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Crimean–Congo Hemorrhagic Fever Virus:
interferon-induced antiviral mechanisms and immune evasion strategies

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Till min underbara familj

ABSTRACT

The *Bunyaviridae* family is the largest virus family consisting of more than 350 viruses. Despite the increasing knowledge regarding the biology of members of this family, little is known about Crimean-Congo hemorrhagic fever virus (CCHFV), a virus classified within the *Nairovirus* genus and the causative agent of a severe hemorrhagic fever in humans with high mortality. The virus is transmitted through the bites of Ixodid ticks or by direct contact with blood or tissues from infected animals. Nosocomial transmission among caregivers has also been reported. CCHFV is endemic in large parts of the world and is regarded as a public health problem in these regions. Research investigations have been limited by the requirement for specialized BSL-4 containment laboratories with high security and the lack of appropriate animal models. As a consequence, the factors determining the pathogenesis of CCHFV are largely unexplored.

Type I Interferons (IFNs) form the first line of defense against a virus infection and is indispensable for the innate immune response in vertebrates. A number of studies have demonstrated that human IFNs have an antiviral effect against a variety of viruses. In this thesis the antiviral effect of IFNs against CCHFV was investigated. In paper II it was demonstrated that IFNs have an antiviral effect against CCHFV and in paper I and II the human MxA protein was identified as a major contributor to these observed interferon-induced effects. However, MxA was found not to be the sole determinant of the observed decrease in virus replication following IFN treatment. The preliminary findings suggest that the dsRNA-dependent protein kinase PKR also contributes to the antiviral effects and most likely other proteins are involved as well.

The importance of the IFN system is illustrated by the numerous viral encoded gene products with IFN antagonistic properties targeting almost all components of the IFN response. Often these antagonists are multifunctional proteins acting on several different pathways of the immune response. In this thesis it was investigated if CCHFV, in conformity with most other viruses, had the ability to interfere with host immune response mechanisms. In paper III it was demonstrated that CCHFV delays the early immune responses, most likely by interfering with the IRF-3 pathway. This in turn has the consequence that secretion of IFNs following infection is a relatively late event.

Recently it was shown that triphosphates in the 5' terminus of viral RNA are key determinants for detection by RIG-I. In paper IV it was shown that some selected viruses, among them CCHFV, avoids RIG-I recognition by processing of their 5'termini to generate monophosphates. These findings not only show that CCHFV can interfere with host antiviral signalling, but also identifies a new strategy by which viruses avoid activation of the innate immune response. If the observed delay in immune responses following a CCHFV infection is the result of the RIG-I avoidance or the combination of avoidance and the expression of an interferon antagonist remains to be investigated.

Taken together, the findings in this thesis show that CCHFV is sensitive to the actions of IFNs and IFN-induced antiviral proteins. However, the virus avoids detection and interferon induction early in the infection and once the virus is replicating, IFN has little effect on virus replication. A better understanding of interactions between the virus and their hosts will facilitate in the future development of improved measurements in the fight against viral infections.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their roman numerals:

I. **Ida Andersson,** Bladh L, Mousavi-Jazi M, Magnusson K-E, Lundkvist Å, Haller O and Mirazimi A. (2004).
    Human MxA protein inhibits the replication of Crimean-Congo Hemorrhagic fever virus.
    J Virol 78(8):4323-4329

II. **Ida Andersson,** Lundkvist Å, Haller O and Mirazimi A. (2006).
    Type I Interferon inhibits Crimean-Congo Hemorrhagic Fever Virus in human target cells.
    J Med Virol. Feb;78(2):216-22

III. **Ida Andersson,** Karlberg H, Mousavi-Jazi M, Martínez-Sobrido L, Weber F and Mirazimi **A**. (2008).
    Crimean-Congo Hemorrhagic Fever Virus delays activation of the innate immune response.
    In press, J Med Virol.

IV. Habjan M, **Ida Andersson**, Klingström J, Schümann M, Martin A, Zimmermann P, Wagner V, Pichlmair A, Schneider U, Mühlberger E, Mirazimi A, and Weber F. (2008).
    Processing of genome 5' termini as a strategy of negative-strand RNA viruses to avoid RIG-I-dependent cytokine induction
    In press, PLoS ONE

    Preliminary results
    Ida Andersson and Mirazimi A. (2008)
    The role of PKR and RNaseL for controlling the replication cycle of Crimean-Congo Hemorrhagic Fever Virus.
    Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADAR-1	RNA specific adenosine deaminase-1
BSL-4	Biosafety level 4
BUNV	Bunyamwera virus
CARD	Caspase reqruitment domain
CARDIF	CARD-adaptor-inducing IFN-β
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
DC	Dendritic cell
DIC	Disseminated intravascular coagulopathy
dsRNA	Double-stranded RNA
DUGV	Dugbe virus
EBOV	Ebola virus
eIF2-α	Eukaryotic initiation factor 2-α
EMCV	Encephalomyocarditis virus
FLUAV	Influenza A virus
GAF	Interferon-γ activated factor
GAS	Interferon-γ activated sequence
GBP-1	Guanylate-binding protein-1
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus-1
HTNV	Hantaan virus
Huh-7	Human hepatoma cells-7
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IFNAR	type I IFN receptor
IL	Interleukin
IPS-1	IFN-β promoter stimulator-1
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor-3
ISRE	Interferon-stimulated regulatory element
Jak	Janus kinases
kDa	kilo dalton
LACV	La Crosse virus
MAVS	Mitochondrial antiviral signalling protein
MDA5	Melanoma differentiation antigen 5
NFκB	Nuclear Factor Kappa B
NiV	Nipah virus
NK cells	Natural killer cells
NLS	Nuclear localization signal
NP	Nucleocapsid protein
NSDV	Nairobi sheep disease virus
NSm	Non-structural protein, M segment
NSs	Non-structural protein, S segment

OAS	Oligoadenylate synthetase
OTU	Ovarian tumor domain
PAMP	Pathogen associated molecular pattern
pDCs	Plasmacytoid dendritic cells
PKR	dsRNA dependent protein kinase
PML	Promyelotic leukaemia protein
PRR	Pattern recognition receptor
RIG-I	Retinoic inducible gene-I
RNAPII	RNA polymerase II
RVFV	Rift Valley fever virus
SARS	Severe acute respiratory syndrome
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
TBE	Tick borne encephalitis
THOV	Thogoto virus
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VISA	Virus induced signalling adaptor
VSV	Vesicular stomatitis virus
2-5 OAS	2'-5' oligoadenylate synthetase
2-5A	2'-5' linked adenylates

BACKGROUND

Introduction

Within the last years there has been an increasing awareness that new diseases are still erupting into our environment at regular intervals. We call these diseases "emerging" infectious diseases and they can be defined as "diseases of infectious origin whose incidence in humans has either increased or threatens to increase in the near future". These infectious agents are often extremely virulent and recent examples include the SARS-Coronavirus and the high pathogenic avian influenza. Increased travelling, escalating population growth with expanding poverty, dramatic changes in the society and in climatic conditions have the consequence that no nation can be completely safe when it comes to human vulnerability to infectious diseases.

During the last decade there have been a growing number of reports describing large outbreaks of an emerging virus named Crimean-Congo hemorrhagic fever virus (CCHFV) in the community or at hospital settings in several parts of Europe. The possible use of CCHFV as a bioterrorism agent, its capacity of human-to-human transmission together with the lack of vaccines and good antiviral treatments makes the virus an important pathogen in a public health perspective. Handling of CCHFV requires specialized BSL-4 containment laboratories, with the highest levels of safety measures. This restriction, together with the lack of an appropriate animal model, have hampered the number of research investigations and resulted in poor knowledge about the virus.

The general aim with this thesis have been to increase the knowledge concerning the pathogenesis of CCHFV by studying the role of interferons in controlling the infection as well as immune evasion strategies adopted by the virus. A better understanding of the interference of CCHFV with innate immune mechanisms is critical for the future design of antiviral treatments and therapies.

Crimean-Congo hemorrhagic fever virus

History

In the 12th century a disease, today considered to be Crimean-Congo hemorrhagic fever, was described in the geographical region now corresponding to Tadzhikistan. It was described as an hemorrhagic disease resulting in the presence of blood in the urine, gums, rectum, vomitus and abdominal cavity and was thought to be transmitted by a louse or a tick [Hoogstraal, 1979]. In modern medicine the disease was first described during the investigation of an outbreak among soviet military personnel on the Crimean peninsula in 1944-1945, which resulted in the name Crimean hemorrhagic fever (CHF). Subsequently it was found that the infectious agent was indistinguishable from a virus found in human isolates from Congo and Uganda [Simpson et al., 1967; Woodall et al., 1967] and the name Crimean-Congo Hemorrhagic fever (CCHF) was established.

Classification and structure

CCHFV is classified within the *Nairovirus* genus of the family *Bunyaviridae*, a large virus family comprising over 350 arthropod-borne viruses. The other genera of the family include *Orthobunyavirus, Phlebovirus, Hantavirus* and *Tospovirus* (Table 1). Thirty-four described viruses are found within the *Nairovirus* genus and these viruses are further classified into seven serogroups. The only three members of this genus known to cause disease in humans are CCHFV, Dugbe virus (DUGV) and Nairobi sheep disease virus (NSDV).

Virions within the family are spherical, approximately 100 nm in diameter and contain a lipid bilayered envelope [Whitehouse, 2004]. The genome is composed of three single stranded RNA segments of negative sense, designated the small (S), medium (M) and large (L) segments. The S and the M segments together encode three structural proteins: a nucleocapsid protein (NP) on the S segment and two envelope glycoproteins on the M segment, named Gn and Gc in regards to their relative location to the amino or carboxy terminus. The L segment encodes a viral RNA-dependent RNA polymerase [Schmaljohn and Hooper, 2001] (Figure 1). Some

Genus	Virus
Orthobunyavirus	Bunyamwera (BUNV)
	La Crosse virus (LACV)
Phlebovirus	Rift Valley fever virus (RVFV)
	Uukuniemi virus (UUKV)
	Sandfly Sicilian fever virus (SFSV)
Nairovirus	Crimean-Congo hemorrhagic fever virus (CCHFV)
	Dugbe virus (DUGV)
	Nairobi sheep disease virus (NSDV)
Hantavirus	Hantaan virus (HTNV)
	Puumala virus (PUUV)
	Sin Nombre virus (SNV)
Tospovirus	Tomato Spotted Wilt virus (TSWV)

Table 1. *Representative members of the five different genera in the Bunyaviridae family.*

members of the family also encode a non-structural protein on their S segments, termed NSs, and/or a non-structural protein on their M segments, termed NSm.

The RNA segments are complexed with the nucleocapsid protein to form individual S, M and L ribonucleocapsids [Bishop, 1996]. Base-pairing of the terminal nucleotides is predicted to form a stabile panhandle structure, shaping the ribonucleocapsids into non-covalently closed circular structures. For a virion to be infectious it must contain at least one ribonucleocapsid of each segment, though the number of nucleocapsids may vary between virions [Schmaljohn and Hooper, 2001].

