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To my family

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ABSTRACT

Filovirus family consist of highly pathogenic negative-strand RNA viruses that are characterized by their filamentous virion structure. The main focus in previous research has been on ebolaviruses and marburgviruses due to the large epidemics caused by them. Host innate immune system and interferon responses play a vital role in restriction of viral infections. One of the reasons for the high lethality of filovirus infections is their ability to effectively suppress host antiviral responses by different viral proteins and mechanisms.

The aims of this project was to systematically analyze innate immune antagonistic functions of filovirus proteins and identify filovirus encoded proteins that specifically inhibit interferon (IFN) gene expression in model cell systems. The study showed that filoviruses interfere with host innate immune responses by inhibiting retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) RIG-I and melanoma differentiation-associated protein 5 (MDA5). Several filovirus VP24 proteins inhibit the expression of type I and III IFN genes.

In Study I we produced recombinant proteins for Ebola virus and compared the adjuvant effect of AS03 and FCA in immunizing rabbits and guinea pigs to find that both systems are comparable in antibody response. Study II showed a new function for EBOV VP24 as it inhibits type III IFN-λ1 gene expression. In Study III we expanded these findings to cover nine filoviruses and compared their inhibition of IFN-ß and IFN- λ1 promoter activation and localization in the nucleus. Differences considering VP24 localization and inhibition of interferon gene expression were found between filovirus VP24 proteins.

In summary, these results add to the knowledge on basic filovirus biology and open a door for future drug development as VP24 plays a significant role in filovirus life cycle and could be utilized as a target protein for further innovations.

KEYWORDS: Filovirus, Ebola virus, Marburg virus, RIG-I, MDA5, type I interferon, type III interferon, VP24

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TIIVISTELMÄ

Filovirukset ovat negatiivi-juosteisia RNA viruksia, joiden viruspartikkelit ovat rakenteeltaan filamenttisia. Aiempi tutkimus on keskittynyt pääasiassa ebola-viruksiin ja marburgviruksiin, sillä nämä viruslajit ovat aiheuttaneet ajoittain laajojakin epidemioita paikallisesti. Luontainen immuniteettivaste ja interferoni-vaste ovat keskeisessä roolissa virusinfektion torjunnassa. Filovirusten vaarallisuus pohjautuukin näiden reaktioiden vaimentamiseen.

Väitöskirjan tavoite on systemaattisesti tarkastella filovirusten proteiinien aiheuttamia spesifisiä vaikutuksia interferonin geenieksпression vaimentamissa. Tutkimus osoitti filovirusten heikentävän luontaista immuniteettivastetta vähentä-mällä RIG-I:n kaltaisten reseptorien aktivoitumista johtaen heikentyneeseen interferonivasteeseen. Monet filoviruksen VP24 proteiinit vaikuttivat tyypin I ja III interferoni-geenieksпressioon.

Ensimmäisessä osatyössä tuotimme Ebolaviruksen proteiineja ja vertailimme kahden eri adjuvanttimenetelmän (AS03 ja FCA) eroja immunisoiduissa kaneissa ja marsuissa. Molemmat menetelmät tuottivat tehokkaasti vasta-aineita. Toisessa osatyössä havaitsimme uuden mekanismin EBOV VP24 proteiinille, sillä VP24 esti tyypin III IFN-λ1 geenieksпressiota. Kolmannessa osatyössä laajensimme tutkimusasetelmaamme kaikkiin saatavilla oleviin filovirus VP24 proteineihin ja tutkimme näiden vaikutusta IFN-ß ja IFN-λ1 vasteeseen. Lisäksi selvitimme VP24 proteiinien tumalokalisaation ja interferoni geenieksпressioiden erot filovirus VP24 proteiinien välillä.

Väitöskirjan havaitsemat uudet ilmiöt tuovat filovirusten elämänkaareen ja molekyylibiologiaan uutta tutkimustietoa. Nämä mekanismit tarjoavat paremman mahdollisuuden tulevaisuuden lääkekehityksen kannalta, sillä VP24 on mahdollinen spesifi kohde tulevaisuuden lääkeinnovaatioissa.

AVAINSANAT: Filovirus, Ebola virus, Marburg virus, RIG-I, MDA5, tyypin I interferoni, tyypin III interferoni, VP24

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Abbreviations

ATP	adenosine triphosphate
AS03	adjuvant system 03
BDBV	Bundibugyo virus
BOMV	Bombali virus
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
DC	dendritic cell
DIC	disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxiribonucleic acid
DRC	Democratic Republic of Congo
ds	double-stranded
EBOV	Zaire ebola virus
EDTA	ethylenediaminetetraacetic acid
ELISA	the enzyme linked immunosorbent assay
EVD	Ebola virus disease
FBS	fetal bovine serum
FCA	Freund's complete adjuvant
GAPDH	glyceraldehyde 3-phophate dehydrogenase
GFP	green fluorescent protein
GP	glycoprotein
GST	glutathione S-transferase
HCV	hepatitis C virus
HEK293	human embryonic kidney 293 cell
HuH7	human hepatoma 7 cell
IFA	immune fluorescent assay
IFN	interferon
IFNAR	interferon-alpha/beta receptor alpha chain
IgG	immunoglobulin G
IgM	immunoglobulin M

IKK	inhibitor of kappaB kinase
IL	interleukin
IPTG	isopropyl-beta-thiogalactopyranoside
IRF3	interferon regulatory factor 3
IRF7	interferon regulatory factor 7
JAK-STAT	Janus kinase–signal transducer and activator of transcription
kD	kilodalton
KPNA	karyopherin alpha
LLOV	Llovio virus
MAPK	mitogen activated protein kinase
MARV	Marburg virus
MAVS	mitochondrial antiviral signaling protein
MCP	monocyte chemoattractant protein
MDA5	melanoma differentiation-associated protein 5
MEF	mouse embryonic fibroblast
MEGA 7	Molecular Evolutionary Genetics Analysis Computing Platform 7
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLAV	Mengla virus
MOF	multiorgan failure
mRNA	messenger ribonucleic acid
MUSCLE	Multiple Sequence Comparison by Log Expectation
mut	mutant
MVD	Marburg virus disease
MxA	myxovirus resistance protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
NLR	nucleotide-binding oligomerization domain-containing like receptors
NLS	nuclear localization site
NOD	nucleotide-binding oligomerization domain-containing
NP	nucleoprotein
NPC1	Niemann-Pick disease type C1
ORF	open reading frames
PACT	PKR activating protein
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	protein kinase R
PRR	pattern recognition receptor
RAVV	Ravn marburg virus

RdRp	RNA-dependent RNA polymerase
RESTV	Reston ebola virus
RIG-I	retinoic acid-inducible gene
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RSV	rous sarcoma virus
RT	room temperature
RT-PCR	Real time polymerase chain reaction
Sf9	Spodoptera frugiperda
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGP	soluble glycoprotein
ssGP	small soluble glycoprotein
ss	single-stranded
STAT	signal transducer and activator of transcription
SUDV	Sudan ebola virus
TAFV	Tai Forest ebola virus
TBK1	TANK binding kinase 1
TIM-1	type I membrane protein
TNF	tumor necrosis factor
TLR	Toll-like receptor
TRAF	tumor necrosis factor receptor-associated factor
UTR	untranslated region
VP	viral protein
WHO	World Health Organization
WB	western blot
wt	wild type
XILV	Xilang virus

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Krister Melén, Laura Kakkola, Felix B. He, Kari Airenne, Olli Vapalahti, Helen Karlberg, Ali Mirazimi & Ilkka Julkunen. Production, purification and immunogenicity of recombinant Ebola virus proteins – A comparison of Freund's adjuvant and adjuvant system 03. *J. Virol. Methods*. 2017;242:35–45. doi: 10.1016/j.jviromet.2016.12.014.

II Felix B. He, Krister Melén, Sari Maljanen, Rickard Lundberg, Miao Jiang, Pamela Österlund, Laura Kakkola & Ilkka Julkunen. Ebolavirus protein VP24 interferes with innate immune responses by inhibiting interferon-λ1 gene expression. *Virology* 2017;509:23–34. doi:10.1016/j.virol.2017.06.002.

III Felix B. He, Hira Khan, Moona Huttunen, Pekka Kolehmainen, Krister Melén, Sari Maljanen, Mengmeng Qu, Miao Jiang, Laura Kakkola & Ilkka Julkunen. Filovirus VP24 proteins differentially regulate RIG-I dependent type I and III interferon promoter activation. *Frontiers in Immunology* 2022 Jan 05; 12:694105

1 Introduction

Filoviruses are unsegmented viruses that belong to the order *Mononegavirales* and the *Filoviridae* family is one of the eight mononegaviral families (Afonso et al., 2016). Typical characteristics of these viruses are their filamentous virion structure, long genomes containing overlapping genes, transcriptional initiation and termination signals and unique structural proteins. Filoviruses have a helical nucleocapsid which also contains several viral proteins that regulate RNA transcription and replication. The filovirus genome encodes seven different proteins, which all have a specific role in virus life cycle (Beniac, Lamboo, & Booth, 2018). Currently there are six filovirus genera: *Ebolavirus*, *Marburgvirus*, *Cuevavirus*, *Dianlovirus*, *Striavirus* and *Thamnovirus*. Altogether these include 12 virus species and some of them are highly pathogenic to humans (Burk et al., 2016; Kuhn et al., 2010). The first reported filovirus outbreak occurred in August 1967 and it was caused by Marburg virus (Slenczka & Klenk, 2007). Since then, there have been number of filovirus outbreaks near equatorial Africa mainly caused by ebolaviruses (Burk et al., 2016).

