka S, Sakai R, Mitani K, Yoshida M, Taniguchi T, Yazaki Y, Hirai H. 1996. A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon-regulated genes. *Mol Cell Biol* 16: 1283-94
135. Kimura T, Kadokawa Y, Harada H, Matsumoto M, Sato M, Kashiwazaki Y, Tarutani M, Tan RS, Takasugi T, Matsuyama T, Mak TW, Noguchi S, Taniguchi T. 1996. Essential and non-redundant roles of p48 (ISGF3 gamma) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. *Genes Cells* 1: 115-24
136. Negishi H, Ohba Y, Yanai H, Takaoka A, Honma K, Yui K, Matsuyama T, Taniguchi T, Honda K. 2005. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci U S A* 102: 15989-94
137. Tsujimura H, Tamura T, Kong HJ, Nishiyama A, Ishii KJ, Klinman DM, Ozato K. 2004. Toll-like receptor 9 signaling activates NF-kappaB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells. *J Immunol* 172: 6820-7
138. Tailor P, Tamura T, Kong HJ, Kubota T, Kubota M, Borghi P, Gabriele L, Ozato K. 2007. The feedback phase of type I interferon induction in dendritic cells requires interferon regulatory factor 8. *Immunity* 27: 228-39

139. Bailey CM, Hendrix MJ. 2008. IRF6 in development and disease: a mediator of quiescence and differentiation. *Cell Cycle* 7: 1925-30
140. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. 1998. How cells respond to interferons. *Annu Rev Biochem* 67: 227-64
141. Li H, Gade P, Xiao W, Kalvakolanu DV. 2007. The interferon signaling network and transcription factor C/EBP-beta. *Cell Mol Immunol* 4: 407-18
142. Takaoka A, Yanai H. 2006. Interferon signalling network in innate defence. *Cell Microbiol* 8: 907-22
143. Katze MG, Fornek JL, Palermo RE, Walters KA, Korth MJ. 2008. Innate immune modulation by RNA viruses: emerging insights from functional genomics. *Nat Rev Immunol* 8: 644-54
144. Boo KH, Yang JS. 2010. Intrinsic cellular defenses against virus infection by antiviral type I interferon. *Yonsei Med J* 51: 9-17
145. Schulz O, Pichlmair A, Rehwinkel J, Rogers NC, Scheuner D, Kato H, Takeuchi O, Akira S, Kaufman RJ, Reis e Sousa C. 2010. Protein kinase R contributes to immunity against specific viruses by regulating interferon mRNA integrity. *Cell Host Microbe* 7: 354-61
146. Malathi K, Dong B, Gale M, Jr., Silverman RH. 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature* 448: 816-9
147. Fensterl V, Sen GC. 2009. Interferons and viral infections. *Biofactors* 35: 14-20
148. D'Cunha J, Ramanujam S, Wagner RJ, Witt PL, Knight E, Jr., Borden EC. 1996. In vitro and in vivo secretion of human ISG15, an IFN-induced immunomodulatory cytokine. *J Immunol* 157: 4100-8
149. Sadler AJ, Williams BR. 2008. Interferon-inducible antiviral effectors. *Nat Rev Immunol* 8: 559-68
150. Espert L, Degols G, Gongora C, Blondel D, Williams BR, Silverman RH, Mechti N. 2003. ISG20, a new interferon-induced RNase specific for single-stranded RNA, defines an alternative antiviral pathway against RNA genomic viruses. *J Biol Chem* 278: 16151-8
151. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23: 19-28
152. Uematsu S, Sato S, Yamamoto M, Hirotani T, Kato H, Takeshita F, Matsuda M, Coban C, Ishii KJ, Kawai T, Takeuchi O, Akira S. 2005. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. *J Exp Med* 201: 915-23
153. Honda K, Yanai H, Mizutani T, Negishi H, Shimada N, Suzuki N, Ohba Y, Takaoka A, Yeh WC, Taniguchi T. 2004. Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc Natl Acad Sci U S A* 101: 15416-21
154. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, Terai K, Matsuda M, Inoue J, Uematsu S, Takeuchi O, Akira S. 2004. Interferon-alpha induction