Genome and replication

The viral glycoproteins are believed to recognize the receptor on target cells before the virus is internalized by receptor mediated endocytosis. The cellular receptor for CCHFV is currently unknown. Viral replication of the CCHFV genome occurs in the
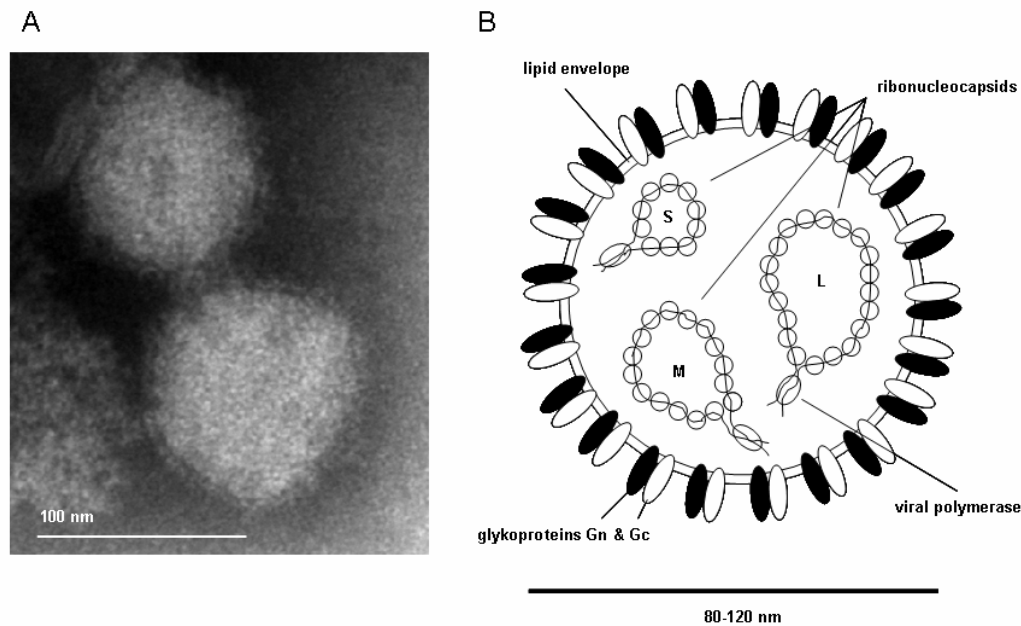
Figure 1. *Electron microscopy image of CCHFV particles (by Kjell-Olof Hedlund) (A) and a schematic drawing of the CCHFV virion (B).*

cytoplasm and virion maturation is believed to occur by budding through the endoplasmatic reticulum into cytoplasmatic vesicles in the Golgi region. These vesicles subsequently fuse with the plasma membrane to release the virions [Whitehouse, 2004].

The NP encoded on the S segment comprises 482 amino acids and is the most abundant protein in infected cells. NP interacts with the viral RNA and together they form the ribonucleocapsids. The interaction of the NP with the RNA is not completely understood. During infection, the NP is targeted to the perinuclear region of infected cells in the absence of native RNA segments in an actin dependent manner [Andersson et al., 2004b].

The M-segment of the nairoviruses encodes the glycoproteins and are 30-50% larger than corresponding segment found in other genera [Elliott, 1990]. The glycoproteins are encoded as a polyprotein, which initially undergo proteolytic cleavages to yield two precursor proteins, pre-Gn (140 kDa) and pre-Gc (85 kDa). These proteins are further processed to generate the mature glycoproteins Gn (37 kDa) and Gc (75 kDa), which form the major structural glycoprotein components of the virus protruding from the envelope [Sanchez et al., 2002]. The cleavages also generate the secreted

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glycoproteins GP38, GP85 and GP160 and possible also a mucin-like protein [Sanchez et al., 2006]. In addition, it was recently shown that CCHFV encodes an NSm protein, generated through C-terminal cleavage of the pre-Gn [Altamura et al., 2007]. The functional implication of this protein is however not yet known.

The site of budding is defined by retention of the glycoproteins in the Golgi membranes [Haferkamp et al., 2005]. It has been found that the Gn and Gc have to interact and form hetero-oligomers for proper transport of both proteins to Golgi. Without the presence of Gn, individually expressed Gc is retained in the ER.

The RNA dependent RNA polymerase, encoded on the L segment, is the largest of the viral proteins and responsible for replication and transcription of the vRNA and cRNA. The protein is composed of 3944 amino acids and contains an ovarian tumor (OTU)-like protease motif in the N-terminus and a RNA polymerase catalytic domain in the central part of the protein [Frias-Staheli et al., 2007; Honig et al., 2004; Kinsella et al., 2004]. The L genome segment of CCHFV is twice the size compared to L segments from other bunyaviruses.

Manifestation of disease

Humans are the only known host, besides newborn mice, in which disease is manifested and a human infection often results in a severe hemorrhagic fever [Whitehouse, 2004]. The typical course of CCHF chares many characteristics with other viral hemorrhagic fever viruses. The progression of disease is rapid and can be divided into four distinct phases, the incubation, the pre-hemorrhagic, the hemorrhagic and the convalescence phases. The incubation period varies between 1 to seven days, depending on the route of transmission. The incubation time following a tick bite can be as short as 1-3 days and appears to be somewhat longer after contact with infected tissues and in cases of nosocomial transmission [Hoogstraal, 1979; Swanepoel et al., 1987]. The incubation time has been found to be shorter in fatal cases, perhaps due to exposure of a higher viral dose [Nabeth et al., 2004].

The pre-hemorrhagic period lasts on average 3 days and starts with an abrupt onset of fever, myalgia, dizziness, headache and vomiting. [Bakir et al., 2005; Hoogstraal, 1979; Swanepoel et al., 1989].

The hemorrhagic period that follows is short and characterized by bleedings from various sites. The most common reported sites of hemorrhage are the nose, gastrointestinal system, urinary and respiratory tract [Ergonul, 2006]. Enlarged spleen

and liver are reported to occur in one third of the patients [Hoogstraal, 1979]. The average case fatality rate ranges are often cited at 10% to 50% and most deaths occur during the second week of illness [Nichol, 2001]. The mortality rate appears to be higher following a nosocomial infection than after a tick bite and the reason for this may simply be explained by differences in viral doses. The level of viremia is also important when predicting the prognosis of the disease. A high virus titer, exceeding 10^9 genomes per ml of plasma, is more often associated with fatal cases than lower levels of virus [Cevik et al., 2007]. Furthermore, in fatal cases there is little evidence of antibody responses.

The convalescence in survivors starts 10-20 days after onset of illness and typical sequelae are weakness, loss of hair, headache, dizziness, nausea, loss of hearing, loss of memory and poor appetite. Some of these complaints may persist for a year or more. Relapse of the infection does not occur [Whitehouse, 2004]. It must be noted that the durations and symptoms in the phases mentioned, may vary significantly between different individuals.

Pathogenesis

The pathogenesis of CCHFV is poorly understood. However, in conformity with other hemorrhagic fever virus infections, it has been observed that the endothelium plays an important role in CCHFV infections. "Capillary toxicosis" was in fact the name used by soviet scientists for CCHF [Hoogstraal, 1979]. Endothelial damage would account for the characteristic rash and contribute to platelet aggregation and activation of the coagulation cascade. Fatal cases of CCHF show a strong coagulation system dysfunction early in the infection with a high score for disseminated intravascular coagulation (DIC) as well as increased levels of pro-inflammatory cytokines such as IL-6 and TNF-α [Ergonul et al., 2006].

The characteristic damage of endothelial cells observed in hemorrhagic fevers may either be a direct result of the virus infection or caused by host immune responses [Schnittler and Feldmann, 2003]. For Ebola virus, much of the cellular damage and coagulopathy induced during infection are the result of host induced responses [Geisbert et al., 2003]. For Dengue virus the release of cytokines cause redistribution of tight junction proteins and vascular leakage [Talavera et al., 2004]. For CCHFV, no direct effects on the integrity of tight junctions in MDCK-1 cells have been

observed [Connolly-Andersen et al., 2007], but the possible role of immune mediated effects during the course of a CCHFV infection remains to be elucidated.

Similarities are today observed between various hemorrhagic fever viruses and the septic shock caused by certain bacterial strains [Bray and Mahanty, 2003; Geisbert et al., 2003; Mahanty and Bray, 2004]. It has been proposed that the cytokine storm found in viral hemorrhagic fever patients is similar to the lipopolysackaride induced shock that occurs following infections with gram-negative bacteria [Geisbert and Jahrling, 2004].

Epidemiology

CCHFV usually circulates unnoticed in nature in an enzoonotic tick-vertebrate-tick cycle. In endemic areas of Europe, Africa and Asia, viremia and antibody production has been documented in a long list of domestic and wild vertebrates including cattle, horses, sheep, goats, pigs, camels, donkeys, hedgehogs, mice and dogs [Nalca and Whitehouse, 2007], but there is no evidence that the virus actually causes disease in these animals. Reptiles and birds, with the exception of ostriches, appears to be refractory to infection [Vorou et al., 2007]. Birds may however play a role in the transportation of CCHFV infected ticks between different countries. Migration of birds have for example been suggested to be the cause of the 2002 outbreak in Turkey [Karti et al., 2004]. Infected ticks may in addition be transferred from endemic to non-endemic areas by the trade of livestock.

CCHF is endemic in large parts of the world and has one of the most extensive geographic ranges of the tick-borne viruses causing disease. The virus and/or the disease have been reported from more than 30 countries in Africa (Uganda, South Africa, Tanzania, Nigeria, Senegal, etc.), Asia (Pakistan, China, Kazakhstan, etc.) and the Middle East (Iran, Iraq, Oman, Saudi Arabia, etc.). CCHFV is mainly transmitted by the Ixodid tick, particularly from the genus *Hyalomma*, and the known geographic occurrence of CCHF coincides well with the global distribution of the ticks. Virus have been isolated from both eggs and unfed immature stages of ticks, showing evidence of both transovarial (from adult to the egg stage) and transstadial (from larvae to nymph to adult) transmission [Watts et al., 1988].

In an epidemiological view, CCHF cases are mainly found in farmers, abattoir workers and veterinarians, working groups regularly exposed to ticks. Viremic blood from infected domestic animals also provides a great risk during slaughter. There is

however no risks associated with the meat itself due to post-slaughter acidification of the tissues. The male to female ratio of reported cases varies between countries and reflects the distribution of the respective sex in the professions having most contact with animals [Ergonul, 2006].

The potential for human-to-human transmission of CCHF put health care workers in great danger. Nosocomial outbreaks with high mortality have repeatedly been reported in hospitals, coinciding with outbreaks in the general population [Ergonul, 2006]. The most dangerous situations are associated with the caretaking of patients which have not yet been diagnosed with CCHF, especially if the patient is in the need of surgery or is bleeding from the gastrointestinal tract [Shepherd et al., 1985].

In areas with a temperate climate, CCHF cases normally occur from spring to early autumn when the activity of the ticks are high [Papa et al., 2002; Papa et al., 2004]. Changes in the climate may pose a threat in the future, since a higher temperature may increase the reproduction of the tick population and result in a higher incidence of tick-borne infections [Gubler et al., 2001].

Prevention and control

The different options for treatment of CCHF are limited and general supportive therapy is often the best approach. Early remedies include administration of thrombocytes, fresh frozen plasma, erythrocyte preparations and the use of the nucleoside analogue Ribavirin.

Ribavirin is the only drug that has been used in the treatment of viral hemorrhagic fevers, including Lassa fever and CCHF [Ergonul, 2007]. Ribavirin has been found to have antiviral activity against CCHFV *in vitro* when the drug was tested against isolates from Nigeria, Uganda, China And South Africa [Paragas et al., 2004]. The drug has also been found to have an effect *in vivo*. In a study done in suckling mice, Ribavirin reduced virus growth in the liver, reduced mortality and extended the mean time to death [Tignor and Hanham, 1993]. However, no randomised clinical trials have been performed for the use of Ribavirin to treat CCHF, but promising results have been obtained in the treatment of many infected patients [Fisher-Hoch et al., 1995; Mardani et al., 2003; Tang et al., 2003].