Filovirus infection in humans is often characterized by a systemic virus replication leading to severe outcomes such as liver failure, coagulation abnormalities and multiorgan failure. These symptoms derive from an interaction of the virus and host cells which leads to a dysregulation of inflammatory mediators, disrupted homeostasis, and impaired host immune responses (Feldmann & Geisbert, 2011). Active virus replication is associated with the ability of filoviruses to inhibit host innate immune responses, mainly interferon (IFN) responses. The production of type I and III interferons is a vital innate immune response towards viral infections, which filovirus infection disrupts leading to a weaker antiviral response (Basler & Amarasinghe, 2009). RIG-I-like receptors (RLRs), consisting of RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated 5) and LGP2 (laboratory of genetics and physiology 2), are signaling molecules in the production of interferons (Brisse & Ly, 2019).

This study aimed to evaluate the effects of filovirus proteins on host cell innate immune responses. In this study, efficient expression constructs for different filovirus proteins were created and specific antibodies against viral proteins were

prepared. These reagents enabled the identification of filovirus proteins that interfere with the activation of innate IFN responses and revealed the molecular mechanisms of IFN antagonistic actions of these proteins.

2 Review of the Literature

2.1 Filoviruses

Viruses with single-stranded negative-sense RNA genomes are divided into three subgroups based on their genome structure being as multisegmented, circular or unsegmented. The family *Filoviridae* is characterized by unsegmented negative-sense RNA genome and belongs to the order *Mononegavirales* (Li et al., 2015). Filoviruses obtained their name due to their macroscopic appearance as filamentous viruses and by their genomic sequence. Also, the virus family is characterized by long genomes (≈19 kb) containing overlapping genes, transcription initiation and termination signals and unique structural proteins without obvious homologs in other mononegaviruses (Kuhn et al., 2010). The structure of filoviruses are formed by five structural proteins VP24, VP30, VP35, RNA-dependent RNA polymerase (L-protein) and nucleocapsid protein (NP) and also a single glycoprotein spike on the surface of the virus (Ponce, Kinoshita, & Nishiura, 2019).

Filoviruses are currently divided in six genera: *Ebolavirus*, *Marburgvirus*, *Cuevavirus*, *Dianlovirus*, *Striavirus* and *Thamnovirus*. Altogether, there are 12 virus types identified in this family. Out of the 12 viruses, there is evidence that six viruses act as human pathogens. Two of the filovirus infection related diseases, Ebola virus disease (EVD) and Marburg virus disease (MVD), are specifically recognized by the World Health Organization. Filovirus outbreaks occur unfrequently and for the past 50 years there have been 37 outbreaks identified and 11 of them have included more than 100 cases. (Burk et al., 2016).

The virus interacts with its host by entering through mucous membranes and infecting a variety of different cells, for example macrophages and dendritic cells. Filoviruses are entered the cell by endocytosis which is followed by a fusion of viral and endosomal membranes, and release of viral nucleocapsid into the cell cytoplasm. In the cytoplasm mRNAs are synthesized from the viral RNA template. The virus continues to spread in the host cells due to a suppression of innate immune response, especially targeting type I and III interferon gene expression. This will eventually lead to multiorgan failure which is a common end point in EVD patients (Elliott, Kiley, & McCormick, 1985; Feldmann & Geisbert, 2011; Mühlberger, 2007; Mühlberger, Lötfering, Klenk, & Becker, 1998; Ponce et al., 2019).

Due to the high fatality rates of EVD and MVD outbreaks, Ebola virus and Marburg virus have been the main focus of filovirus research. Yet the reasons for occasional filovirus emergence in human and other mammal populations is still unknown. A better understanding of the epidemiology, pathogenesis and immunity of these viruses is needed.

2.1.1 History of Filoviruses

Infectious diseases have been the determinant factor of survival throughout human evolution. Infectious diseases are caused by micro-organisms, which cause damage on organs and tissue when entering the host cells (Nii-Trebi, 2017). Approximately 25% of all deaths worldwide are caused by infectious diseases (Fauci, Touchette, & Folkers, 2005). Filovirus disease outbreaks have been of public health concern over the past 50 years, due to their sporadic appearance and unpredictability as they are zoonotic viruses. These viruses are transmitted from wildlife reservoirs to humans and then by human-to-human transmissions.

The first discovery of filovirus outbreak was in 1967 in Germany and former Yugoslavia. A previously unknown pathogen, later named as Marburg virus (MARV), caused high fever of laboratory workers after handling tissues and organs of grivets (*Chlorocebus aethiops*) imported from Uganda (Languon & Quaye, 2019; Martini, Knauff, Schmidt, Mayer, & Baltzer, 1968). After the first outbreak there have been 13 outbreaks. Most of the outbreaks have occurred in Africa. Some outbreaks which were not initially recognized in Africa were later traced back there.

The other important virus in the filovirus family, Ebola virus, was discovered in 1976. There were two simultaneous outbreaks during the same year: one in Sudan and one in Democratic Republic of Congo (DRC) (Team, Branch, Division, Control, & Eradication, 1978). The outbreak in Sudan occurred in four different towns: Nzara, Maridi, Tembura, and Jub. Nzara was reported as the source of the outbreak since almost 40% of the workers of a cotton factory were infected. It was thought that the primary infection was due to bats hanging from beams of the factory (Breman et al., 2016; Team et al., 1978). Since then, there has been numerous outbreaks of EVD. So far there has been 37 outbreaks, which have been caused by different ebolavirus species. Bundibugyo virus (BDBV), Ebola virus (EBOV), Sudan virus (SUDV) and a single case of Taï Forest virus (TAFV) have been shown to cause human filovirus disease as EVD (Burk et al., 2016; Languon & Quaye, 2019).

Bats have been a suspected natural reservoir of filoviruses as the virus persists in them and viruses can be potentially transmitted to potential human hosts (Hayman, 2016). Even though most of the epidemics have been in Africa, filovirus specific antibodies have been detected around the world, including Bangladesh, Philippines, Singapore, China, Spain and Hungary (Kemenesi et al., 2018; Laing et al., 2018;

Negredo et al., 2011; Olival et al., 2013; Taniguchi et al., 2011; Yang et al., 2019). Altogether, the epidemics have one common factor: humans interact without any protection with possible pathogen carriers or hunt wild animals. These actions pose a threat of cross-species filovirus transmission (Kurpiers et al., 2015).

2.1.2 Taxonomy and structure of filoviruses

The members of the family *Filoviridae* are characterized by their variously shaped, often filamentous, enveloped virions. Filovirus genome is approximately 15–19 kb long, linear, negative-sense, non-segmented RNA. Filovirus group is assigned in the order of Mononegavirales for their unsegmented RNA genome (Afonso et al., 2016). The most recent taxonomy of filoviruses was published in 2019 which defined six genera of viruses in filovirus family including 12 species: Bundibugyo virus (BDBV), Tai Forest virus (TAFV), Zaire Ebola virus (EBOV), Bombali virus (BOMV), Sudan virus (SUDV), Reston virus (RESTV), Llovio virus (LLOV), Marburg virus (MARV), Ravn virus (RAVV), Menglà virus (MLAV), Xilang virus (XILV) and Huangjiao virus (HUJV) (Kuhn et al., 2019) (Table 1.).

Table 1. Taxonomy of filoviruses.

Family	Genus	Species	Virus	Host	Human pathogen
Filoviridae	Cuevavirus	Llovio cuevavirus	Llovio virus (LLOV)	Mammalian	No
	Marburgvirus	Marburg marburgvirus	Marburg virus (MARV)	Mammalian	Yes
			Ravn virus (RAVV)	Mammalian	Yes
	Ebolavirus	Bundibugyo ebolavirus	Bundubugyo virus (BDBV)	Mammalian	Yes
		Bombali ebolavirus	Bombali virus (BOMV)	Mammalian	No
		Zaire ebolavirus	Ebola virus (ZEBOV)	Mammalian	Yes
		Reston ebolavirus	Reston virus (RESTV)	Mammalian	No
		Sudan ebolavirus	Sudan virus (SUDV)	Mammalian	Yes
		Tai Forest ebolavirus	Tai Forest virus (TAFV)	Mammalian	Yes
	Dianlovirus	Měnglà dianlovirus	Měnglà virus (MLAV)	Mammalian	No
	Striavirus	Xīlǎng striavirus	Xīlǎng virus (XILV)	Piscine	No
	Thamnovirus	Huángjiāo thamnovirus	Huángjiāo virus (HUJV)	Piscine	No

The ribonucleoprotein (RNP) complexes are composed of genomic RNA, the structural proteins nucleoprotein (NP), polymerase co-factor viral protein 35 (VP35), transcriptional activator (VP30), RNP-associated protein (VP24) and RNA-dependent RNA polymerase (L). VP40 protein forms a layer under the envelope and acts as a matrix protein. Glycoprotein (GP) forms spikes on the virion surface (Bharat et al., 2011; Kirchdoerfer, Wasserman, Amarasinghe, & Saphire, 2017; Sugita, Matsunami, Kawaoka, Noda, & Wolf, 2018). The genomes of filoviruses are organized like most of the other *Mononegavirales* genomes: 3′-NP-VP35-VP40-GP-VP30-VP24-L-5’. Filoviruses differ from each other by having additional genes

between the common order. Replication and transcription promoters are included in terminal leader and trailer sequences. Marburgvirus genomes consist of seven continuous open reading frames (ORFs), which are responsible for encoding the structural proteins. Ebolavirus and Cuevavirus genomes encode the homologues of Marburgvirus structural proteins except the difference in GP where Marburgvirus genome encodes GP subunits GP1 and GP2 and Ebolavirus/Cuevavirus genome encodes additional soluble GP (sGP). The new members of the family, Dianlovirus, Striavirus and Thamnovirus encode some homologues of Marburgvirus proteins while the other proteins have remained uncharacterized (Kuhn et al., 2019; Shi et al., 2018).