through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* 5: 1061-8

155. Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Hacker G, Mann M, Karin M. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439: 204-7
156. Hoshino K, Sugiyama T, Matsumoto M, Tanaka T, Saito M, Hemmi H, Ohara O, Akira S, Kaisho T. 2006. IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. *Nature* 440: 949-53
157. Kawai T, Akira S. 2006. Innate immune recognition of viral infection. *Nat Immunol* 7: 131-7
158. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, Akira S. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 171: 4304-10
159. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol* 5: 503-7
160. Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, Perry A, Cheng G. 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* 439: 208-11
161. Saha SK, Pietras EM, He JQ, Kang JR, Liu SY, Oganesyan G, Shahangian A, Zarnegar B, Shiba TL, Wang Y, Cheng G. 2006. Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *EMBO J* 25: 3257-63
162. Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, Kawai T, Hoshino K, Takeda K, Akira S. 2004. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* 199: 1641-50
163. Perry AK, Chow EK, Goodnough JB, Yeh WC, Cheng G. 2004. Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. *J Exp Med* 199: 1651-8
164. Brandenburg B, Zhuang X. 2007. Virus trafficking - learning from single-virus tracking. *Nat Rev Microbiol* 5: 197-208
165. Crozat K, Beutler B. 2004. TLR7: A new sensor of viral infection. *Proc Natl Acad Sci U S A* 101: 6835-6
166. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. 2007. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* 315: 1398-401
167. Saitoh T, Akira S. 2010. Regulation of innate immune responses by autophagy-related proteins. *J Cell Biol* 189: 925-35

168. Wolff T, Ludwig S. 2009. Influenza viruses control the vertebrate type I interferon system: factors, mechanisms, and consequences. *J Interferon Cytokine Res* 29: 549-57
169. Basler CF, Amarasinghe GK. 2009. Evasion of interferon responses by Ebola and Marburg viruses. *J Interferon Cytokine Res* 29: 511-20
170. Lee HR, Kim MH, Lee JS, Liang C, Jung JU. 2009. Viral interferon regulatory factors. *J Interferon Cytokine Res* 29: 621-7
171. Perdiguero B, Esteban M. 2009. The interferon system and vaccinia virus evasion mechanisms. *J Interferon Cytokine Res* 29: 581-98
172. Marshall EE, Geballe AP. 2009. Multifaceted evasion of the interferon response by cytomegalovirus. *J Interferon Cytokine Res* 29: 609-19
173. Fields BN, Knipe DM, Howley PM. 2007. *Fields virology*. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. 2 v. (xix, 3091, 86 p.) pp.
174. Herfst S, Mas V, Ver LS, Wierda RJ, Osterhaus AD, Fouchier RA, Melero JA. 2008. Low-pH-induced membrane fusion mediated by human metapneumovirus F protein is a rare, strain-dependent phenomenon. *J Virol* 82: 8891-5
175. Schowalter RM, Smith SE, Dutch RE. 2006. Characterization of human metapneumovirus F protein-promoted membrane fusion: critical roles for proteolytic processing and low pH. *J Virol* 80: 10931-41
176. Spann KM, Tran KC, Chi B, Rabin RL, Collins PL. 2004. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. *J Virol* 78: 4363-9
177. Melchjorsen J, Jensen SB, Malmgaard L, Rasmussen SB, Weber F, Bowie AG, Matikainen S, Paludan SR. 2005. Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J Virol* 79: 12944-51
178. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994-7
179. Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, Randall RE. 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci U S A* 101: 17264-9
180. Berghall H, Siren J, Sarkar D, Julkunen I, Fisher PB, Vainionpaa R, Matikainen S. 2006. The interferon-inducible RNA helicase, mda-5, is involved in measles virus-induced expression of antiviral cytokines. *Microbes Infect* 8: 2138-44
181. Yount JS, Gitlin L, Moran TM, Lopez CB. 2008. MDA5 participates in the detection of paramyxovirus infection and is essential for the early activation of dendritic cells in response to Sendai Virus defective interfering particles. *J Immunol* 180: 4910-8

182. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. 2001. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J Virol* 75: 10730-7
183. Sun Q, Sun L, Liu HH, Chen X, Seth RB, Forman J, Chen ZJ. 2006. The specific and essential role of MAVS in antiviral innate immune responses. *Immunity* 24: 633-42
184. Bhoj VG, Sun Q, Bhoj EJ, Somers C, Chen X, Torres JP, Mejias A, Gomez AM, Jafri H, Ramilo O, Chen ZJ. 2008. MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. *Proc Natl Acad Sci U S A* 105: 14046-51
185. Schlender J, Hornung V, Finke S, Gunthner-Biller M, Marozin S, Brzozka K, Moghim S, Endres S, Hartmann G, Conzelmann KK. 2005. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. *J Virol* 79: 5507-15
186. van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, Osterhaus AD, Fouchier RA. 2004. Antigenic and genetic variability of human metapneumoviruses. *Emerg Infect Dis* 10: 658-66
187. Huck B, Scharf G, Neumann-Haefelin D, Puppe W, Weigl J, Falcone V. 2006. Novel human metapneumovirus sublineage. *Emerg Infect Dis* 12: 147-50
188. Liao S, Bao X, Liu T, Lai S, Li K, Garofalo RP, Casola A. 2008. Role of retinoic acid inducible gene-I in human metapneumovirus-induced cellular signalling. *J Gen Virol* 89: 1978-86
189. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med* 7: 719-24
190. Ji W, Chen ZR, Wang YQ. 2009. [Comparison of the clinical manifestation and lung function between RSV and hMPV lower respiratory tract infection]. *Zhonghua Er Ke Za Zhi* 47: 71-3
191. Earp LJ, Delos SE, Park HE, White JM. 2005. The many mechanisms of viral membrane fusion proteins. *Curr Top Microbiol Immunol* 285: 25-66
192. Pichlmair A, Reis e Sousa C. 2007. Innate recognition of viruses. *Immunity* 27: 370-83
193. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, Taniguchi T. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434: 772-7
194. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, Diamond MS, Colonna M. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A* 103: 8459-64
195. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential

function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5: 730-7
196. Foy E, Li K, Sumpter R, Jr., Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M, Jr. 2005. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc Natl Acad Sci U S A* 102: 2986-91
197. Sumpter R, Jr., Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M, Jr. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 79: 2689-99
198. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, Xiang Y, Bose S. 2009. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 10: 1073-80
199. Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314: 997-1001
200. Ablasser A, Poeck H, Anz D, Berger M, Schlee M, Kim S, Bourquin C, Goutagny N, Jiang Z, Fitzgerald KA, Rothenfusser S, Endres S, Hartmann G, Hornung V. 2009. Selection of molecular structure and delivery of RNA oligonucleotides to activate TLR7 versus TLR8 and to induce high amounts of IL-12p70 in primary human monocytes. *J Immunol* 182: 6824-33
201. Hornung V, Schlender J, Guenthner-Biller M, Rothenfusser S, Endres S, Conzelmann KK, Hartmann G. 2004. Replication-dependent potent IFN-alpha induction in human plasmacytoid dendritic cells by a single-stranded RNA virus. *J Immunol* 173: 5935-43
202. Guiducci C, Ott G, Chan JH, Damon E, Calacsan C, Matray T, Lee KD, Coffman RL, Barrat FJ. 2006. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J Exp Med* 203: 1999-2008
203. Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, Chang B, Duramad O, Coffman RL. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* 202: 1131-9
204. Deffrasnes C, Hamelin ME, Boivin G. 2007. Human metapneumovirus. *Semin Respir Crit Care Med* 28: 213-21
205. Biacchesi S, Skiadopoulos MH, Yang L, Lamirande EW, Tran KC, Murphy BR, Collins PL, Buchholz UJ. 2004. Recombinant human Metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate. *J Virol* 78: 12877-87
206. Schickli JH, Kaur J, Ulbrandt N, Spaete RR, Tang RS. 2005. An S101P substitution in the putative cleavage motif of the human metapneumovirus fusion protein is a major determinant for trypsin-independent growth in vero cells and does not alter tissue tropism in hamsters. *J Virol* 79: 10678-89