There are no effective vaccines available for CCHF. Nevertheless, experimental vaccines have been reported and has shown high antibody levels when distributed in human volunteers [Whitehouse, 2004]. Recently, DNA vaccines for CCHFV, Tick

borne encephalitis virus (TBEV), Rift Valley fever virus (RVFV) and Hantaan virus (HTNV) were tested in mice, either individually or in combination [Spik et al., 2006]. The DNA vaccine expressing CCHFV Gn and Gc elicited antibodies, but its protective efficacy could not be demonstrated due to the lack of challenge animal models for CCHFV.

Antibodies towards CCHFV are typically not observed until 5-9 days after the onset of illness, and are in general not found at all in patients who succumb to the disease. Therefore, the use of antibodies from survivors or animals might be of therapeutic importance. Possible benefits have been reported [Hoogstraal, 1979], but further studies are required to assess the usefulness.

Host responses to viral infections

Innate immune responses

We encounter potential pathogens routinely, but often our innate or unspecific immune response is sufficient to control the infection and prevent us from getting ill. This non-specific immune system includes anatomical barriers (such as the skin, the low pH of the gastrointestinal tract and elevated body temperature in cases of fever), secreted molecules (for example interferons, cytokines and nitric oxide) and cellular factors (i.e. NK cells, dendritic cells and macrophages). In cases when these initial innate defences are breached, our acquired, adaptive immune response is stimulated to mediate antigen specific responses with the property of protection against re-infection by the same pathogen.

The interferon (IFN) system is one of the major players in the innate defence against all kinds of viruses. Infected cells synthesize and secrete IFNs that circulates the body in order to induce an antiviral state in uninfected cells to prevent further growth and spread of these dangerous intruders.

Interferons

IFNs were first described when Isaacs and Lindenmann in 1957 discovered that a secreted factor from influenza virus infected chick cells could transfer a virus-resistant state to other cells [Isaacs and Lindenmann, 1957]. Since then the IFN system has been studied in extensive detail.

The IFNs are a multigene family of inducible cytokines [Roberts et al., 1998; Stark et al., 1998] and can be grouped into three subtypes; type I, type II and type III, based on their sequence and recognition by specific receptors. Type I includes 13 subtypes of IFN-α, one subtype of IFN-β and others such as IFN-ε, -κ, -ω and –τ. Type II includes IFN-γ and type III the recently discovered IFN-λ, [Ank et al., 2006; Pestka et al., 2004]. IFN-λ can be subdivided in IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B).

Production of IFN is rapidly induced following a virus infection. IFN-α and -β can be produced by all nucleated cells, with plasmacytoid dendritic cells (pDCs) as the main producer [Asselin-Paturel and Trinchieri, 2005]. IFN-γ is synthesized only by certain cells of the immune system including natural killer (NK)-cells, CD4 Th1 cells and

cytotoxic suppressor cells [Young, 1996]. All studies investigating IFN-λ has so far indicated a simultaneous expression of IFN-λ and type I IFNs, but many questions about IFN-λ still remains to be answered [Ank et al., 2006].

Recognition of viral infection and interferon induction

Prior to the year 2000 no receptors that linked recognition of viral structures to the induction of IFNs had been identified. After years of intense research it is now clear that two receptor systems, recognizing nucleic acids, are responsible for most of the virus detection and subsequent interferon induction. The receptors are referred to as "pattern recognition receptors" (PRR), recognizing highly conserved "pathogen associated molecular patterns" (PAMPs) on the surface or within the microbes.

Extracellular and endosomal recognition

One of the PRR systems is the Toll-like receptors (TLRs), recognizing different types of nucleic acid, enough to cover detection of almost all types of viruses. The most important TLRs for virus recognition are TLR-3 (recognizing dsRNA, a common feature of RNA as well as DNA viruses), TLR-7 and TLR-8 (recognizing ssRNA and viruses with ssRNA genomes) and TLR-9 (recognizing unmethylated CpG motifs common in DNA viruses) [Akira and Takeda, 2004]. The TLRs are primarily expressed by dendritic cells (DCs) and macrophages and activation of TLR-3, -7 and -9 in these cells can induce expression of enormous amounts of IFN. TLR-7 and -9 are found intracellular in the endosomal compartment and TLR-3 in the plasma membrane as well as in the endosomes. Hence, these receptors recognize extracellular virus material or viral nucleic acids generated by uncoating and degradation of incoming viruses. This feature enables the development of an IFN response without the need of viral replication. The intracellular TIR domain of TLR-3, -7 and -9 recruits cytoplasmic signalling molecules to activate down-stream signalling pathways. TLR-3 uses TRIF and TLR-7 and -9 utilize MyD88 as adaptor proteins and down-stream signalling ultimately leads to transcriptional activation of the IFN-β promoter by NFκB and IRFs.

Intracellular recognition

Intracellular recognition is managed by the other receptor system, consisting of two cytosolic receptors, the caspase recruitment domain (CARD)-containing RNA helicases retinoic inducible gene-I (RIG-I) [Yoneyama et al., 2004] and melanoma differentiation antigen 5 (MDA5) [Andrejeva et al., 2004]. RIG-I and MDA5 share similar signalling features and structural homology [Yoneyama et al., 2005], but recent findings suggest that these two helicases discriminate between different viral ligands to initiate innate immune responses. Signalling through RIG-I is triggered by a number of RNA viruses, as well as by synthetic, in vitro transcribed RNA. In addition, RIG-I has recently been found to recognize RNA bearing 5'triphoshorylated ends [Hornung et al., 2006; Pichlmair et al., 2006]. By contrast, MDA-5 is triggered by picornaviruses and the synthetic RNA polymer poly I:C [Loo et al., 2008]. A third member of the helicase family, LGP2 has also been described. This protein lacks the CARD domain and possibly functions as a negative regulator of IFN production [Yoneyama et al., 2005].

Four groups independently and simultaneously identified the link between the RIG-I and MDA5 and downstream signalling molecules, a CARD-containing adaptor protein named IFN-β promoter stimulator-1 (IPS-1) [Kawai et al., 2005], mitochondrial antiviral signalling protein (MAVS) [Seth et al., 2005], virus induced signalling adaptor (VISA) [Xu et al., 2005] or CARD-adaptor-inducing IFN-β (CARDIF) [Meylan et al., 2005]. This protein has been found to be linked to the mitochondrion, an interaction essential for its function, and it will therefore be referred to as MAVS in this text. MAVS function downstream of RIG-I and MDA5 and upstream of NFκB and IRF phosphorylation [Seth et al., 2005].

Interferon-β promoter activation

Both signalling through TLRs, as well as through RIG-I/MDA5, ultimately lead to the activation of a limited set of transcription factors. The most important ones are NFκB, IRF-3 and ATF-2/c-Jun. NFκB is normally held in the cytoplasm in an inactive state by inhibitory proteins. Upon virus infection these inhibitors are degraded and NFκB can be translocated into the nucleus [Sharma et al., 2003]. Similarly, IRF-3 also resides in the cytoplasm, but upon viral challenge IRF-3 is phoshorylated by the kinases TBK-1 and Iκκε, dimerized and translocated to the nucleus [Fitzgerald et al., 2003].
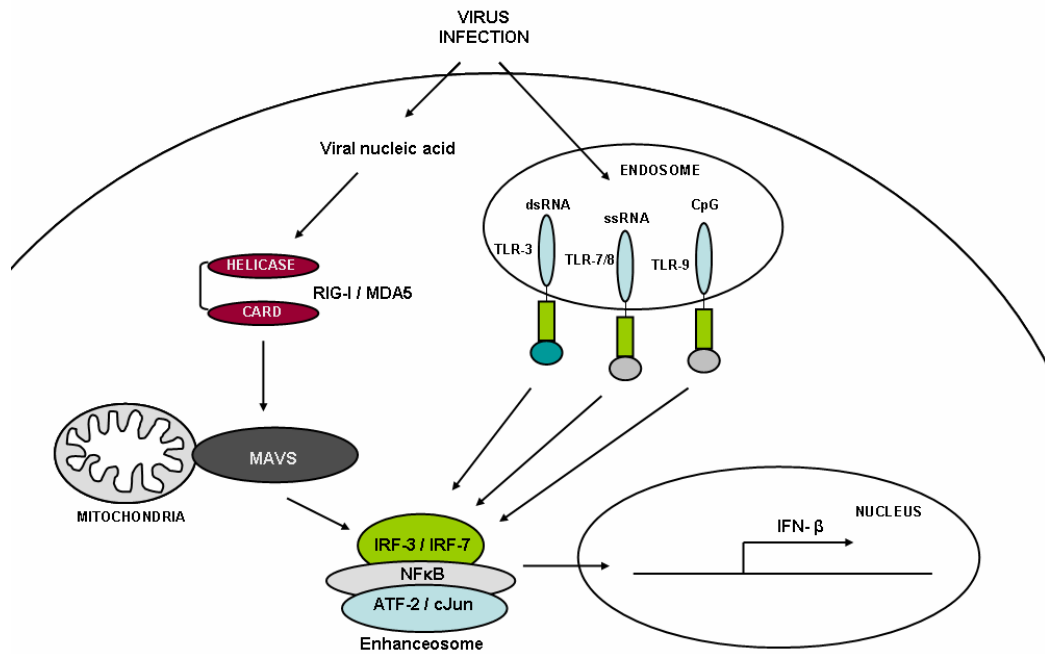
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Figure 2. *A schematic summary of the two main pathways for recognition of viral infection, namely the RIG-I/MDA5 and the TLR pathways, and the subsequent induction of the IFN-β promoter. For details, see text.*

ATF2/c-Jun is found in the nucleus and activated upon phoshorylation by stress kinases. When activated, these three factors form a multiprotein complex, called the enhanceosome [Maniatis et al., 1998], which assembles around the IFN-β promoter to promote transcriptional activation. Viral recognition and IFN-β promoter induction is summarized in figure 2.

The TLR-pathway operates mainly in pDCs to detect viral nucleic acids in endocytosed material while RIG-I is found in most other cell types [Seth et al., 2006]. This indicates that RIG-I and TLR-pathways are not redundant, but instead have evolved independently to mediate antiviral defences in different cell types and against different viruses [Kato et al., 2005].

Interferon signalling

All three classes of IFNs signal through distinct receptors. Type I IFNs signal through the IFN-α/βR or, a homodimer consisting of the two subunits IFN-αR1 and IFN-αR2, while IFN-γ (Type II) uses a tetrameric receptor composed of two IFNGR1 and two

IFNG2 subunits. Finally, the type III IFNs, IFN-λ uses a receptor complex consisting of IL-10R2 and IL-28R1 chains.

The different types of IFNs share the same basic downstream signalling patterns, namely the Jak-Stat pathway. Simplified, upon binding of the IFN to its receptor, Janus kinases (Jaks) are activated to phosphorylate their downstream substrates, members of the Stat family of signal transducers and activators of transcription, most commonly Stat1 and Stat2. Tyrosine phoshorylation of these Stats, lead to the formation of two main transcriptional activator complexes, the IFN-stimulated gene factor 3 (ISGF3) and the IFN-γ activated factor (GAF). In general, type I IFNs activates the formation of ISGF3 more strongly than type II IFNs, while type II IFNs mainly induces GAF activation. The ISGF3 and GAF complexes are then translocated to the nucleus where they bind to their specific DNA sequences containing specific motifs, IFN-stimulated regulatory elements (ISRE) or IFN-γ-activated sequence (GAS) respectively. The binding to these motifs stimulate the transcriptional activation of a large number of interferon stimulated genes (ISGs) to initiate biological activities such as induction of an antiviral state [Pestka et al., 2004; Takaoka and Yanai, 2006] The downstream signalling pathways of IFN-λs remain to be investigated in further detail (Figure 3).