2.1.3 Epidemiology

The outbreaks caused by filoviruses have been a public health concern for over 50 years. They cause spontaneous and unpredictable outbreaks. The clinical picture in MVD or EVD outbreaks are very severe since the average lowest case fatality rate in the outbreaks have been around 30% and the highest approximately 80% (Burk et al., 2016). Filoviruses are zoonotic pathogens, which means that they most likely originate from wildlife reservoirs such as bats, rodents or fish which cause spillover infections in humans. This is followed by human-to-human transmission and the pathogen can spread in the population (Hartman, Towner, & Nichol, 2010).

MVD and EVD epidemics have to meet certain criteria in order for them to be called outbreaks. Firstly, there has to be a suspected case (alive or dead) with distinct symptoms of EVD or MVD, such as high fever. Secondly, a known or a proven contact with a suspected, probable or confirmed, ebolavirus or marburgvirus case with a sick or dead animal with the symptoms of bloody diarrhea, bleeding from gums, bleeding into skin, or bleeding into eyes or urine. A suspected case means non-diagnosed disease with a link with a confirmed case (positive laboratory result). The usual laboratory tests include reverse transcriptase-polymerase chain reaction (RT-PCR) or the detection of IgM antibodies against marburgviruses or ebolaviruses (World Health Organization, 2014).

2.1.3.1 Marburgvirus outbreaks

The first case of MVD (1967) led to 31 reported cases of MVD and 7 deaths. After the discovery of the virus, there has been 13 outbreaks of MVD most of which have occurred in Africa. The outbreaks have been caused by both members of the *Marburgvirus* genus: MARV and RAVV (Languon & Quaye, 2019).

Later confirmed cases of MVD occurred in 1975 (South Africa), 1980 (Kenya), 1987 (Kenya) and 1990 (Russia). Those outbreaks were small with less than 5

confirmed cases and the outbreaks led to one human casualty each. In 1975, 1980 and 1987 the outbreaks were closely related to bats as the index cases (a patient in an outbreak who is first noticed by the health authorities) were in direct contact with bats either by accident or by visiting forest areas or caves where the primary infection occurred. Also, the cause of 1987 outbreak was the discovery of a new Marburg-like virus, later named as Ravn virus (Johnson et al., 1996; Smith et al., 1982; Trappler et al., 1975). The case of MVD in Russia in 1990 was a laboratory acquired infection when the worker contracted the disease from a virus positive serum sample (Languon & Quaye, 2019).

The largest outbreaks in MVD history occurred in 1998 and 2005. It started in 1998 in the DRC when a group of gold miners reported to have been in contact with animal excretions in the mine. The miners reported to have seen different animals such as rodents, bats, frogs, shrews, cockroaches and moth flies in the mines. None of the workers reported to have been in direct contact or bitten by the animals. However, the positive cases were microbiologically confirmed to be infected by RAVV and it was discovered that there were at least nine lineages of the virus in circulation among the infected patients (Bausch et al., 2006). The outbreak came to an end in 2000 when the mine got flooded. Altogether during 1998-2000 the outbreak in DRC led to 154 reported cases with 128 deaths. The largest outbreak from MVD took place in 2004 in Angola and lasted until 2005. During this time, due to the lack of modern facilities and epidemiological strategies together with the problems in the surveillance and identification of the pathogens with poor tracing of the cases led to an unknown index case and the lack of epidemiological study. The Angola outbreak was estimated to include 252 cases of MVD and 227 deaths (Towner et al., 2006).

The other MVD outbreaks occurred in the timespan of 2007 to 2017. All these small outbreaks were moderate or small and all took place or originated in Uganda. In 2007 there were four confirmed MVD cases (MARV and RAVV) which traced back again to the mines where the workers got exposed to bats' excreta (Adjemian et al., 2011). In 2008, there were two separate cases of MVD as a tourist from the United States of America and a tourist from the Netherlands visited Uganda viewing wildlife and visiting caves. The American tourist survived while the Dutch tourist died (Leggiadro, 2010; Timen, 2009). The latest outbreaks were noticed in 2012, 2014 and 2017 which have altogether led to 18 confirmed cases and eight deaths. The origin of the infection in these outbreaks have again been traced to mines and bats (Knust et al., 2015; Nyakarahuka et al., 2017, 2018).

2.1.3.2 Ebolavirus outbreaks

The first reported cases of EVD were in 1976 when almost simultaneously there were two outbreaks caused by different ebolaviruses: SUDV and EBOV. These outbreaks occurred in Sudan and DRC and led to 602 confirmed cases and 431 deaths (Burke, Declerq, & Ghysebrechts, 1978; Team et al., 1978). The primary source of the virus was suggested to be bats that were hanging from beams in a cotton factory in Sudan (Team et al., 1978). Since then, there has been a total of 37 EVD outbreaks. The relatively large and fatal outbreaks in the beginning of 1976 was followed by a single case in England where a researcher was accidentally infected by a needle stick during the transfer of homogenized liver sample from a SUDV-infected rodent. The researcher survived (Bowen & Lloyd, 1977). In the next year 1977 a 9-year-old girl was infected by EBOV and later died (Heymann et al., 1980). The next outbreak in 1979 occurred again in Nzara, Sudan, in the same village where the first ever outbreak of Ebola virus occurred. The index case was reported to have been working in the same cotton factory where the Ebola virus was first discovered, all other cases were originated from human transmissions. The outbreak of SUDV led to 34 cases and 22 deaths during that year (Baron, McCormick, & Zubeir, 1983).

The next confirmed EVD cases took place in the United States of America in 1989 and 1990 when there was an EVD outbreak in primates housed in quarantine facilities in Virginia and Pennsylvania. This outbreak was traced to the importation of cynomolgus monkeys (*Macaca fascicularis*) from the Philippines which were infected by RESTV (CDC, 1990). Seven animal handlers had serological evidence of an infection with very mild symptoms and all workers recovered (Laing et al., 2018). In 1994 there were two outbreaks, one in Gabon (EBOV) and one in Côte d' Ivoire (Taï Forest virus, TAFV). The Gabon outbreak most probably originated from a disruption of the forest canopy by gold mining activities which led to exposure of litter and animal contact. The outbreak caused 52 cases and 31 deaths (Georges et al., 1999). A single case of TAFV originated from a researcher carrying a chimpanzee which later led to the isolation of a new Ebola virus (Le Guenno et al., 1995). A larger outbreak occurred in 1995 in DRC where 315 cases were reported and 250 patients died from the EVD caused by EBOV (Khan et al., 1999).

During the years 1996 to 2000 there were multiple outbreaks. In 1996 and 1997 two separate EBOV outbreaks in Gabon where 37 cases and 60 cases led to 21 and 45 deaths in their respective outbreaks. Both outbreaks likely originated from a close contact between humans and wild animals such as chimpanzees (Georges et al., 1999). Later that year the first outbreak in Southern parts of Africa occurred in Gabon in South Africa when a nurse was exposed to a sick medical personal's blood. In addition, in 1996 a Russian scientist was infected by a laboratory acquired infection of EBOV and died (Languon & Quaye, 2019).

In year 2000 there was one of the largest outbreaks during that time in Uganda where 425 reported cases were identified with 224 deaths. Even though the index case was not known, it was speculated that most of the secondary infections were contracted in the funeral of the index case (Okware et al., 2002). Since then, there has been several SUDV and EBOV outbreaks in the African region. In 2001 to 2003 in Gabon and DRC there were multiple EVD outbreaks originating from the contacts between humans and gorillas, chimpanzees, monkeys, forest duikers or porcupines ("Outbreaks of Ebola haemorrhagic fever, Congo and Gabon, October 2001-July 2002" 2003). In 2004, a SUDV outbreak occurred in Southern Sudan where a hunter killed a baboon and was in contact with fresh monkey meat ("Outbreak of Ebola haemorrhagic fever in Yambio, south Sudan, April - June 2004" 2005). In the same year there was a laboratory acquired infection in Russia. In 2005 and 2007 two outbreaks of EBOV took place in DRC where the primary source of the virus was probably a direct contact with blood of bats from the food market (Languon & Quaye, 2019). In 2007 a new ebolavirus species was discovered in a town of Bundibugyo, where 131 cases were confirmed and later the virus was named as Bundibugyo virus (BDBV). The BDBV outbreak led to 42 deaths (Towner et al., 2008). Between 2008 to 2013 there were multiple EBOV, SUDV and BDBV outbreaks in Africa, which all led to casualties. Most outbreaks were traced back to a possible zoonotic exposure (Languon & Quaye, 2019).