207. Schowalter RM, Chang A, Robach JG, Buchholz UJ, Dutch RE. 2009. Low-pH triggering of human metapneumovirus fusion: essential residues and importance in entry. *J Virol* 83: 1511-22
208. Mach N, Gillessen S, Wilson SB, Sheehan C, Mihm M, Dranoff G. 2000. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 60: 3239-46
209. Ulbrandt ND, Ji H, Patel NK, Riggs JM, Brewah YA, Ready S, Donacki NE, Folliot K, Barnes AS, Senthil K, Wilson S, Chen M, Clarke L, MacPhail M, Li J, Woods RM, Coelingh K, Reed JL, McCarthy MP, Pfarr DS, Osterhaus AD, Fouchier RA, Kiener PA, Suzich JA. 2006. Isolation and characterization of monoclonal antibodies which neutralize human metapneumovirus in vitro and in vivo. *J Virol* 80: 7799-806
210. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* 198: 1043-55
211. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, Ludwig H, Sutter G, Suzuki K, Hemmi H, Sato S, Yamamoto M, Uematsu S, Kawai T, Takeuchi O, Akira S. 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* 7: 40-8
212. Roberts ZJ, Goutagny N, Perera PY, Kato H, Kumar H, Kawai T, Akira S, Savan R, van Echo D, Fitzgerald KA, Young HA, Ching LM, Vogel SN. 2007. The chemotherapeutic agent DMXAA potently and specifically activates the TBK1-IRF-3 signaling axis. *J Exp Med* 204: 1559-69
213. Rutledge RG, Cote C. 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Res* 31: e93
214. Goodbourn S, Randall RE. 2009. The regulation of type I interferon production by paramyxoviruses. *J Interferon Cytokine Res* 29: 539-47
215. Ramachandran A, Horvath CM. 2009. Paramyxovirus disruption of interferon signal transduction: STATus report. *J Interferon Cytokine Res* 29: 531-7
216. Lo MS, Brazas RM, Holtzman MJ. 2005. Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. *J Virol* 79: 9315-9
217. Dinwiddie DL, Harrod KS. 2008. Human metapneumovirus inhibits IFN-alpha signaling through inhibition of STAT1 phosphorylation. *Am J Respir Cell Mol Biol* 38: 661-70
218. Bao X, Liu T, Spetch L, Kolli D, Garofalo RP, Casola A. 2007. Airway epithelial cell response to human metapneumovirus infection. *Virology* 368: 91-101
219. Biacchesi S, Pham QN, Skiadopoulos MH, Murphy BR, Collins PL, Buchholz UJ. 2006. Modification of the trypsin-dependent cleavage activation site of the human metapneumovirus fusion protein to be trypsin independent does not increase replication or spread in rodents or nonhuman primates. *J Virol* 80: 5798-806