One of the earliest synthesized ISGs is IRF-7. When this transcription factor is transcribed and phoshorylated by TBK-1 and Iκκε kinases, it regulates transcription of all the IFN-α genes to induce production of a full range of type I IFNs. Plasmacytoid dentritic cells (pDCs) produce high levels of type I IFNs and are considered to be the main producer in response to viral infection [Colonna et al., 2004]. pDCs recognize viruses mainly via TLR-7, TLR-8 and TLR-9 located in the endosomes and downstream signalling from these TLRs leads to the activation of IRF-7. The pDCs express high levels of IRF-7 constitutively, in contrast to the normal inducible expression in other cell types. This constant expression of IRF-7 is considered to be the major factor for the rapid and potent induction of a full-blown interferon response by these cells.

IFNs activate the expression of several hundred ISG products which have antiviral, antiproliferative, and immunomodulatory functions. Among the best studied IFN-induced antiviral proteins we find the Mx-proteins, the dsRNA-dependent protein kinase (PKR) and the $2',5'$ oligoadenylate synthetases 2-5 OAS / RNaseL system. Other frequently mentioned proteins with potential antiviral effects includes P56 [Hui et al., 2003], ISG20 [Espert et al., 2003], promyelotic leukaemia protein (PML)

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[Regad and Chelbi-Alix, 2001], ISG15 [Lenschow et al., 2007] and guanylate-binding protein 1 (GBP-1) [Anderson et al., 1999].
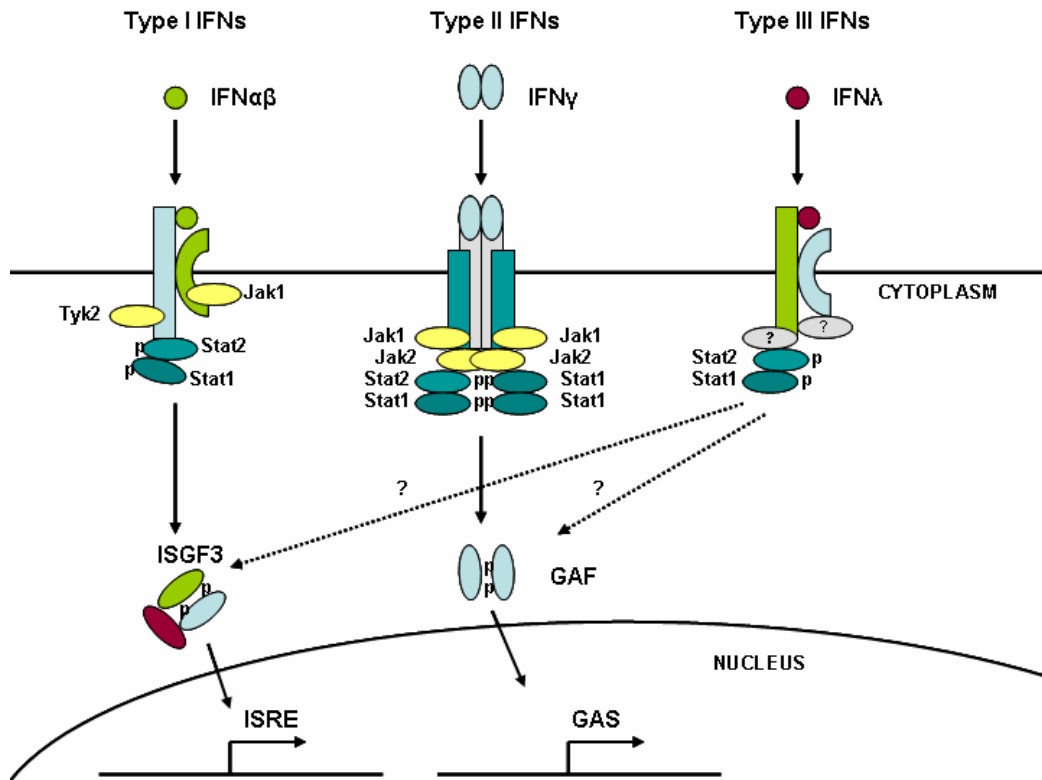

Figure 3. *Signalling though the Jak-Stat pathway by the Type I, II and III IFNs.*

Interferon induced antiviral proteins

Mx-proteins

The Mx-proteins were discovered 25 years ago when an inbred mouse strain showed an unusual high degree of resistance against influenza A virus (FLUAV) [Horisberger et al., 1983]. Later it was revealed that this resistance was mediated by a single gene, called Mx1, located on chromosome 16 [Reeves et al., 1988]. The mouse genome also contains a second Mx gene, Mx2, closely linked to Mx1 and mapped on the same chromosome [Staeheli and Sutcliffe, 1988]. The expression of the Mx1 gene is transient and rapidly induced in the nucleus upon stimulation with IFNs type I (IFN-α and -β) [Haller, 1981] while Mx2 is expressed in the cytoplasm. Interestingly, the antiviral specificity of rodent Mx-proteins coincide with the cellular location of the proteins. The nuclear Mx1 protein provides resistance against FLUAV and Thogoto virus, two viruses that replicate in the nucleus. Instead the cytoplasmic Mx2 protein confers resistance against viruses replicating in the cytoplasm, such as viruses belonging to the *Bunyaviridae* family [Haller et al., 1998]. Notably, most of the inbred mouse strains carry defective Mx1 alleles and are highly susceptible against infection by mouse adapted FLUAV [Staeheli et al., 1988].

Today we know that Mx-proteins are present in a number of species, including humans. The human homologue to Mx1 is called MxA and is localized to the cytoplasm. A second related cytoplasmic Mx protein, termed MxB, has also been identified. MxA and MxB are mapped on the human chromosome 21, which is homologous to chromosome 16 in mice [Horisberger et al., 1988]. Gene expression of both proteins is normally silent, but rapidly induced upon stimulation with IFN type I or type III [Kotenko et al., 2003; Meager et al., 2005]. In contrast to the mouse Mx1 protein, human MxA has an antiviral activity against a broad range of RNA and DNA viruses irrespective of their intracellular replication site, including bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, picornaviruses, and Hepatitis B virus, a DNA virus with a genomic RNA intermediate [Andersson et al., 2004a; Bridgen et al., 2004; Chieux et al., 2001; Frese et al., 1996; Frese et al., 1995; Gordien et al., 2001; Haller et al., 1998; Kanerva et al., 1996; Landis et al., 1998; Peltekian et al., 2005]. However, the human MxB protein lacks detectable antiviral activity.

The antiviral mechanisms of MxA have been studied extensively during the recent years, but are still not completely understood. MxA is thought to act by binding to essential virus components and prevent them from participating in their normal functions. For La Crosse virus (family *Bunyaviridae*), MxA was found to physically interact with the nucleocapsid component of the viral nucleocapsids, forming protein complexes in the perinuclear area, thereby preventing the nucleocapsid from taking part in the process of viral RNA synthesis [Kochs et al., 2002b]. It is reasonable to speculate that a similar mechanism can be applied for MxA in the antiviral effect against other viruses replicating in the cytoplasm. In the case of orthomyxoviruses, replicating in the nucleus, MxA multimers are instead thought to recognize nucleocapsids in the cytoplasm and prevent their normal transport into the nucleus [Kochs and Haller, 1999].

The Mx-proteins belongs to a family of conserved high molecular weight GTPases, and to the superfamily of dynamin-like GTPases, which are normally involved in intracellular transport processes [Sever et al., 2000]. The Mx-proteins have a relatively low affinity for GTP and a high rate of intrinsic GTP hydrolysis [Staeheli et al., 1993]. The Mx proteins have a GTPase domain in their N-terminus, a central interactive domain and an effector domain in the C-terminus. The nuclear localization signal (NLS) present in some of the rodent forms of Mx is localized to the C-terminus [Haller et al., 2007].

The dynemin-like GTPases are known to self assemble into highly ordered oligomers. This feature is shared by the Mx proteins, which have been found to assemble into ring-like and helical structures [Kochs et al., 2002a; Melen et al., 1992; Nakayama et al., 1993]. This assembly appears to be critical for the GTPase activity, stability and recognition of viral target structures. Mouse Mx1 and human MxA both show a typical granular staining pattern in the nucleus or cytoplasm in IFN treated cells. These granules are thought to consist of Mx multimers, which provide stability and storage for the proteins. Monomeric forms of MxA is rapidly degraded [Janzen et al., 2000].

The importance of the Mx-proteins in the antiviral defence has been demonstrated in several mouse models. By disrupting the Mx1 gene the mice died rapidly following challenge with mouse adapted FLUAV [Haller, 1981]. The importance of MxA was proven by transgenically introducing MxA into mice lacking endogenous Mx expression. This turned these animals resistant to challenge with Thogoto virus (THOV), and less susceptible against FLUAV and Vesicular stomatitis virus (VSV)

[Pavlovic et al., 1995]. Furthermore, MxA was found to be a powerful interferon induced antiviral agent on its own when IFNAR-/- animals could regain their resistance against THOV, La Crosse virus and Semliki Forest virus, when constitutive MxA expression was introduced [Hefti et al., 1999].

PKR

The double-stranded RNA dependent protein kinase (PKR) is an enzyme with multiple effects which plays a critical role in antiviral host defence mechanisms [Garcia et al., 2006]. PKR has a translational regulatory function and is a member of the alpha subunit of eukaryotic initiation factor 2 (eIF2-α)-specific kinase subfamily [de Haro et al., 1996]. As might be expected for an inhibitor of translation, PKR is associated to ribosomes [Zhu et al., 1997].

In non-stressed cells PKR normally resides in the cytoplasm in a monomeric state and acts as a censor of stress signals such as dsRNA. PKR can be activated in response to dsRNA of cellular, viral or synthetic (poly I:C) origin with a size greater than 30 bp. The dsRNA molecules are recognized and bound to the N-terminal of PKR, which lead to activation of the protein through autophoshorylation and homodimerization. Once activated, the PKR function as a serine/threonine kinase and phoshorylates eIF2 on its α-subunit. In mammals, eIF2 normally delivers Met-tRNAi to the ribosomes to initiate translation in a GTP dependent manner. Once translation is initiated, eIF2 is released from the initiation complex and GTP is hydrolysed. Inactive eIF2-GDP molecules are then regenerated to eIF2-GTP molecules in a reaction catalysed by eIF2B. When eIF2 is phoshorylated at residue S-51 on the α-subunit, it binds strongly to the catalysator eIF2B, thereby preventing GTP regeneration by eIF2B and inhibiting translation. PKR is also activated by other factors than dsRNA, such as oxidative stress, cytokines and growth factors [Garcia et al., 2006].

The importance of PKR is shown by the long list of viral antagonists targeting this protein. These antagonists act by interfering with PKR activation, inhibiting PKR dimerization, synthesizing PKR pseudosubstrates or by degrading PKR. Excellent examples of PKR antagonists are the E3L protein of Vaccinia virus (binds dsRNA and directly interacts with PKR), NS1 protein of FLUAV (sequesters dsRNA and directly interacts with PKR) and the Tat protein of HIV-1 (a PKR pseudosubstrate which also directly interacts with PKR) [Garcia et al., 2006].

In addition to its functions in translational inhibition, PKR operates as a PRR for dsRNA and is involved in signal transduction through the IκB/NFκB pathway [Kumar et al., 1994]. Furthermore, PKR can act as a mediator of virus induced apoptosis [Balachandran et al., 2000].