The largest outbreak in the filovirus history occurred during December 2013 to March 2016 in West Africa which led to 28 616 cases of EVD caused by EBOV. The outbreak led to 11 310 deaths making EVD as one of the deadliest zoonotic pathogens. The index case was a 2-year-old boy from a small village in Guinea. It is believed that the boy had been infected by insectivorous bats (Marí Saéz et al., 2015). The outbreak quickly got out of control and spread to neighboring countries, Liberia and Sierra Leone. This was also the first time when the transmission of the EVD was very effective and sporadic cases among the travelers were reported in Italy, Mali, Nigeria, Senegal, Spain, the United Kingdom and the United State of America (Languon & Quaye, 2019). In the meantime, while there was the largest outbreak in West Africa, a new outbreak occurred in DRC (Maganga et al., 2014).

Since the West African outbreak of EVD, DRC has been the risk area for EVD. Since 2017 several outbreaks of EVD have been a problem in DRC even though it has been reported that the outbreaks have not been connected with each other. Due to limited services and resources, the index cases of these outbreaks are yet to be identified. The most recent outbreak started in August 2018 in the Province of North Kivu only a week after the previous outbreak had been declared over. (Barry et al., 2018; Languon & Quaye, 2019). According to WHO, the latest EVD outbreak was declared over in June 2020 and altogether 3481 cases were reported with 2299 deaths.

Table 2. Known filovirus outbreaks.

Year	Country	Virus	Cases and case fatality	
1967	Germany and Yugoslavia	MARV	31	(23%)
1975	South Africa	MARV	3	(33%)
1976	Sudan	SUDV	284	(53%)
1976	DRC	EBOV	318	(88%)
1976	England	SUDV	1	(0%)
1977	DRC	EBOV	1	(100%)
1979	Sudan	SUDV	34	(65%)
1980	Kenya	MARV	2	(50%)
1987	Kenya	RAVV	1	(100%)
1989	Philippines and United States	RESTV	7	(0%)
1990	Russia	MARV	1	(100%)
1994	Gabon	EBOV	52	(60%)
1994	Côte d' Ivoire	TAFV	1	(0%)
1995	DRC	EBOV	315	(79%)
1996	Gabon	EBOV	37	(57%)
1996	Gabon	EBOV	60	(75%)
1996	South Africa	EBOV	2	(50%)
1996	Russia	EBOV	1	(100%)
1998	DRC	MARV & RAVV	154	(83%)
2000	Uganda	SUDV	425	(53%)
2001	Gabon and DRC	EBOV	124	(78%)
2002	DRC	EBOV	143	(90%)
2003	DRC	EBOV	35	(83%)
2004	Sudan	SUDV	17	(41%)
2004	Russia	EBOV	1	(100%)
2004	Angola	MARV	252	(90%)
2005	DRC	EBOV	12	(83%)
2007	DRC	EBOV	264	(70%)
2007	Uganda	BDBV	131	(32%)
2007	Uganda	RAVV & MARV	4	(25%)
2008	Uganda	MARV	1	(0%)
2008	Uganda	MARV	1	(100%)
2008	Philippines	RESTV	6	(0%)
2008	DRC	EBOV	32	(47%)
2011	Uganda	SUDV	1	(100%)
2012	Uganda	MARV	15	(27%)
2012	Uganda	SUDV	11	(36%)
2012	DRC	BDBV	36	(36%)
2012	Uganda	SUDV	6	(50%)
2013	Multiple countries	EBOV	28,616	(40%)
2014	DRC	EBOV	69	(71%)
2014	Uganda	MARV	1	(100%)
2017	Uganda	MARV	3	(100%)
2017	DRC	EBOV	8	(50%)
2018	DRC	EBOV	54	(61%)
2018	DRC	EBOV	3470	(66%)

2.1.4 Ebolavirus genome and protein functions

Ebolaviruses are filamentous, enveloped RNA viruses that contain up to 19 kilobase long genome. The genome encodes seven to nine proteins from one genomic negative sense RNA segment (Elliott et al., 1985). There are short extragenic regions

in both ends of the genome called leader and trailer sequences, which contain replication and transcription promoters as well as encapsidation signals. The initiation of viral RNA synthesis by RNA-dependent RNA polymerase (RdRp) L, is signaled by the leader region. There are also signals in the leader region that direct packaging of full-length, negative strand copies of the viral genome in nucleocapsids. The promoter of genome replication is located in the trailer region at the 3' end of the antigenomic RNA (Crary et al., 2003).

Figure 1. Schematic illustration of EBOV genome. In general, filovirus genome are organized as mononegavirus genomes with a general gene order.

The seven proteins encoded by ebolaviruses are GP, RdRp (L), NP, and four viral proteins (VPs): 24, 30, 35, and 40. The gene order of EBOV genome is NP, VP35, VP40, GP/sGP, VP30, VP24, and L (Kawaoka, 2005; Kuhn et al., 2019) (Fig. 1.). The ribonucleoprotein complexes (RNP) consist of NP and VP30 both of which interact and protect the viral RNA. RNPs also include VP24, VP35 and RdRp (L) (Volchkov et al., 1999). Each protein has specific functions during the viral life cycle. GP is the type 1 transmembrane protein and it is the only surface protein on the viral envelope and is required for the binding and fusion mediating the entry of the virus into the host cell (Carette et al., 2011). EBOV GP is heavily N- and O-glycosylated. The protein is cleaved into two subunits (GP1 and GP2) on the viral surface and it exits as a trimeric protein complex (peplomers). Besides the full-length GP, the Ebola virus encodes two other forms of the GP: nonstructural soluble glycoprotein (sGP) and small soluble GP (ssGP). sGP and ssGP have been suggested to function as decoy molecules and neutralize EBOV GP-specific antibodies (Mehedi et al., 2011). L-polymerase is the main component of the RdRp complex. VP24 is essential in virion assembly and it antagonizes type I and III IFN responses. VP30 is involved in viral mRNA synthesis and it may regulate transcriptase and replicase functions of the RdRP complex (Messaoudi et al., 2015). VP35 is a polymerase cofactor and it interacts with the RdRp complex and also inhibits IFN production while VP40 is a matrix protein and required in virion assembly and budding (Rivera & Messaoudi, 2016) (Table 3.).

Table 3. Known filovirus protein functions.

Ebola virus proteins	Protein functions
VP24	- Minor matrix protein, virion assembly - Inhibits type I and type III interferon (IFN) gene expression - Inhibits type I and III IFN signaling reducing the expression of IFN-induced genes (blocks STAT1/2 nuclear import)
VP30	- Minor nucleoprotein - Transcription activator
VP35	- Polymerase co-factor - Binds dsRNA and inhibits type I IFN production - Inhibits dendritic cell maturation - Blocks IKKε/TBK1 activation and IRF3 phosphorylation
VP40	- Viral matrix protein - Required in virion assembly and budding
NP	- Structural protein of nucleocapsid complex - Catalyzes viral replication and transcription of the RNA genome
GP	- Viral envelope glycoprotein - Attachment to host cell surface - Mediates virus entry - Target of anti-GP neutralizing antibodies
sGP (ssGP)	- Soluble glycoprotein (small soluble GP) - Possible decoy of anti-GP antibodies
L polymerase	- Viral RNA-dependent RNA polymerase

2.1.5 Virus life cycle

Ebolaviruses attach on the target cell membrane via surface protein GP. So far there is no evidence of a specific viral receptor for ebolaviruses. It has been suggested that integrins, C-type lectins, and TIM-1 may serve as cellular receptors (Mühlberger, 2007). Also, endosomal protein NPC1 has been shown to attach to GP through domain C which triggers membrane fusion (Wang et al., 2016). After the initial binding of the virus on the host cell membrane, virions enter the cell via endocytosis. This is followed by the fusion of viral and endocytic membranes, and the release of the RNP into the cytoplasm (Ito et al., 2001; Watanabe et al., 2000). Subsequently, in the cytoplasm the L-polymerase binds to the leader region of the viral RNA and begins to transcribe individual genes sequentially in a 3′ to 5′ direction. The synthesis of viral mRNAs is detectable approximately 6-7 h after the primary infection

(Sanchez & Kiley, 1987). Out of the seven viral genes, NP is the most abundantly transcribed gene and L-polymerase is the least transcribed. The transcription is dependent on transcription factor VP30 (Mühlberger et al.,1999). Continued transcription and translation of virus genes leads to continuously increasing quantity of viral proteins, especially NP, which in turn results in greater rate of viral replication (Mühlberger et al., 1998, 1999). The transcribed viral mRNAs are 5′-capped and 3′-polyadenylated by the L-protein. At the same time, replication of negative-sense viral genomic RNA results in full-length positive-sense antigenomic RNAs which serves as templates for the synthesis of additional negative-sense viral genomes (Rivera & Messaoudi, 2016). Ultimately, when the amount of negative-sense genomes and viral proteins increase, the assembly and budding of the newly formed viruses takes place at the plasma membrane with the aid of newly formed nucleocapsids, VP24, VP40 and GP (Rivera & Messaoudi, 2016).

2.2 Genus Ebolavirus

There are six ebolaviruses: Bundibugyo virus (BDBV), Bombali virus (BOMV), Ebola virus (EBOV), Sudan virus (SUDV), Reston virus (RESTV) and Taï Forest virus (TAFV). Out of the six ebolaviruses, four are known human pathogens and only BOMV and RESTV have not caused disease in humans (Burk et al., 2016; Languon & Quaye, 2019). So far the largest outbreak of filovirus infections were caused by EBOV in West Africa, in Guinea, Sierra Leone, and Liberia in 2014–2015 (Sylvain Baize et al., 2014). Ebolavirus particles have a uniform diameter of 80 nm with a varying up to 10 000-14 000 nm in length (Feldmann & Geisbert, 2011). EBOV is classified as a biothreat pathogen of category A due to its high case-fatality rate and limited treatment and vaccination options. It is also a potential bioterrorism agent and considered as one of the deadliest human pathogens (Borio et al., 2002).