220. Wathelet MG, Lin CH, Parekh BS, Ronco LV, Howley PM, Maniatis T. 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. *Mol Cell* 1: 507-18
221. Fontana JM, Bankamp B, Rota PA. 2008. Inhibition of interferon induction and signaling by paramyxoviruses. *Immunol Rev* 225: 46-67
222. Brzozka K, Finke S, Conzelmann KK. 2005. Identification of the rabies virus alpha/beta interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. *J Virol* 79: 7673-81
223. Brzozka K, Finke S, Conzelmann KK. 2006. Inhibition of interferon signaling by rabies virus phosphoprotein P: activation-dependent binding of STAT1 and STAT2. *J Virol* 80: 2675-83
224. Bao X, Liu T, Shan Y, Li K, Garofalo RP, Casola A. 2008. Human metapneumovirus glycoprotein G inhibits innate immune responses. *PLoS Pathog* 4: e1000077
225. Herfst S, de Graaf M, Schickli JH, Tang RS, Kaur J, Yang CF, Spaete RR, Haller AA, van den Hoogen BG, Osterhaus AD, Fouchier RA. 2004. Recovery of human metapneumovirus genetic lineages a and B from cloned cDNA. *J Virol* 78: 8264-70
226. Barnes BJ, Kellum MJ, Field AE, Pitha PM. 2002. Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes. *Mol Cell Biol* 22: 5721-40
227. Mancl ME, Hu G, Sangster-Guity N, Olshalsky SL, Hoops K, Fitzgerald-Bocarsly P, Pitha PM, Pinder K, Barnes BJ. 2005. Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms. Multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. *J Biol Chem* 280: 21078-90
228. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, Ortmann WA, Koeuth T, Gonzalez Escribano MF, Pons-Estel B, Petri M, Daly M, Gregersen PK, Martin J, Altshuler D, Behrens TW, Alarcon-Riquelme ME. 2006. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat Genet* 38: 550-5
229. Dideberg V, Kristjansdottir G, Milani L, Libioulle C, Sigurdsson S, Louis E, Wiman AC, Vermeire S, Rutgeerts P, Belaiche J, Franchimont D, Van Gossum A, Bours V, Syvanen AC. 2007. An insertion-deletion polymorphism in the interferon regulatory Factor 5 (IRF5) gene confers risk of inflammatory bowel diseases. *Hum Mol Genet* 16: 3008-16
230. Barnes BJ, Moore PA, Pitha PM. 2001. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. *J Biol Chem* 276: 23382-90
231. Yasuda K, Richez C, Maciaszek JW, Agrawal N, Akira S, Marshak-Rothstein A, Rifkin IR. 2007. Murine dendritic cell type I IFN production induced by

human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *J Immunol* 178: 6876-85
232. Park JH, Kim YG, McDonald C, Kanneganti TD, Hasegawa M, Body-Malapel M, Inohara N, Nunez G. 2007. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J Immunol* 178: 2380-6
233. McCarthy JV, Ni J, Dixit VM. 1998. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. *J Biol Chem* 273: 16968-75
234. Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416: 194-9
235. Leber JH, Crimmins GT, Raghavan S, Meyer-Morse NP, Cox JS, Portnoy DA. 2008. Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. *PLoS Pathog* 4: e6
236. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. 1998. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity* 8: 297-303
237. Lee TH, Huang Q, Oikemus S, Shank J, Ventura JJ, Cusson N, Vaillancourt RR, Su B, Davis RJ, Kelliher MA. 2003. The death domain kinase RIP1 is essential for tumor necrosis factor alpha signaling to p38 mitogen-activated protein kinase. *Mol Cell Biol* 23: 8377-85
238. Ting AT, Pimentel-Muinos FX, Seed B. 1996. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J* 15: 6189-96
239. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* 4: 387-96
240. Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA. 2005. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. *J Biol Chem* 280: 36560-6
241. Chen W, Lam SS, Srinath H, Jiang Z, Correia JJ, Schiffer CA, Fitzgerald KA, Lin K, Royer WE, Jr. 2008. Insights into interferon regulatory factor activation from the crystal structure of dimeric IRF5. *Nat Struct Mol Biol* 15: 1213-20
242. Pandey AK, Yang Y, Jiang Z, Fortune SM, Coulombe F, Behr MA, Fitzgerald KA, Sassetti CM, Kelliher MA. 2009. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. *PLoS Pathog* 5: e1000500
243. Stockinger S, Materna T, Stoiber D, Bayr L, Steinborn R, Kolbe T, Unger H, Chakraborty T, Levy DE, Muller M, Decker T. 2002. Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes. *J Immunol* 169: 6522-9
244. Stetson DB, Medzhitov R. 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24: 93-103