2-5 OAS / RNAse L

Cellular and extracellular exonucelases are important proteins in the protection against pathogens. The first IFN-activated RNase to be discovered was RNAse L, a cytosolic endoribonuclease, present in most cell types and activated by short oligoadenylates produced by the 2'-5' oligoadenylate synthetase (2-5 OAS). 2-5 OAS is activated by dsRNA produced during viral infection, and converts ATP to pyrophosphate and 2'-5' linked adenylates (2-5A) [Kerr and Brown, 1978]. The 2-5As are recognized and bound in the cytoplasm by RNAse L, converting the monomeric enzyme from a latent form into a potent dimeric exoribonucleas, resulting in the degradation of single stranded viral and cellular RNAs. RNAse L is activated by subnanomolar concentrations of 2-5A resulting in cleavage of ssRNA [Floyd-Smith et al., 1981]. IFN signalling induces transcription of 2-5 OAS gene due to an ISRE in the promoter [Rutherford et al., 1988] and hence, cells exposed to IFNs have elevated levels of 2-5 OAS, contributing to the induction of an antiviral state.

Many, but not all, RNA and DNA viruses are inhibited by RNAse L [Silverman, 2007]. The antiviral effect of RNAse L occurs through a combination of mechanisms with the cleavage of RNA substrates as the common determinant. Besides the direct antiviral effects, RNAse L also mediates apoptosis and regulates cell proliferation.

ISG20

There are evidence of alternative antiviral pathways beyond MxA, PKR and RNAse L. This has been illustrated by the fact that mice, with deficiencies in all three pathways, still are able to mount a limited antiviral defence [Zhou et al., 1999]. At least a part of this antiviral effect has been addressed to the interferon induced 3'-5' exonuclease ISG20 [Espert et al., 2003]. Human ISG20 was first identified when the ISG20 gene expression was increased following IFN treatment [Gongora et al., 1997]. ISG20 is a 3'-5'exonuclease transcriptionally activated by type I and type II IFNs and

with a confirmed preference for single stranded RNA over single stranded DNA [Nguyen et al., 2001]. ISG20 is found both in the cytoplasm as well as the nucleus. Over-expression of ISG20 in HeLa cells confers resistance to some RNA viruses including FLUAV, vesicular stomatitis virus (VSV), enchephalymyocarditis virus (ECMV) [Espert et al., 2003]. In addition, we have observed that ISG20 has a substantial antiviral effect against CCHFV (unpublished data). Exact how ISG20 affect virus replication is at the moment not known.

The induction of type I IFNs and the subsequent generation of the antiviral state is schematically illustrated in figure 4.
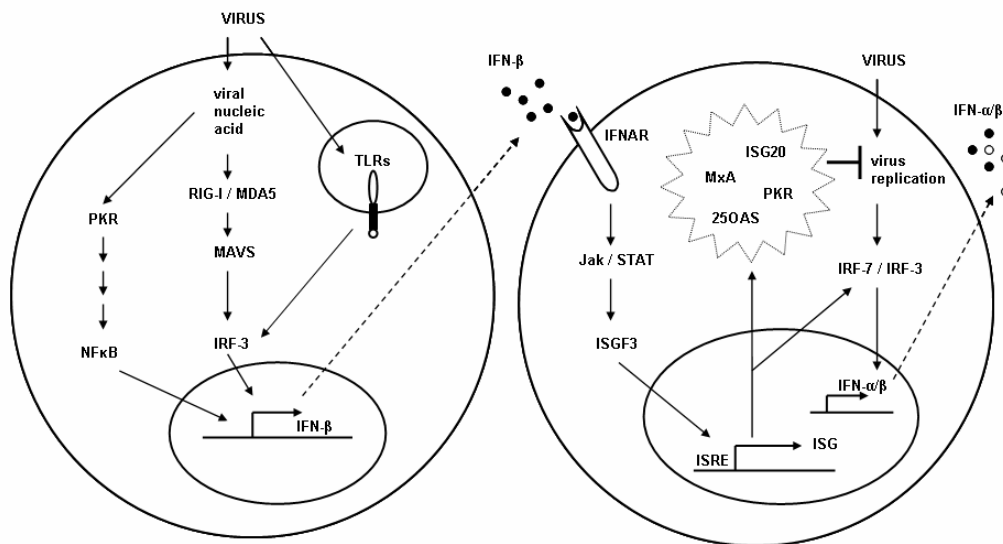

Figure 4. The induction, signalling and action of type I IFNs following a viral infection. Intra- or extracellular recognition of viral nucleic acid by the RIG-I/MDA5, PKR and TLR pathways lead to the activation of transcription factors which in turn activate the IFN-β promoter. Secreted IFN-β binds to the type I IFN receptor (IFNAR) on neighbouring cells and signals through the Jak/Stat pathway to induce expression of a number of ISGs and induce an antiviral state. The most extensively studied ISGs are the antiviral proteins MxA, PKR, 25OAS and ISG20 and also IRF-7, which enhances the innate response by promoting the expression of several IFN-α subtypes. Adapted from [Haller et al., 2006] with permission.

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Interferon antagonists

There is a continuous battle going on between the hosts and the viruses. Viruses need to use the host replication machinery for multiplication to ensure transmissions to new hosts and survival as a population. For this to be achieved in the presence of the powerful innate immune response, most viruses have evolved strategies to inhibit or down-regulate the IFN response. Most viruses, if not all, are equipped with antagonists designed to subvert the host IFN response by various means. The antagonists are often multifunctional proteins and have the capacity to interfere with many different signalling pathways. Luckily for us humans, our IFN system is most often able to come out as winners and fight off the infections, despite these often quite potent antagonists.

The list of viral encoded IFN antagonists are continuously growing and these proteins can target almost all components of the IFN system, ranging from inhibition of IFN induction and IFN signalling to direct inhibitory effects of antiviral effector proteins. In addition, a general block in cellular translation is used by some viruses. To illustrate the many different targets for viral antagonists, some selected examples of inhibitors at different levels are given below.

Viral inhibition of IFN induction

A prominent example of a viral protein which inhibits IFN induction is the NS1 protein of FLUAV. It binds to both dsRNA and ssRNA and thereby prevents recognition of infection and subsequent interferon induction [Garcia-Sastre et al., 1998; Lu et al., 1995]. In addition, NS1 interacts with RIG-I and thereby inhibits the RIG-I mediated pathway of IFN-β induction. [Mibayashi et al., 2007]. The VP35 protein of Ebola virus is another viral protein that binds dsRNA [Cardenas et al., 2006] as well as the E3L protein of poxviruses [Xiang et al., 2002]. The large poxviruses can in addition afford to secrete soluble IFN-binding proteins which compete with cellular receptors for the secreted IFN [Alcami et al., 2000]. The phosphoprotein P, an essential component of many viral polymerases, is the main antagonist for many negative-strand RNA-viruses. For example, the P-protein of Rabies virus has been found to inhibit TBK-1 mediated phosphorylation of IRF-3 [Brzozka et al., 2005], the P-protein of Ebola (VP35) interferes with IRF-3 activation

[Basler et al., 2003] and the P protein of Borna virus binds directly to TBK-1 and reduce its activity [Unterstab et al., 2005].

Viral inhibition of IFN signalling

When it comes to viral inhibition of IFN signalling, a frequent target is the Jak-STAT pathway, signalling down-stream the IFN receptors to induce transcription of antiviral genes. For example, Herpes simplex virus-1 (HSV-1) induces the suppressor SOCS-3, which down-regulate STAT and Jak phosphorylation [Yokota et al., 2004], The V proteins of some paramyxoviruses induce ubiquitinylation and degradation of STAT1 molecules [Yokota et al., 2004]. The NS4B protein of flaviviruses blocks IFN induced signal transduction cascades by inhibiting phosphorylation and nuclear translocation of STAT1 [Munoz-Jordan et al., 2005; Munoz-Jordan et al., 2003]

Viral inhibition of IFN effector proteins

Another antagonistic strategy is to directly interfere with the antiviral functions of selective IFN-induced proteins. IFN-induced PKR and RNAse L proteins need to be activated by dsRNA, a feature which make them vulnerable to viral antagonists. The RNA binding proteins expressed by some viruses, such as VP35 and NS1 proteins mentioned above, are able to prevent the activation of PKR and 2-5 OAS/RNase L systems, by sequestering the activating dsRNA molecules.

Some viruses encode proteins which directly bind to or inactivate PKR, such as the vaccinia virus K3L gene product [Davies et al., 1992]. Poliovirus induce degradation of PKR [Black et al., 1993]. RNAse L can also be directly targeted by different antagonists. For example, EMCV induce the expression of a cellular RNase L inhibitor [Martinand et al., 1998] and HSV-1/2 induce the synthesis of 2-5A derivatives that binds to and inactivate RNAse L [Cayley et al., 1984].

No specific inhibitors of the Mx proteins have been identified to this date. The activity of the Mx proteins is not modulated by dsRNA and the proteins are not posttranslationally modified. One way of avoiding Mx activation is simply by preventing IFN induction.

General inhibition of host gene expression or protein translation

General inhibition of host gene transcription or cellular protein synthesis is used as an antagonistic strategy by many viruses causing acute infections. Many host-cell functions are of course affected by this general inhibition, but the main reason why viruses have evolved this mechanism is specifically to circumvent IFN induction. This can be illustrated by the Bunyamwera virus (BUNV), where the NSs protein inhibits cellular mRNA transcription. Nonetheless, the NSs only provides a growth advantage in IFN competent cells and mutants lacking NSs is pathogenic in IFNAR-/- mice and grow to high titers in cells devoid of IFN production [Weber et al., 2002; Young et al., 2003]. Another prominent example of a virus interfering with these processes is VSV [Ferran and Lucas-Lenard, 1997]. There are many disadvantages associated with preventing host cell transcription or translation. For example, the infected cells will eventually die and only provide the virus with a limited time to replicate. Perhaps this is the reason why more sophisticated antagonistic mechanisms have evolved.

Antagonists encoded by members of the *Bunyaviridae* family

IFN antagonism has been demonstrated for many of the members of the *Bunyaviridae* family and in most cases the antagonistic properties has been addressed to the non-structural proteins (NSs) encoded on the S segment, which show a great difference in size, amino acid sequence and coding strategies [Elliott, 1990].

The S segment of Rift Valley fever virus (RVFV), *Phlebovirus* genus, encodes a non-structural protein on the S segment which functions as an interferon antagonist [Bouloy et al., 2001]. The NSs is encoded in the genomic sense, while the nucleocapsid protein is encoded for in the antigenomic sense [Giorgi et al., 1991]. RVFV replicates in the cytoplasm, but NSs forms filamentous structures in the nucleus [Yadani et al., 1999]. The NSs protein has been found not to interfere with the normally important transcription factors IRF-3, NFκB or AP-1, but instead exert its antiviral action by inducing a general inhibition of the host cell transcriptional machinery [Billecocq et al., 2004]. NSs activity is mediated by preventing the assembly of the multisubunit complex of the transcription factor TFIIH, an essential cofactor for the cellular RNA polymerase II (RNAPII) [Le May et al., 2004].

Recently, it was also found that NSs interacts with SAP-30 (Sin3A associated protein 30), a subunit of a transcription repressor complexes [Le May et al., 2008].