2.2.1 Ebola virus disease and transmission

In humans EVD is clinically a severe disease characterized by high fever with diarrhea, vomiting and hemorrhagic manifestations, and suppressed immune responses which will possibly lead to sepsis-like symptoms and shock (Feldmann & Geisbert, 2011). The disease caused by Ebola virus targets numerous tissues in the human body. Infection also targets many cell types which ultimately leads to dysregulation of inflammatory mediators, disrupted homeostasis, and impaired host immune responses. Above that, EVD usually leads to the insufficiency of coagulation and vascular system leading to multiorgan failure and disseminated intravascular coagulation (DIC) (Borio et al., 2002; Feldmann & Geisbert, 2011).

The majority of ebolavirus outbreaks have been caused by EBOV and the outbreaks have been located in either Central or West Africa. EBOV has a suspected natural reservoir in animals, mainly bats, but humans transmit the infection via blood, serum or bodily secretions (Feldmann & Geisbert, 2011). Studies have shown that patients that have recovered from the primary infection of EVD excrete the virus for several weeks or even months via genital tract, especially semen (Deen et al., 2017). Humans are considered as the end hosts of Ebolaviruses (Groseth, Feldmann, & Strong, 2007). Many ecological studies speculate the possible reservoir of filoviruses, especially since ebolaviruses are so lethal and usually emerge suddenly. To date there are no firm links to natural reservoirs of ebolaviruses, but many studies suggest that most likely bats and rodents play a significant role in virus transmission as many outbreaks were traced back to caves and mines and to contact with either litter or raw meat (Berge, Bowong, Lubuma, & Manyombe, 2018; Leroy et al., 2005; Morvan et al., 1999; Swanepoel et al., 1996). Strong evidence of transmitting the disease has been shown between fruit bats and their close contact with humans or as they are being hunted for food. Ebola virus may reside silently in natural reservoirs and be activated from a stimulus such as stress, coinfection, pregnancy, ecological changes and a change in food habits (Gupta et al., 2004; Strong et al., 2008).

In the outbreaks of EVD the dominant mode of transmission has been from human-to-human either through mucosa or lacerations (Fischer et al., 2015). The average incubation time when considering infections in human-to-human transmissions have been approximately 9–10 days (Ajelli et al., 2015). A deeper analysis of the source of infective virus between the patient and the secondary case(s) indicate that infectious body fluids play a big part of transmissions in EVD (Dowell et al., 1999). A large meta-analysis done on secondary transmissions showed that less than 1% cases were infected by Ebola virus when the person was not in direct contact with an EVD patient (Dean, Halloran, Yang, & Longini, 2016).

Once the patient has been in contact with a virus positive patient and transmission has occurred, symptoms most likely arise in 4–10 days after the exposure. Although there is a wide variation in incubation time ranging from 2 days to even 21 days, the common incubation time is around 10 days (Jeffs, 2006; Kortepeter, Bausch, & Bray, 2011). Typically, symptoms begin with flu-like symptoms, and they progress to much more severe symptoms later, such as fever, myalgia, and chills. There are also gastrointestinal symptoms such as diarrhea and vomiting, and after this phase the disease may progress to even more fatal symptoms such as hemorrhagic complications, anuria, dysthesia, and sepsis-like symptoms resulting in multiorgan failure. Other symptoms may include headache, profound weakness, coughing, and rhinorrhea. There may also be cardiovascular symptoms which often leads to a septic shock and edema (Feldmann & Geisbert, 2011; Hartman et al., 2010; Jeffs, 2006). EVD patients are also characterized by some hematological

changes. The changes in laboratory parameters include leukopenia, decreased neutrophil counts, and increase in liver enzymes. When the infection progresses, patients often show thrombocytopenia, prolonged prothrombin time, and activated partial thromboplastin time which will ultimately lead to DIC and multiorgan failure followed by death (Feldmann & Geisbert, 2011). Patients who survive EVD have been developing long-term symptoms and disorders such as recurrent hepatitis, myalgia, arthralgia, prolonged hair loss, psychosis, and uveitis as 75% of survivors report at least one cardinal symptom after recovery (Feldmann & Geisbert, 2011; Hartman et al., 2010; Tozay et al., 2020).

2.2.2 Diagnosis and treatment

The standard test for diagnosing EVD has been real-time RT-PCR test, which was the primary laboratory test also in the West African outbreak during the years of 2013–2016. Typically high levels of viral RNA are detected 3–6 days after the onset of symptoms in patients' blood (De La Vega et al., 2015; Towner et al., 2004). The negative result in the first RT-PCR test does not exclude the presence of Ebola virus, and patients with suspected Ebola virus infection should be tested several times within 72 hours from the first sample (Rieger et al., 2016). The severity of the disease has a close link between viral load and fatal infections tend to show 10–100 fold higher serum viral loads than the survivors (Faye et al., 2015). ELISA-based EBOV-specific IgG and IgM antibody detection methods have also been developed but these serological methods have a bigger role in to diagnosing paucisymptomatic or asymptomatic Ebola virus infections (Glynn et al., 2017). Nevertheless, due to limited recourses in the epidemic areas, EVD is often diagnosed based on anamnestic information and patient's symptoms (Goeijenbier et al., 2014).

Fortunately, promising therapeutic alternatives have been investigated. The major focus on drug development has been aiming to reduce viral replication to limit cytokine storm and to let the innate and adaptive immune responses to clear the infection. Promising therapeutic alternatives of antiviral compounds and humanized monoclonal neutralizing antibody cocktails have been studied and the latter has been used in EVD patients (Goeijenbier et al., 2014; Malvy et al., 2019). Due to the severity of EVD, WHO declared that it is ethical to use experimental drugs to treat and prevent the disease. However, to date, there are no clinically proven efficient therapeutics to treat EVD and the main treatment for EVD is still supportive care and possibly intensive care.

2.2.3 Vaccine development

Rapid diagnosis and treatment are the cornerstones for reducing mortality in epidemic areas. Another effective way to reduce EVD epidemics is to use vaccines in high-risk areas. Vaccine development targeting filoviruses and especially Ebola virus has been active for decades and after the West African outbreaks the development has been accelerated (Venkatraman et al., 2018). The development of vaccines is a long process and must show good efficacy in experimental EVD models (Geisbert et al., 2002). To date, there are two licensed Ebola vaccines that were qualified by WHO and other health agencies.

A recombinant vesicular stomatitis virus Indiana strain vectored vaccine (Ervebo, rVSV-EBOV), containing EBOV GP, was shown promising results as it was found to be immunogenic in phase 1 trials. The vaccine was found to cause arthritis in Switzerland but this side effect was not observed in African population (Agnandji et al., 2016). The testing of rVSV-EBOV continued to phase 3 trial in Guinea using ring vaccination strategy (inhibiting the spread of disease by only vaccinating the close contacts that are most likely to be infected) (Henao-Restrepo et al., 2015). The trial reported highly effective results as the efficacy was 100% (P=0.0045) (Gsell et al., 2017). The rVSV-EBOV vaccine has been used in the recent outbreak in the DRC as part of WHO's Expanded Access Framework. The strategy for vaccination in the area was a modified ring vaccination as primary contacts and health-care workers were vaccinated but also individuals that might come into contact with EVD patients were also offered the vaccine (Malvy et al., 2019). Ervebo was licensed in November 2019 by European Medicines Agency and prequalified by WHO.

Another licensed vaccine called Zabdeno/Mvabea (Ad26-ZEBOV/MVA-BN-Filo) was authorized in May 2020 and the vaccine is delivered in two doses. Zabdeno is administered first and Mvabea is given approximately 8 weeks later as a second dose. Zabdeno component is a monovalent replication-incompetent adenoviral vector serotype 26 (Ad26) vaccine, which encodes the full-length GP of the EBOV Mayinga variant and Mvabea component consists of a modified Vaccinia Ankara virus (MVA) encoding GPs from EBOV, SUDV, and Marburg virus (MARV), and TAFV nucleoprotein (Tomori & Kolawole, 2021)

Even though remarkable leaps have been taken in the field of ebolavirus vaccines, the long-term durability and immune response to the vaccination remains to be studied (Huttner et al., 2018; Huttner & Siegrist, 2018). rVSV-EBOV works efficiently in emergency situations as the primary strategy for vaccination, when the strategy has been ring vaccination, and providing at least a short-term immunity. Most likely several licensed vaccines will pass the clinical trials within the next decade and the common questions considering safety, duration of immunity, monovalent or polyvalent and storage conditions will be resolved.

2.3 Innate immunity in Ebolavirus infection

Animal species have many natural barriers, such as physical layers (skin, epithelial surfaces), cellular sensors, innate cytokines and innate immune cells, to prevent infections. Unlike many other animal species, humans are susceptible to Ebola virus infections since human cells express presumed Ebola virus receptors, C-type lectins, glycosaminoglycans, phosphatidylserine and TIM and TAM receptors (Davey et al., 2017). Filoviruses in general can infect a broad range of cells including endothelial and epithelial cells, hepatocytes, fibroblasts, and antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) (Olejnik et al., 2011). Ebola virus especially targets macrophages and DCs which acts as bridges between innate and adaptive immunity. EBOV infection disrupts virus-specific responses and prevents or delays antigen presentation to T cells (Geisbert, Hensley, et al., 2003).