245. Stockinger S, Reutterer B, Schaljo B, Schellack C, Brunner S, Materna T, Yamamoto M, Akira S, Taniguchi T, Murray PJ, Muller M, Decker T. 2004. IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. *J Immunol* 173: 7416-25
246. O'Connell RM, Vaidya SA, Perry AK, Saha SK, Dempsey PW, Cheng G. 2005. Immune activation of type I IFNs by Listeria monocytogenes occurs independently of TLR4, TLR2, and receptor interacting protein 2 but involves TNFR-associated NF kappa B kinase-binding kinase 1. *J Immunol* 174: 1602-7
247. Huye LE, Ning S, Kelliher M, Pagano JS. 2007. Interferon regulatory factor 7 is activated by a viral oncoprotein through RIP-dependent ubiquitination. *Mol Cell Biol* 27: 2910-8
248. Balkhi MY, Fitzgerald KA, Pitha PM. 2008. Functional regulation of MyD88-activated interferon regulatory factor 5 by K63-linked polyubiquitination. *Mol Cell Biol* 28: 7296-308
249. Balkhi MY, Fitzgerald KA, Pitha PM. 2010. IKKalpha negatively regulates IRF-5 function in a MyD88-TRAF6 pathway. *Cell Signal* 22: 117-27
250. Lin R, Yang L, Arguello M, Penafuerte C, Hiscott J. 2005. A CRM1-dependent nuclear export pathway is involved in the regulation of IRF-5 subcellular localization. *J Biol Chem* 280: 3088-95
251. Lee FS, Peters RT, Dang LC, Maniatis T. 1998. MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. *Proc Natl Acad Sci U S A* 95: 9319-24
252. Troester MA, Hoadley KA, Sorlie T, Herbert BS, Borresen-Dale AL, Lonning PE, Shay JW, Kaufmann WK, Perou CM. 2004. Cell-type-specific responses to chemotherapeutics in breast cancer. *Cancer Res* 64: 4218-26
253. Vicente D, Montes M, Cilla G, Perez-Yarza EG, Perez-Trallero E. 2006. Differences in clinical severity between genotype A and genotype B human metapneumovirus infection in children. *Clin Infect Dis* 42: e111-3
254. Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, Sturfelt G, Jonsen A, Rantapaa-Dahlqvist S, Moller B, Kere J, Koskenmies S, Widen E, Eloranta ML, Julkunen H, Kristjansdottir H, Steinsson K, Alm G, Ronnblom L, Syvanen AC. 2005. Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. *Am J Hum Genet* 76: 528-37
255. Rueda B, Reddy MV, Gonzalez-Gay MA, Balsa A, Pascual-Salcedo D, Petersson IF, Eimon A, Paira S, Scherbarth HR, Pons-Estel BA, Gonzalez-Escribano MF, Alarcon-Riquelme ME, Martin J. 2006. Analysis of IRF5 gene functional polymorphisms in rheumatoid arthritis. *Arthritis Rheum* 54: 3815-9
256. Kristjansdottir G, Sandling JK, Bonetti A, Roos IM, Milani L, Wang C, Gustafsdottir SM, Sigurdsson S, Lundmark A, Tienari PJ, Koivisto K, Elovaara I, Pirttila T, Reunanen M, Peltonen L, Saarela J, Hillert J, Olsson T, Landegren U, Alcina A, Fernandez O, Leyva L, Guerrero M, Lucas M, Izquierdo G, Matesanz F, Syvanen AC. 2008. Interferon regulatory factor 5 (IRF5) gene

variants are associated with multiple sclerosis in three distinct populations. *J Med Genet* 45: 362-9