The NSs of the Bunyamwera virus (BUNV), *Orthobunyavirus* genus, function as an interferon antagonist by blocking transcriptional activation of IFN-α/β [Bridgen et al., 2001; Weber et al., 2002] without interfering with the activation of IRF-3 [Kohl et al., 2003]. BUNV NSs causes modifications to the phosphorylation state of the RNAPII and thereby impairs host cell mRNA synthesis [Thomas et al., 2004]. Furthermore, BUNV NSs has been found to interact with MED8, a component of the Mediator complex, which regulates RNAPII [Leonard et al., 2006]. BUNV NSs also counteracts the induction of apoptosis by inhibiting IRF-3 mediated cell death [Kohl et al., 2003]. La Crosse Virus (LACV), also a member of the *Orthobunyavirus* genus, encodes an NSs protein which suppresses the type I IFN system in mammalian cells [Blakqori et al., 2007].

For a long time it was thought that members of the *Hantavirus* genus did not encode a non-structural protein on their S-segments. Today it is known that Tula and Puumala hantaviruses have a functional NSs protein, which weakly inhibit the activation of the IFN-β promoter [Jaaskelainen et al., 2007; Jaaskelainen et al., 2008].

For members of the *Nairovirus* genus, no additional NSs proteins have been identified to this date. However, for CCHFV, the S segment encodes an additional open reading frame, in a positive sense orientation, which is conserved in almost all CCHFV strains [Hewson et al., 2004], but a corresponding peptide product has so far not been identified. This might suggest the presence of a yet undiscovered protein which possibly could function as an antagonist. In addition, it was recently reported that the L segment of CCHFV contains an Ovarian Tumor (OTU) domain providing the virus with a strategy for immune evasion [Frias-Staheli et al., 2007]. This domain is also present in the L-proteins of Dugbe virus and Nairobi sheep disease virus, but in none of the other genera of the *Bunyaviridae* family [Honig et al., 2004]. The OTU containing protease hydrolyses ubiquitin and ISG15 from conjugated cellular target proteins. ISG15 is an interferon induced molecule with antiviral properties, and by viral targeting of this molecule the host antiviral response may be subverted.

1200 hours in the P4 lab….

SPECIFIC AIMS

- To study the antiviral activity of the interferon-induced human MxA protein against CCHFV **(Paper I).**

- To examine the antiviral potential of IFN-α against CCHFV in human target cells **(Paper II).**

- To investigate if CCHFV has the ability to interfere with early immune responses and hence prevent secretion of IFNs following infection **(Paper III)**.

- To study the role of 5'triphosphates in RIG-I/MDA5-dependent activation of the IFN-β promoter in response to negative stranded RNA viruses **(Paper IV)**.

- To study the role of PKR and RNAse L in the IFN-induced inhibition of CCHFV replication **(Preliminary results, manuscript)**.

RESULTS

Paper I

Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus

The human MxA protein is an important mediator of IFN-induced antiviral effects against a number of viruses belonging to different families, including representative members of the *Bunyaviridae*, *Orthomyxoviridae*, *Rhabdoviridae*, *Paramyxoviridae*, *Picornaviridae* and *Hepadnaviridae* families [Bridgen et al., 2004; Chieux et al., 2001; Frese et al., 1996; Frese et al., 1995; Gordien et al., 2001; Haller et al., 1998; Kanerva et al., 1996; Landis et al., 1998; Peltekian et al., 2005]. Despite the immense knowledge regarding the antiviral action of MxA against different viruses, no data was published for CCHFV in this regard.

To investigate the antiviral potential of the human MxA protein against CCHFV, we used Vero cells, permanently transfected with plasmids expressing either the wild-type human MxA protein (VA9, VA3 and VA12) or cells transfected with a plasmid expressing a mutant form of MxA (VA[E645R]). Cells transfected with a plasmid expressing the neomycin resistance gene (VN36 and VN41) were used as controls. When these different cells were infected with CCHFV, viral progeny titers obtained from MxA expressing cells were up to 1000-fold lower compared to the control cells, showing that wt human MxA has an antiviral activity against CCHFV (Figure 1, paper I).

MxA generally appears to interfere with early steps in the virus replication cycle. It inhibits primary transcription of VSV, measles and THOV [Kochs and Haller, 1999; Pavlovic et al., 1990; Schneider-Schaulies et al., 1994]. For other viruses, such as the members of the *Bunyaviridae* family, the block also seem to occur early but rather at the level of genome amplification than transcription [Kochs et al., 2002b]. For CCHFV viral protein synthesis was comparable between the different cells up to twelve hours after the infection, indicating that primary transcription and translation was unaffected (Figure 2, paper 1). However, the amounts of vRNA produced in MxA expressing cells were found to be significantly lower for both the S and the M

segment, compared to the control cells, indicating that MxA blocks CCHFV at the level of genome replication (Figure 3, paper I).

Normally, MxA can be detected as punctuate granula in the cytoplasm of stimulated cells. When investigating the pattern of MxA expression in an immunofluorescence assay, we found that MxA was sequestered to the perinuclear region in infected cells where it perfectly co-localized with the CCHFV NP. This phenomenon had previously also been observed for LACV [Kochs et al., 2002b]. In addition, in a co-immunoprecipitation analysis, CCHFV NP and MxA were found to interact with each other (Figure 4, paper I).

The mutant form of MxA, MxA(E645R), has a single amino acid change in the carboxy terminal effector domain. MxA(E645R) had previously been found to have lost its antiviral activity against La Crosse and VSV [Kochs et al., 2002b], while still remaining active against FLUAV and Thogoto virus [Frese et al., 1995; Zurcher et al., 1992]. The present study showed that the mutant had lost its antiviral activity against CCHFV. No reduction in progeny virus titers were observed in comparison to titers obtained from the control cell lines (Figure 1, paper I). Furthermore, the mutant form remained in the small granulas distributed all over the cytoplasm of the infected cells and showed neither co-localization nor co-immunoprecipitation with the CCHFV NP (Figure 4, paper I). The fact that a single amino acid substitution in the MxA protein results in a complete loss of activity demonstrates the high specificity of the antiviral activity. Recognition of NP by MxA relies on specific protein-protein interactions, which seem to be important for the antiviral activity.

For LACV, another member of the *Bunyaviridae* family, it has been proposed that MxA exerts its antiviral action by wrapping around incoming nucleocapsids sequestering the protein in the perinuclear region, thereby preventing the nucleocapsid protein from taking part in the generation of new viral particles [Kochs et al., 2002b]. It is known that newly synthesized NP is needed for the polymerase to shift from the transcription to the replication mode [Schmaljohn, 1996]. In view of the fact that MxA interacts with the CCHFV NP and is localized to the same intracellular region, it is reasonable to speculate that a similar mechanism of action is applied by MxA against CCHFV (Figure 5).
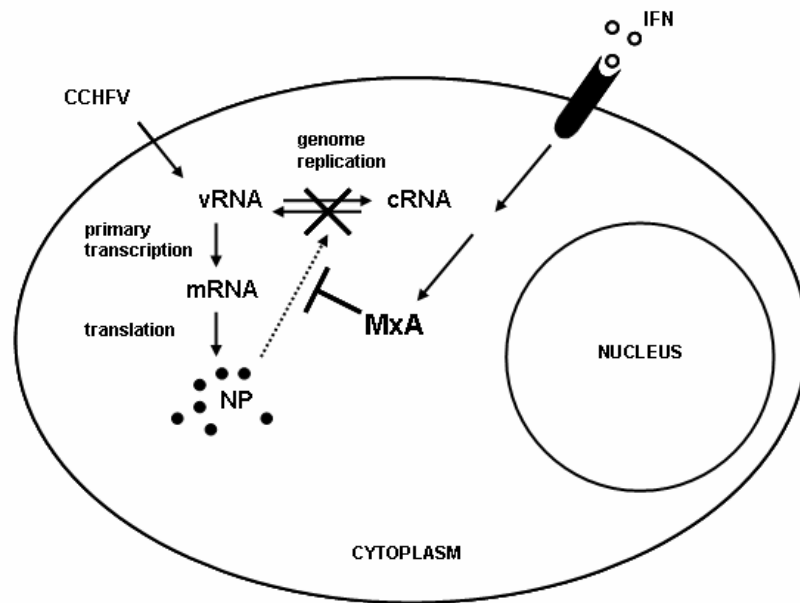
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Figure 5. *Proposed antiviral mechanism for the human MxA protein against CCHFV (Paper I). MxA expression is induced upon stimulation by type I and III IFNs. MxA binds to and sequesters the NP to the perinuclear region of the infected cell. Normally, NP is involved in the switch of the viral polymerase from transcription to replication mode. However, the MxA-mediated sequestration of NP to the perinuclear region depletes the cytoplasm of free NP, prevents the polymerase switch and causes a block in viral genome replication. This mechanism has previously been suggested for THOV [Kochs et al., 2002b].*

Paper II

Type I Interferon inhibits Crimean-Congo hemorrhagic fever virus in human target cells

The type I IFN system is the first line of defence against viral infections. As a response to the infection, virus infected cells synthesize and secrete IFN-α/β to warn surrounding cells from the intruders. Once secreted, the IFNs circulate in the body to induce an antiviral state in susceptible cells, leading to limitations in further growth and spread of the virus. The IFN response is absolutely indispensable for vertebrates in the control of viral infections. This can be illustrated in knock-out mice with deletions in their IFN-α/β receptor, making them unable to respond to IFN stimulation. These mice quickly succumb to viral infections despite the fact that their adaptive immune system is otherwise intact [Hwang et al., 1995].

In this study we were interested to investigate the antiviral potential of type I IFNs on the replication of CCHFV in human target cells. For this purpose we used primary human umbilical vein endothelial cells (HUVECs) and human hepatoma cells (Huh-7). HUVECs were chosen because the endothelium has been shown to be involved in the hemorrhagic fever caused by CCHFV, and Huh-7 because hepatocytes is regarded as one of the target cells for CCHFV [Swanepoel et al., 1989]. First of all, both cell types were shown to support CCHFV replication (Figure 1, paper II). When the cells were treated with different concentrations of human IFN-α prior to infection, a decrease in CCHFV NP expression and viral progeny titers were observed in a dose dependent manner in both of the cell types (Figure 2A and B, paper II). Huh-7 cells were more permissive to the infection than the HUVECs as illustrated by the 1000-fold higher output of virus in the mock treated control cultures. On the other hand, the Huh-7 cells were protected by the IFN pre-treatment to a lesser degree. Real-Time PCR detecting S segment vRNA confirmed the data from the titrations and the western blots. In IFN-treated cells the amounts of vRNA were significantly reduced in comparison to the controls (Figure 2C, paper II). Taken together, these results show that human IFN-α has an antiviral effect against CCHFV in the investigated target cells.

Type I IFNs induce the expression of numerous proteins with antiviral activity. One of the best studied antiviral proteins is the MxA protein. In paper I we showed that

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over-expression of the MxA protein lead to a reduction in CCHFV replication and in this study we observed an increase in MxA protein expression following treatment with increasing concentrations of IFN-α (Figure 2A, paper II). Therefore, we wondered whether endogenously produced MxA played a role in the IFN induced antiviral effects we observed in our target cells. To address this question, we decided to use a siRNAs approach to knock-down the MxA protein expression. Four different siRNAs targeted to different regions on the MxA gene were synthesized and tested for their silencing ability. A mixture of the four siRNAs were shown to give the strongest silencing effect (Figure 3, paper II). Due to toxic effects of the transfection media to the HUVECs, the Huh-7 cells were used in the siRNA experiments.