Invading and replicating EBOVs are recognized by the host cell via pattern recognition receptors (PRRs) (Fig. 2A.). PRRs recognize pathogens via pathogen-associated molecular patterns (PAMPs) and activate host innate immune responses. RNA virus infection activates PRRs such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide -binding oligomerization domain-containing (NOD)-like receptors (NLRs). Cell membrane-associated the Toll-like receptors (TLR) 7 and 8 are activated by ssRNA leading to the activation and nuclear translocation of transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor 7 (IRF7), and mitogen-activated protein kinase (MAP) kinase-activated transcription factors (MAPK TFs). TLR3, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated antigen 5 (MDA5) are activated by viral ss/dsRNA molecules leading to activation and nuclear translocation of IRF3 (and IRF7), NF-kB and MAPK transcription factors (Jensen & Thomsen, 2012; Jiang et al., 2015, 2011). Activation of these signaling pathways leads to the production of type I interferons α and β (IFN-α/β) and type III interferons (IFN-λs). Secretion of type I and III IFNs and binding to IFN-receptors result in the activation of JAK–STAT (Janus kinase-signal transducer and activator of transcription) signaling pathway which ultimately leads to the enhanced expression of IFN-stimulated genes (Schoggins, 2014). Enhanced production of type I and III IFNs result in further immunological responses and enhanced expression of PRRs and adaptor molecules, which ultimately leads to the antiviral response (Fig. 2B).

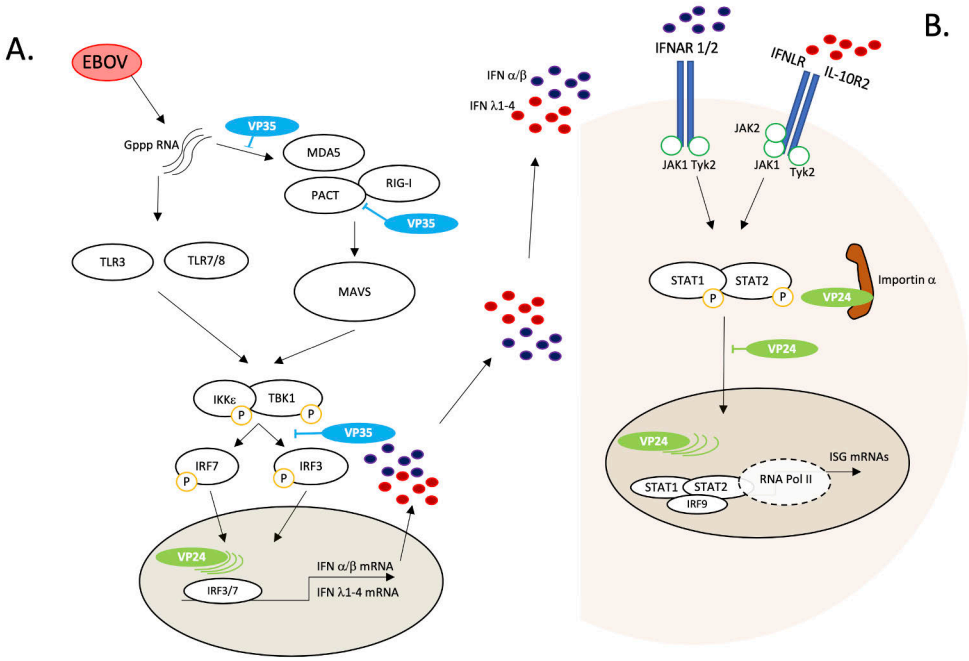

Figure 2. Illustration of the inhibition of type I and III interferons and overall antiviral response. In panel A, two of the seven proteins encoded by EBOV have shown inhibitory effect in previous in vitro studies: VP35 and VP24. In panel B, VP24 blocks the nuclear import of phosphorylated STAT1-STAT2 dimers.

2.3.1 Effects on cytokine gene expression

Filoviruses prefer to replicate in macrophages, dendritic cells, and epithelial cells in nonhuman primates. The unbalanced immune response is at least partly due to the malfunction of these cell types (Geisbert, Hensley, et al., 2003; Geisbert, Young, et al., 2003). There is strong evidence of the link between poor prognosis of EVD patients and excessive cytokine and chemokine production (Reynard et al., 2019). The ability of the virus to spread and infect many different organs is due to its preference to infect macrophages and dendritic cells as these cells travel via blood and lymphatic system to other organs (Geisbert, Young, et al., 2003). The infection of organs leads to strong inflammatory responses and the release of pro-inflammatory cytokines and chemokines, such as interleukin-1β (IL-1β), IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1α (MIP1α), MIP1β, and tumor necrosis factor (TNF) as well as to the release of reactive oxygen species and nitric oxide (S. Baize et al., 2002; Messaoudi et al., 2015; Villinger et al., 1999).

The production of MIP1α and MCP1 is followed by a positive feedback loop where enhanced cytokine production attracts even more monocytes and

macrophages to the infection site, thus enabling virus to infect more cells (Geisbert, Young, et al., 2003). EBOV infection of dendritic cells leads to impaired antigen presentation to T cells. This is due to the inhibition of upregulation of CD40, CD80, CD86, and major histocompatibility complex (MHC) class II molecules, essential factors for the maturation and antigen presentation of dendritic cells (Lubaki et al., 2013; Mahanty et al., 2003). The common characteristic of filovirus infection is lymphopenia, which presents as the lack of $CD4^+$ and $CD8^+$ T cells and natural killer (NK) cells in the blood stream. In addition to defects in cell-mediated immune responses, the reduced levels of $CD4^+$ T cells may result in reduced levels of virus specific immunoglobulin M (IgM) and IgG antibodies (Sylvain Baize et al., 1999; Bradfute et al., 2007; Reed et al., 2004).

The burst of immunological mediators, better known as a "cytokine storm", has been suggested to cause the lethality of EVD (Feldmann & Geisbert, 2011; Wauquier et al., 2010). Cytokine storm is caused by the virus-induced overproduction of pro- and anti-inflammatory factors consisting of IFNs, TNFs, interleukins and chemokines (Reynard et al., 2019; Tisoncik et al., 2012). The exact mechanisms leading to cytokine storm is yet to be discovered but it is likely that the production of pro- and anti-inflammatory mediators occurs in other cell types than macrophages and DCs. This is thought by the fact that the infection of macrophages and DCs suppresses their cytokine and chemokine production, and these cell types play a significant role in antiviral response (Younan et al., 2017). However, previous studies suggested that certain viruses or bacteria can trigger cytokine storm through activation of T-cell receptors and CD28 and/or PAMP recognition pathways (Arad et al., 2011; Scherer et al., 1993).

2.3.2 Downregulation of antiviral responses

2.3.2.1 Effects of type I and III interferon gene expression by Ebolavirus proteins

As stated before, RIG-I-like receptors including RIG-I and MDA5 pathways play a dominant role in innate immune responses to RNA viruses. Both pathways recognize viral RNA and activate interferon gene expression by first activating mitochondrial antiviral signaling proteins (MAVS) which is located in mitochondrial membranes. This leads to a recruitment of tumor necrosis factor receptor-associated factor (TRAF) proteins. TRAF proteins activate inhibitor of kappaB kinases (IKKα/β/γ/ε) and TANK binding kinase 1 (TBK1) followed by phosphorylation of MAVS. Phosphorylated MAVS binds to IRF3 (Liu et al., 2015). Phosphorylated IRF3s are then dimerized and the dimerized complexes are translocated in the nucleus. The

nuclear binding of IRF3 dimers bind to the promoters of type I and type III IFN leading to the expression and secretion of interferons.

One of the main mechanisms to avoid the host antiviral response is by blocking the expression of IFN and IFN-induced genes (Basler & Amarasinghe, 2009). It has been shown that type I and III IFNs (IFN α/β/λ) have a major role in antiviral response in viral infections (Schneider, Chevillotte, & Rice, 2014). Type I and III IFNs are produced by activating RIG-I-like receptors (RLR) and Toll-like receptors (TLR) leading to signaling cascades downstream (Jensen & Thomsen, 2012)

Currently, EBOV VP24 and VP35 have been shown to interfere with the activation of innate immune responses (Messaoudi et al., 2015). VP35 has been shown to interfere with RIG-I signaling pathway and to inhibit the maturation of dendritic cells by preventing the expression of MHC class I and class II and the costimulatory molecules CD40, CD80, and CD86. This impairs antigen presentation to $CD8^+$ and $CD4^+$ T cells and disrupts the link between innate and adaptive immunity (Yen et al., 2014). The interaction between PKR activator PACT and RIG-I is disrupted which results in abnormal activation of RIG-I ATPase (Luthra et al., 2013). VP35 also increases the SUMOylation of IRF7 which leads to the reduced activity of IRF7 and decreased gene expression of IFN-α/β and IFN-λ (Chang et al., 2009; Prins et al., 2009). VP24 is expressed in the cell cytoplasm and nucleus and it inhibits RIG-I-induced IFN gene expression. The mechanism of nuclear interference of IFN gene expression by VP24 is currently unidentified.

2.3.2.1.1 Downregulation of IFN-induced antiviral responses by Ebola virus proteins

One of the main reasons for the lethality of EVD is the ability of EBOV to replicate in many different cell types. EBOV also inhibits host innate immune responses. Several mechanisms have been shown to reduce the antiviral response in EBOV infected cells. After being produced by virus infected cells, IFNs mediate their antiviral actions by binding to their specific receptors: IFN-α/β bind to IFNAR1 and IFNAR2 and IFN-λ1-4 bind to cell-specific receptors composed of IFNLR and IL-10R2 receptor chains. The binding of IFN-α/β/λ to their receptors leads to the activation of the JAK-STAT signaling pathway followed by phosphorylation, via phosphotyrosine-SH2 interactions, and dimerization of STAT1 and STAT2 (Platanias, 2005; Schoggins, 2014). The dimerization leads to exposure of nuclear localization signal (NLS) on STAT1 and STAT2 (Melén et al., 2003; Melén, Kinnunen, & Julkunen, 2001). STAT1 and STAT2 NLSs interact with transport factor importin α (also called karyopherin alpha (KPNA) proteins), which mediate their nuclear import. In the nucleoplasm activated STAT1-STAT2 complexes bind on the promoter elements of IFN-stimulated genes (Melen et al., 2001; McBride &

Reich, 2003). Ebola virus VP24 interacts with antiviral defense system by interfering the nuclear translocation of STAT1 and STAT2 dimers. VP24 has an NLS which mediates binding to importin α molecules followed by nuclear translocation of VP24. Binding of VP24 to importin α prevents the interaction of STAT1-STAT2 complexes with the NLS-binding armadillo domains of importin α which leads to inhibition of nuclear import of STAT1-STAT2 and reduced transcription of IFN-stimulated genes (Xu et al., 2014).

2.3.2.1.2 Effects on translation pathways

Filoviruses have been shown to inhibit the innate immune responses of host cell resulting in effective replication using the host cell machinery. Filoviruses produce mRNAs with long 5′ and 3′ untranslated regions (UTRs). The upstream open reading frames (uORFs) of Ebola virus proteins, VP24 and, VP30, and L-polymerase mRNAs are marked by the upstream AUGs (uAUGs) that are located in the UTRs (Shabman et al., 2013). In normal conditions uORF of L mRNA suppresses the expression of primary L ORF. This suppression requires uAUG. During cell stress, such as an infection, general translation is in general decreased due to phosphorylation of the translation initiation factor eIF-2α. This decreased translational activity allows cells to recover from stress situations. In viral infections this mechanism impairs virus replication. However, in the conditions of stress, cellular mRNAs with uORFs show efficient translation (Wek et al., 2006). A recent study demonstrated the role of L 5′UTR and L uORF in stress conditions and suggested that due to Ebola virus uORF, the virus is able to maintain the production of its L-polymerase even during stress and eIF-2α phosphorylation (Shabman et al., 2013).

3 Aims

The main objectives of this study were to provide better understanding of filovirus life cycle, pathogenesis, and virus–host interactions at molecular level. The aim was to systematically study the effects of filovirus proteins on innate immune and antiviral response. A better understanding of the mechanisms of virus – host interactions will provide us novel means to modulate the immune system and develop better modalities of prevention and treatment of virus infections.

The specific research objectives were:

1. To express Ebola virus proteins, create research tools and antibodies for further virus – host interaction studies. To compare the immunogenicity of different Ebola virus proteins in relevance to vaccine development.
2. To identify Ebola virus proteins that potentially interfere with RIG-I pathway regulated interferon gene expression and reveal the molecular mechanisms of action of Ebola virus interferon antagonists such as viral protein 24 (VP24).
3. To systematically compare the ability of VP24 proteins of nine mammalian filovirus species in their ability to interfere with RIG-I signaling and interferon gene expression.

4 Materials and Methods

4.1 Cell cultures, constructs and reagents

4.1.1 Cell cultures, transfections and cell stimulations

In Study I, human hepatoma Huh7 cells were maintained in continuous culture in minimum Eagle's medium- α (Invitrogen Corp., Carlsbad, CA, USA). The growth media included 0.6 µg/ml penicillin, 60 µg/ml streptomycin and 10% fetal calf serum (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) (Nakabayashi et al.,1982). For baculovirus expression, *Spodoptera frugiperda* (Sf9) (ATCC: CRL-1711) cells were cultured in TNM-FH medium (Sigma-Aldrich Co. LLC., St. Louis, MO, USA). Vero cells (African green monkey kidney epithelial cells) were grown in Dulbecco's modified Eagle's medium (DMEM). In study II and III, human embryonic kidney 293 cells and Huh7 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with HEPES (MP Biomedicals). The immortalized RIG-$I^{+/+}$/$MDA5^{+/+}$ (wild type), RIG-$I^{-/-}$/$MDA5^{+/+}$, RIG-$I^{+/+}$/$MDA5^{-/-}$ and RIG-$I^{-/-}$/$MDA5^{-/-}$ mouse embryonic fibroblast (MEF) cell lines were generated from E12.5-14.5 embryos and maintained as described previously (Mäkelä et al., 2015). In all studies the cell media included 10% heat inactivated fetal bovine serum (FBS; Biowest), penicillin/streptomycin and L-glutamine. Sf9 cell media also included amphotericin B 2.5 µg/ml and 10% fetal calf serum (Sigma-Aldrich Co.) or in EX-C420 Serum-Free Medium (Sigma-Aldrich Co.) as recommend by the manufacturer.

The transfections of HEK293 and Huh7 cells were carried out with TransIT-LT1 Reagent (Mirus) according to manufacturer's instructions (Mirus). In studies II–III the double transfections were carried out by first transfecting plasmids with TransIT-LT1 reagent followed by transfection with stimulatory low molecular weight polyI:C (10µg/well in 12-well plates; LMW polyI:C; InvivoGen) with Lipofectamine2000 (Invitrogen). In study I the stimulation of HEK293 cells were also carried by infecting the cells with Sendai virus (Cantell strain). The virus strain was propagated in 9 days old embryonated chicken eggs and titrared in dendritic cells (Österlund et al., 2005). HEK293 cells were infected in different time points at multiplicity of infection (MOI) 5. MxA promoter activation was carried out by adding IFN-α2b (IntronA; Merck Sharp & Dohme Limited) in indicated concentrations into the

media. In study III the ssRNA mimic of Ebolavirus was transfected to MEF cells using Lipofectamine3000 transfection reagent (Invitrogen) according to manufacturers' recommendation.

4.1.2 Plasmids and RNA constructs (Study I–III)

Six genes of EBOV strain H.sapiens-wt/SLE/2014/Makona- G3856.1 (GenBank KM233113.1) encoding all the Ebolavirus proteins (VP24, VP30, VP35, VP40, NP and full-length GP) were synthesized and obtained from GenArt (Thermo Fisher Scientific) (Gire et al., 2014). These genes were subcloned into pcDNA3.1/myc-His(-) A (Life Technologies) expression plasmid (C-terminal myc- and 6xHis-tag). The rest of filovirus VP24 sequences including BOMV (MF319185), BDBV (KC545394), RESTV (KY798006), SUDV (KC545389), TAFV (KU182910), ZEBOV (KM233113), LLOV (NC016144.1), MARV (NC001608.3) and MLAV (KX371887) were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/) and the VP24 genes were synthesized by Geneart (Thermo Fisher Scientific). Filovirus VP24 genes were cloned into an expression plasmid which creates a fusion protein with an N-terminal HA-tag, pEBB-HA-N. BDBV and MARV VP24 genes were also obtained in codon optimized forms due to low expression of authentic sequences (study III). Mutant EBOV VP24 was created by mutating five amino acids into alanine with L201A, E203A, P204A, D205A and S207A amino acid changes.

The ends of N-terminal of EBOV VP24, VP30, VP35, VP40, NP and GP and human importin α5 gene (GenBank: NM002264) were processed by adding a BamHI restriction site and a Kozak consensus sequence, ACC, prior to translation start site (GGA TCC ACC ATG). To the C-terminal ends of structural proteins of EBOV, a second BamHI site, without a stop codon, was added which enables the subcloning of the genes into the BamHI site of the bacterial expression vector pGEX-2T (Amersham Biosciences, Buckinghamshire, U. K.) and the eukaryotic expression vectors pcDNA3.1(−)A-myc-His (Invitrogen) and a prokaryotic expression vector pGEX-2T(+) (GE Healthcare Europe GmbH, Finland; GenBank: U13850.1). For N-terminal HA-tag, filovirus VP24 coding cDNA were subcloned into the BamHI site of the eukaryotic expression vector pEBB-HA.

The plasmids of pIFN-β-Luc and pIFN-λ1-Luc containing the promotor areas for the interferon genes upstream of a firefly luciferase, Renilla luciferase gene under Rous sarcoma virus promoter (RSV-Renilla), pMxA-Luc containing the promotor areas for the genes, wtRIG-I, constitutively active RIG-I (ΔRIG-I), MDA5, wtIRF3, IRF3-5D (constitutively active form of IRF3) and hepatitis C virus nonstructural protein 3/4A (HCV NS3/4A) have been described previously (Holzinger et al., 2007; Jiang et al., 2015; Kaukinen, Sillanpää, Nousiainen, Melén, & Julkunen, 2013; P. I. Österlund, Pietilä, Veckman, Kotenko, & Julkunen, 2007; Ronni et al., 1998).