When the MxA protein expression was knocked-down by siRNA in IFN stimulated cells, the levels of CCHFV NP was increased, compared to the expression in cells that still expressed MxA (Figure 4A, paper II). The levels of NP were comparable between cells transfected with a control siRNA (non-silencing) and the untransfected control, showing that siRNA as such did not affect the increase in NP expression. Furthermore, the silencing of MxA expression was shown to increase progeny viral titers from IFN treated cells. Titers were approximately five times higher when the MxA protein expression was knocked-out, compared to titers from the MxA expressing cells or cells transfected with control siRNA (Figure 4B, paper II). However, the titers were not completely restored, most importantly because other antiviral proteins are induced upon IFN treatment, contributing to the IFN induced antiviral effect against CCHFV and probably in part because the silencing of the MxA gene in our siRNA experiments was not hundred percent.

In this study we identify the human MxA protein as a major contributor to the interferon induced antiviral effect against CCHFV in human target cells.

Paper III

Crimean-Congo hemorrhagic fever virus delays activation of the innate immune response

During the innate immune response to a viral infection, the induction and secretion of IFNs is an essential event. We were therefore interested to measure if IFNs were produced from infected cells during a CCHFV infection. To address this question we used a bioassay based on a recombinant, IFN sensitive New Castle Disease virus (NDV), expressing the green fluorescent protein (GFP). In this bioassay, no biologically active type I IFN were found in supernatants harvested 24 hours post infection from CCHFV infected, IFN competent A549 cells. However, at 48 hours post infection, substantial amounts of IFN were found in the media (Figure 1a and 1b, paper III). Cells transfected with poly I:C were used as a positive control and, as suspected, IFN was detected in the media at both 24 and 48 hours. A serial dilution of IFN-α was used as an assay control (Figure 1c, paper III). RT-PCR analysis of the infected cells supported the findings from the bioassay (Figure 1d, paper III).

IFN is normally induced within hours of a viral infection. Nevertheless, our findings showed that CCHFV induces biologically active IFN relatively late following infection. Other members of the *Bunyaviridae* family have previously been found to down-regulate the IFN response and to be poor inducers of IFN [Billecocq et al., 2004; Weber et al., 2002]. However, no reports were to be found about CCHFV in this regard. In order to investigate if CCHFV had the ability to interfere with IFN responses, we compared the expression of ISG56 following native infections to infections with UV irradiated virus. ISG56 is a highly sensitive IFN-stimulated gene and a useful marker for early immune responses. A549 and Huh-7 cells were infected with native and UV treated virus and analysed with RT-PCR at different times post infection. In both cell types ISG56 mRNA was detected at 3 hours post infection in cells infected with UV treated virus. However, in cells infected with native CCHFV, ISG56 mRNA was not detected until 17 or 24 hours of infection (Figure 2b, paper III).

In addition, we investigated the ability of CCHFV to interfere with IRF-3 nuclear translocation. IRF-3 is an important transcription factor in the induction of IFNs. In an immunofluorescence analysis we found that IRF-3 was translocated to the nucleus

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already after 3 hours in cells infected with UV-irradiated virus. By contrast, in cells infected with the native virus nuclear translocation of IRF-3 was not observed until 24 hours post infection (Figure 3a and 3b, paper III). Taken together, these results suggest that native replicating CCHFV have the ability of interfere with the early IFN response of the host.

We have previously observed that pre-treatment with IFN-α significantly reduces CCHFV progeny virus titers (paper II). In this study we show that CCHFV interferes with the innate immune response and that IFNs are not induced until 48 hours post infection. We were therefore interested to investigate what the outcome would be if the cells were treated with IFN close to or after the time of infection. As previously observed, we found that pre-treatment with IFN-α reduced progeny virus titers approximately one log step. Titers were also strongly reduced when IFN was added two hours before or one hour after the infection. However, when IFN was added after the infection, no reduction of titers was observed (Figure 4a, paper III). These results were supported by a western blot (Figure 4b, paper III).

The results from this study show that replicating CCHFV has the ability to delay the early IFN responses in the host, possibly by interfering with the activation pathway of IRF-3. Furthermore, we show that IFNs are induced relatively late after infection and that once the virus is replicating it is almost insensitive to subsequent IFN treatment.

Paper IV

Processing of genome 5' termini as a strategy of negative-strand RNA viruses to avoid RIG-I-dependent cytokine induction

Double-stranded RNA was for a long time regarded as a key activator of innate immune responses against viruses through recognition by RIG-I and MDA5, and it was widely assumed that production of dsRNA was a feature shared by all viruses. Recently it was shown that dsRNA indeed could be observed for viruses with genomes consisting of positive-strand RNA, dsRNA or DNA. However, no detectable amounts of dsRNA was observed in cells infected with negative stranded RNA viruses [Weber et al., 2006].

Recent data show that genomic single stranded RNA bearing a 5' triphosphate group is sufficient to activate RIG-I dependent IFN-α/β induction [Hornung et al., 2006; Pichlmair et al., 2006]. In these publications the importance of RIG-I in recognizing genomic RNA were demonstrated for the negative stranded RNA viruses FLUAV, rabies virus and VSV.

In the group of negative stranded RNA viruses, several pathogens with extreme virulence are found. In this paper we were interested to investigate if RIG-I and/or MDA5 were required for recognition of a selected group of negative stranded RNA viruses. First, we investigated which of these factors were necessary for recognition of Nipah virus (NiV, family *Paramyxoviridae*) and Ebola Zaire virus (ZEBOV, family *Filoviridae*), two non-segmented viruses. Genomic RNA (vRNA) from both viruses were shown to activate the IFN response when vRNA was transfected into 293T cells, pre-transfected with reporter plasmids under the control of the IFN-β promoter (Figure 1A and 1B, paper IV). vRNA from FLUAV was used as a positive control (Figure 1C, paper IV). The results from the reporter assay were confirmed in an RT-PCR analysis showing that treatment of cells with vRNAs resulted in transcriptional upregulation of the IFN-β gene as well as some IFN-stimulated genes (Figure 1D, paper IV).

When short hairpin RNAs (shRNA), expressed by retroviruses, were used to knock-down RIG-I expression, the activation of the IFN-β promoter decreased following transfection with both NiV and ZEBOV vRNA (Figure 2B, paper IV). However, no

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reduction in reporter activity was observed when MDA5 expression was down-regulated. Since the RIG-I response previously was found to be activated by RNA bearing 5'triphosphates [Pichlmair et al., 2006], shrimp alkaline phosphatase (SAP) was used in an attempt to degrade these triphosphates. When NiV and ZEBOV vRNA were treated with SAP, the particles no longer activated the IFN-β reporter (Figure 2C, paper IV). The effect could be reversed by adding EDTA, an inhibitor of SAP. Taken together, these data suggests that 5'triphosphates on NiV and ZEBOV genomic RNA is the trigger of RIG-I dependent IFN responses.

Next, we decided to study a group of highly virulent negative stranded RNA viruses containing a segmented genome, namely Lassa virus (LASV, family *Arenaviridae*), Rift Valley fever virus (RVFV, family *Bunyaviridae*), Hantaan virus (HTNV, family *Bunyaviridae*) and Crimean-Congo hemorrhagic fever virus (CCHFV, family *Bunyaviridae*). RNAs of LACV and RVFV were found to activate the IFN-β promoter in a 5'triphosphate dependent manner (Figure 3A and 3B, paper IV) as observed for the non-segmented viruses. Surprisingly, RNAs from HTNV and CCHFV did not trigger IFN induction (Figure 3C, paper IV). The identity and integrity of the genomes were confirmed to rule out trivial explanations to the lack of induction (Figure 3D, paper IV).

We assumed that HTNV and CCHFV RNAs contained 5'monophosphates instead of 5'triphosphates. To test this hypothesis, we treated the RNAs with a 5'-3'exonuclease able to digest ssRNA with a 5'monophosphate and then carried out RT-PCR analysis. As suspected, HTNV and CCHFV RNAs were shown to be sensitive to digestion while RVFV RNA was protected (Figure 4A, paper IV). In addition, it was found that Borna disease virus (BDV) avoids RIG-I activation in a similar manner to HTNV and CCHFV (Figure 4B and 4C, paper IV).

Furthermore, we investigated whether non-inducing and inducing vRNAs differed in their ability to bind to RIG-I. For this purpose, an RNA pulldown assay was performed using GFP tagged RIG-I coupled to Sepharose beads. As expected, the vRNA of RVFV was precipitated by the RIG-I beads. By contrast, vRNAs from CCHFV, HTNV and BDV were not bound by RIG-I (Figure 5, paper IV). These findings support our hypothesis that processing of RNA 5' ends enable viral escape of RIG-I signalling.

Taken together, our results establish RIG-I as a key intracellular receptor for recognition of negative strand RNA viruses and suggest cleavage of triphosphates at the 5' end, to generate monophosphates, as a new strategy of viruses to evade the innate immune response. The findings from paper IV are summarized in figure 6.
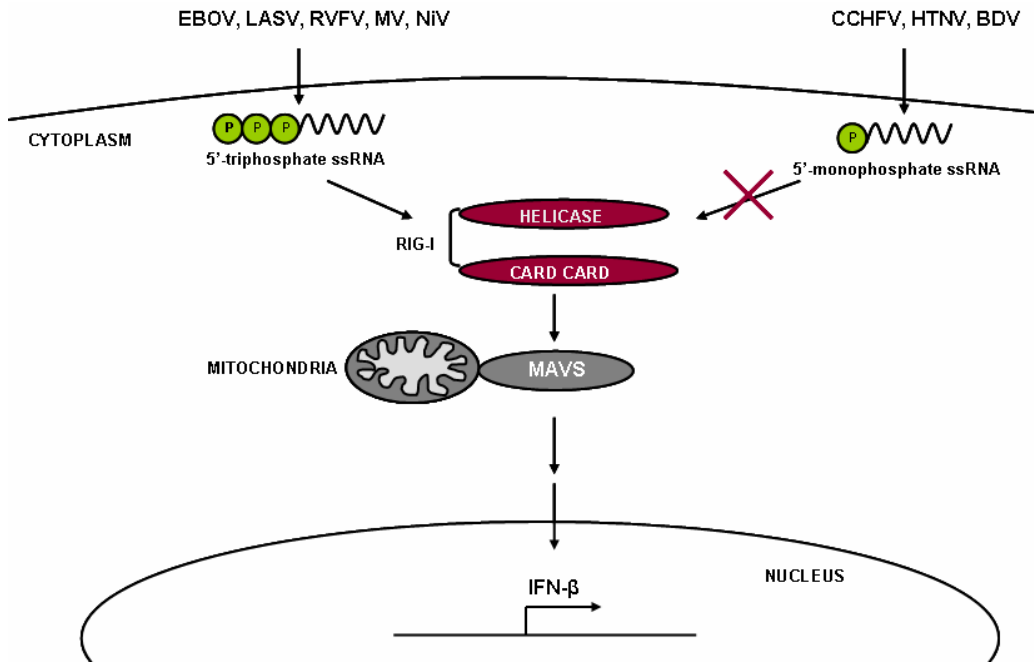

Figure 6. *Summary of the findings in Paper III. EBOV, LASV, RVFV, MV and NiV strongly activated the IFN-β promoter through RIG-I recognition of viral RNA bearing 5'triphoshates. By contrast, CCHFV, HTNV and BDV were found to avoid RIG-I detection and induction of the IFN-β promoter by processing of their 5' RNA termini.*

Preliminary results

The role of PKR and RNAse L for controlling the replication cycle of Crimean-Congo hemorrhagic fever virus

The type I IFNs induce the expression of more than 300 IFN-induced proteins and the most well characterized proteins with antiviral activity are the Mx-proteins, PKR and the 2-5 OAS/ RNAse L system. In paper II and III it was demonstrated that IFNs had an antiviral effect against CCHFV. Furthermore, it was shown that the human MxA protein plays an important role in these IFN-induced effects. However, MxA was suggested not to be the only antiviral protein involved (paper II). With this in mind, the roles played by PKR and RNAse L in the IFN-induced antiviral effects were investigated.

To study the possible involvement of PKR in the IFN-induced antiviral defence against CCHFV, PKR activity was induced by poly I:C and IFN in MEFs derived from wt 129 mice and PKR-/- mice before infection. Following transfection with poly I:C alone and also after the combination of poly I:C transfection and treatment with IFN-α, PKR protein expression increased in the wt MEFs indicating induction of the protein (Figure 1a, preliminary results). The increase in PKR expression coincided with a reduction of the CCHFV NP expression, suggesting that PKR can act antivirally against CCHFV. However, the NP expression remained stable in PKR-/- MEFs despite the different treatments. The fact that CCHFV NP expression did not decrease following treatment with dsRNA and IFN in the PKR-/- cells suggests that PKR is the protein responsible for the observed reduction in the wt cells. When analysing the viral progeny titers, additional proof of the antiviral effect of PKR against CCHFV was found (Figure 1b, preliminary results). The two pre-treatments resulted in a nearly 100 percent inhibition of viral titers in the wt cells in comparison to the control, while the titers were less inhibited in the PKR-/- cells (40 and 78 percent respectively).

In addition, PKR expression was comparable between infected cells without stimulation and in uninfected control cells, suggesting that CCHFV per se does not induce PKR expression (Figure 1a, preliminary results).

To further investigate if PKR was induced following a CCHFV infection, wt MEFs and PKR-/- MEFs were infected with CCHFV and harvested for progeny virus

titration and western blot. No significant differences between CCHFV progeny virus titers from the two cell types were found (Figure 2a, preliminary results). In addition, PKR expression was comparable between infected cells and uninfected control cells, adding further proof to the hypothesis that PKR expression is not induced following CCHFV infection (Figure 2b, preliminary results).

In the RNAse L-/- cells, CCHFV NP expression was decreased following pre-treatment with poly I:C and IFN, compared to the control cells, suggesting that RNAse L has no significant antiviral effect against CCHFV (Figure 3b, preliminary results).

Taken together, our preliminary findings suggest that PKR has an antiviral effect against CCHFV. However, CCHFV infection as such does not seem to induce PKR expression. The role played by RNAse L appears to be of minor importance.

DISCUSSION

The defence against a virus infection is dependent on a rapid and efficient response by the infected host. The type I IFN defence is the first and also the major mechanism to keep viruses at bay and is indispensable for the vertebrate host. In this thesis, the type I IFN response has been discussed in the context of interferon-induced antiviral effects against CCHFV. In paper I and II, we identify the human MxA protein as a major contributor to the interferon induced antiviral effect against CCHFV. However, in our studies MxA was found not to be the sole explanation to the observed reduction in virus replication following IFN treatment. Our preliminary findings show that the PKR protein also contributes to the interferon induced antiviral effects against CCHFV replication, while RNAse L seems to be of minor importance. In addition, we have investigated the antiviral potential of the human ISG20 protein (unpublished data). Transfecting cells with a plasmid encoding the human ISG20 protein prior to infection, resulted in a significant reduction of CCHFV progeny titers compared to cells transfected with a control plasmid. These data suggests that ISG20 also is involved in the antiviral defence against CCHFV. Taken together, our findings implies that several different pathways of antiviral mechanisms exist in the interferon-induced host defence against CCHFV and most likely many more awaits to be discovered (figure 7).

IFNs are used in the treatment of many virus infections and our results might suggest that IFN treatment could be beneficial for CCHF patients. However, in the case of CCHFV, IFN will in most instances be administered after the appearance of symptoms, which probably is too late in the course of infection to be valuable for the outcome of the disease. In paper III we show that IFN has little effects on already replicating CCHFV. Another concern with the usage of IFN is the lack of knowledge regarding whether the hemorrhages associated with CCHFV infection are caused directly by the virus infection or if they are immuno-mediated. It may be possible that the addition of IFN might actually worsen the symptoms. There is however no clinical trials yet that prove either benefits or drawbacks of IFN in the treatment of CCHF patients.
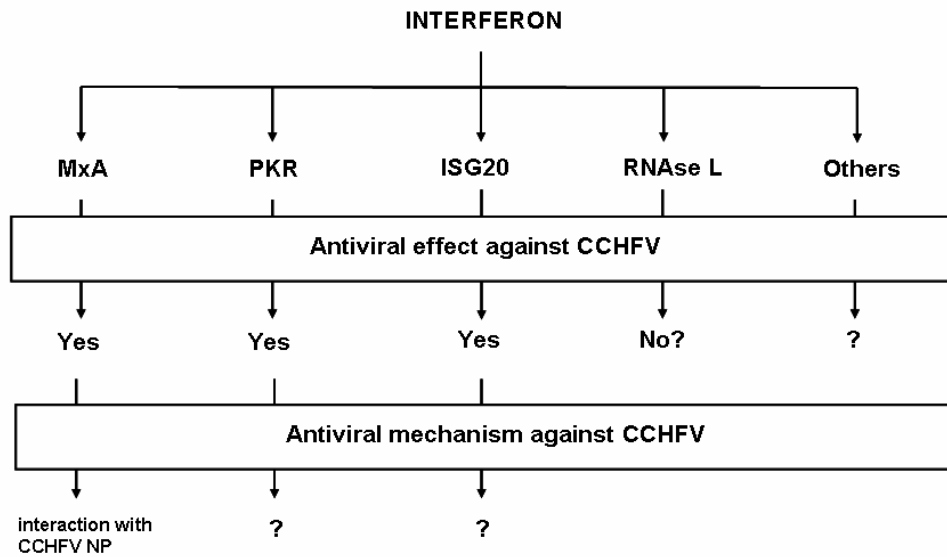
INTERFERON

| MxA | PKR | ISG20 | RNAse L | Others |

Antiviral effect against CCHFV

| Yes | Yes | Yes | No? | ? |

Antiviral mechanism against CCHFV

interaction with
CCHFV NP   ?   ?

Figure 7. Summary of the interferon induced antiviral proteins discussed in this thesis and their antiviral effect against CCHFV.

Viruses have evolved multiple strategies to escape the IFN system and virtually every pathway of the innate immune defence is targeted by viral proteins. In paper III and IV of this thesis, we aimed to investigate if CCHFV, like most other viruses, had the ability to interfere with host immune response mechanisms. Recently, it was shown that 5'triphosphates on viral RNAs are the major determinants for detection by RIG-I [Hornung et al., 2006; Pichlmair et al., 2006]. In paper IV we show that some negative stranded RNA viruses, among them CCHFV, are equipped with the ability to escape RIG-I dependent cytokine induction by removal of these 5'triphosphates and the generation of 5'monophosphates instead. This finding identifies a new strategy by which viruses avoid activation of the innate immune response in order to circumvent the induction of IFNs. The generation of monophoshates appears to occur by different mechanisms in different viruses, but to be driven by the same selection pressure from RIG-I and the antiviral IFN system.

In addition to the findings in paper IV, we show that IRF-3 nuclear translocation and induction of ISGs is delayed in cells infected with replicating CCHFV compared to cells infected with UV irradiated virus (paper III). If this delay simply reflects the avoidance of RIG-I detection and/or is the result of the direct actions of an interferon antagonist encoded by replicating CCHFV is not known at this stage. All genera of the *Bunyaviridae* family, besides the *Nairovirus* genus, encode NSs proteins with

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interferon antagonistic properties regardless of their ability to avoid RIG-I detection. It is therefore tempting to speculate if CCHFV also encodes a non-structural protein. The highly conserved additional open reading frame in the S-segment is certainly worth investigating further. It has also been speculated if the larger size of the genomic RNA segments of the nairoviruses would account for some of the properties that the NSs proteins have in the other genera. In fact, it has recently been reported that an OTU domain is found within the L segment of CCHFV, providing the virus with a strategy for immune evasion [Frias-Staheli et al., 2007].

Taken together, the findings in this thesis show that CCHFV is sensitive to the action of IFNs and IFN-induced antiviral proteins. However, CCHFV have evolved strategies to escape the detection of the immune response by avoiding RIG-I recognition and possibly also by encoding a yet unidentified IFN antagonist. This leads to a lack of IFNs, and consequently a shortage of IFN-induced antiviral proteins, early in infection when they are most urgently needed. Later on, when the CCHFV replication machinery is in progress, IFNs has little or no effect on virus growth and the virus can run its course and cause its devastating disease. However, it has to be kept in mind that all studies in this thesis were carried out using cell lines. IFN responses in infected human individuals remain to be investigated and it can not be excluded that different results will be obtained under these circumstances.

For Ebola virus it has been shown that the development of an antigen specific, cell mediated immune response is the major determinant for the outcome of infection [Zampieri et al., 2007]. A high viremia, lack of detectable antibodies and absence of CD8+T cell activation has been found to be distinguishing features in fatal Ebola cases. For CCHFV, a high viremia and non-existence of antibodies is also associated with a fatal outcome, though the knowledge regarding T-cell mediated responses during CCHF is most limited. Identifying the immunological differences between fatal and non-fatal cases is crucial for the understanding of the pathogenesis of CCHFV and other viral hemorrhagic fever viruses.

To protect the host cells from intruders it is essential that foreign nucleic acids are distinguished from abundant self nucleic acids. Several surveillance systems, mentioned in this thesis, have evolved to detect invaders and activate cellular immune responses. The Toll-like receptors, TLR-3, -7 and -8, recognize exogenous nucleic

acids while RIG-I and MDA5 recognize foreign RNA in the cytoplasm. For the TLRs, the endosomal location seems to be crucial for self versus non-self discrimination. By contrast, RIG-I senses RNAs bearing 5'triphosphates in the cytoplasm, a structure normally not found in cellular proteins. It is therefore speculated that RIG-I function as a self versus non-self recognition receptor. Cellular proteins have a 7-methyl guanosine cap in their 5'ends, which protect them from being recognized. Many mRNAs of viruses infecting eukaryotic cells also contain caps at their 5'ends and poly(A) tails in their 3'ends. Despite this adaptation of several viruses to the host cells, synthesis of viral RNAs most often leads to the formation of transient cytoplasmic RNA intermediates with an uncapped 5'triphosphate end. Interestingly, self-RNAs generated from host DNA dependent RNA polymerase III carry 5' triphosphates [Bowie and Fitzgerald, 2007], indicating that there is more to viral recognition in the cytoplasm than just RIG-I detection of 5'triphosphates.

There are in fact already other known means of viral recognition beyond TLRs and RIG-I/MDA5. Karen Mossman and colleagues have shown that entry of enveloped viruses induce a cellular antiviral state by inducing a subset of ISGs in an IRF-3 dependent but IFN-independent manner [Collins et al., 2004]. Furthermore, they suggest a hypothesis for innate antiviral defences based on the extent of virus exposure [Paladino et al., 2006]. They claim that in the presence of a low amount of viruses, or defective virus particles, an IFN-independent antiviral response is triggered to block virus replication in the infected cell only. This response requires IRF-3 and is proposed to occur in reaction to virus particle entry, before virus replication. When the amount of viruses instead is above a certain threshold, both IRF-3 and NFκB are activated, leading to production of IFNs and a strong immune response. This response signals to surrounding cells to induce an antiviral state and to attract immune cells to the site of infection.

The innate immune defence against viruses consists of many redundant pathways, and we have most likely not identified all of them yet. In the coming years we can expect an increase in the knowledge regarding how viruses interact with their hosts and vice versa. This information will hopefully enable the development of improved measurements in the continuous fight against CCHFV and other viral infections.

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