For the ssRNA mimic of EBOV (KM233113), a minigenome of Ebolavirus (EBOV_negssDNA_GFP, 1940 bp) was designed with a chimeric eGFP gene between 3'UTR leader and 5'UTR trailer regions of the genome. The fragment was cloned into pMKRQ vector backbone (Geneart, Thermo Fisher Scientific) which was then used as a template for the production of EBOV dsDNA by PCR. The ssRNA mimic was then produced by *in vitro* transcription with T7 RNA polymerase, using Ebola minigenome dsDNA plasmid as a template to produce an EBOV minigenome mimic with authentic RNA 3' and 5' ends. In vitro produced ssRNA was then purified and desalted with NAP5 column (GE Healthcare).

The gene constructs for E. coli expression of glutathione S-transferase (GST) fused importins α1, α3, α5, α6 and α7 and the gene construct for baculovirus expression of GST-importin α4 have been described previously (Fagerlund et al., 2005; Melén et al., 2003). NLS (or importin-α interaction site) mutated EBOV VP24 expression plasmid has been described in Study II.

For baculoviral expression pBVboost-STOP and pBVboost-6xHis vectors were created by modifying pBVboost vector (Airenne et al.,, 2003), by replacing the BamHI − HindIII multiple cloning site with a segment 5′-GA TCC TAG ATA GAT AGA −3′ that creates a new BamHI cloning site and a stop codon in 3 reading frames (pBVboost- STOP) and with a segment 5′-GA TCC CAT CAC CAT CAC CAT CAC TAG A-3′ that brings a new BamHI cloning site and C-terminal six histidines and a stop codon (pBVboost-6xHis) to a subcloned insert. The proteins of EBOV (VP24, VP30, VP35, VP40, NP and GP) were inserted into pBVboost and pBVboost-His vectors to produce His-tagged and non-tagged recombinant proteins.

4.1.3 Production of recombinant EBOV proteins (Study I)

The production of EBOV GST-VP24, −VP30, −VP35, −VP40 and −NP fusion proteins at + 30 ºC under 0.2 mM isopropyl-beta-thiogalactopyranoside (IPTG) induction in *E. coli* BL21 cells for 3 h. The collection of *E. coli* cells was done by centrifugation followed by being lysed in 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100, sonicated and clarified by centrifugation at 13,000 rpm for 20 min. 25 mg of soluble cellular protein samples were bound to Glutathione Sepharose 4 B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), washed, and eluted as recommended by the manufacturer for purification. The purified protein samples (10 mg) were mixed in Laemmli sample buffer (Laemmli, 1970) and subjected to preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Model 491 Prep Cell, Bio-Rad Laboratories, Richmond, CA, USA). The cast SDS-PAGE gel was 8% for GST-VP24 and 6% for GST- VP30, −VP35, −VP40 and −NP. Sample fractions (5ml), containing separated proteins, were lyophilized followed by resuspension into 0.5 ml of water. Coomassie

Blue staining on 12% SDS-PAGE was used to verify the purity and quantity of fractions.

The production of EBOV GP-His-tagged protein was carried out by using infected *Sf9* cells with EBOV GP-His expressing baculoviruses for 42h (Airenne et al., 2003). The cells were then harvested, and the cell extract was prepared by mixing the cells in 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% Triton X- 100 and passing the cells through a syringe. Acquired protein (10mg) was mixed in Laemmli sample buffer and subjected to 6% preparative SDS-PAGE.

4.1.4 *In vitro* translation and mutagenesis (Study II–III)

EBOV genes were subcloned into pcDNA3.1/myc-His (-) plasmid enabling the *in vitro* -translation with a TnT Quick Coupled Transcription/Translation Systems kit (Promega). To ensure protein visibility in autoradiography the produced proteins were metabolically labeled with [35S]-methionine/cysteine by Easy Tag Express Protein Labeling Mix (PerkinElmer), separated on 12% SDS-PAGE followed by autoradiography in Biomax XAR Films (Carestream Health). For importing binding assays described below, some filovirus VP24 proteins (BDBV and MLAV) were *in vitro* transcribed and translated using pcDNA3.1+ and some (MARV) into pcDNA3.1+/myc-His B plasmid.

Amino acid mutations of filovirus VP24 proteins were carried out with Phusion Site-Directed Mutagenesis Kit (Thermo Scientific) according to the manufacturer's instructions.

4.1.5 Antibodies

Antibodies used in the study are presented in Table 4.

Table 4. Antibodies used in this thesis.

Antibody	WB	IFA
Rabbit anti-IRF3 (FL-425; Santa Cruz Biotechnology)	1:200	
Rabbit anti- phospho-IRF3 (Ser396) (4D4G; Cell Signaling)	1:1000	
Rabbit anti-c-Myc (A- 14; Santa Cruz Biotechnology)	1:100	
Mouse anti-HA1.1 Epitope Tag (BioLegend)	1:500	
Mouse anti-GAPDH (6C5; Santa Cruz Biotechnology)	1:1000	
Mouse anti-FLAG (M2; Sigma-Aldrich)	1:500	
Anti-TBK1/NAK (Cell Signaling)	1:1000	
Mouse anti-HA1.1 epitope tag (BioLegend)	1:1000	
Mouse anti-GAPDH (Santa Cruz Biotechnology)	1:700	
Rabbit anti-IRF3 in house made at THL	1:200	1:200
Rabbit anti-MDA5 in house made at THL	1:200	
Rabbit anti-RIG-I in house made at THL	1:200	
IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences)	1:15000	
IRDye 680RD goat anti-mouse IgG (LI-COR Biosciences)	1:15000	
Rhodamine Red-X-labeled goat anti-rabbit immunoglobulins (Jackson ImmunoResearch Laboratories)		1:100
Anti-HA, Cell Signaling Technology		1:1600
Alexa Fluor 488 anti-mouse (Thermo Fisher)		1:1000
Alexa Fluor 488 anti-rabbit (Thermo Fisher)		1:1000
Alexa Fluor 568 anti-mouse (Thermo Fisher)		1:1000

4.2 Gene expression studies (Study II–III)

4.2.1 Reporter gene assays

96-well plates were used to grow HEK293 cells. The cells were grown into 80–90% confluency and the cells were transfected with promoter-luciferase constructs (20 ng/ well) together with wtRIG-I, ΔRIG-I or MDA5 expression plasmids (30 ng/well) and filovirus VP24 or hepatitis C virus nonstructural protein (HCV NS3/4A) expression plasmids (3–30 ng/well). For internal transfection efficacy control the transfection plasmid mixture included RSV-Renilla (50 ng/ well) as well. Transfected cells were harvested at indicated time points for Twinlite Dual Luciferase Reporter Gene Assay System (Perkin Elmer) and Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) (Studies I-III) according to the manufacturer's instructions (Fig. 3A.). The firefly luciferase results were normalized with the values of Renilla luciferase. All transfection experiments were performed multiple times and the results have been done in triplicates and repeated at least three times (Fig. 3B.).

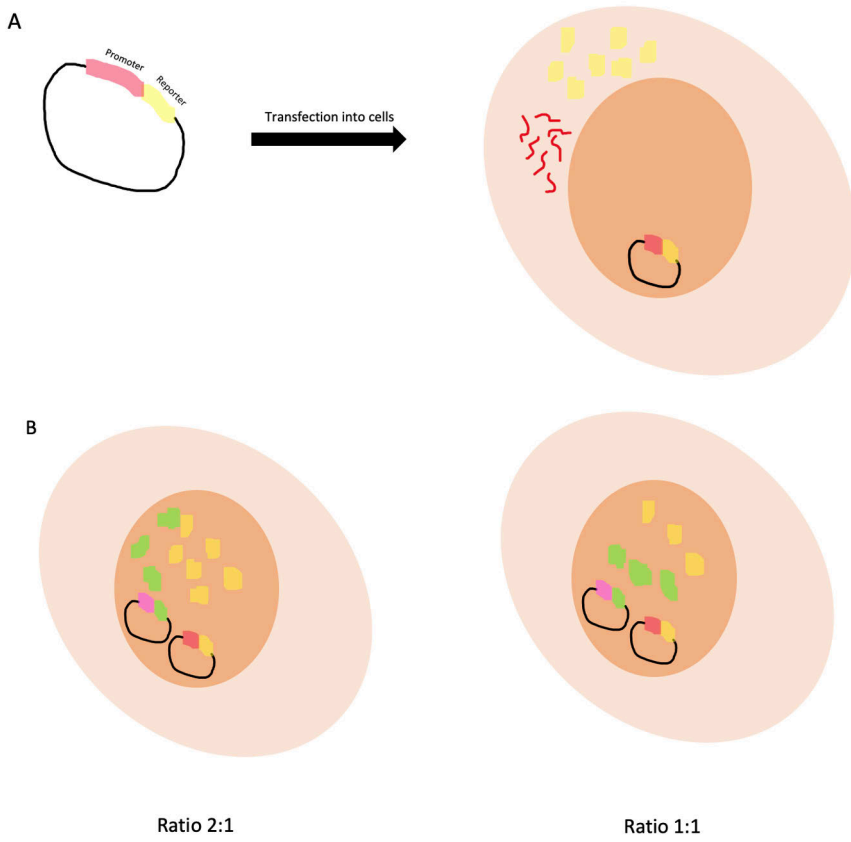

Figure 3. Workflow for reporter gene assay: the assay in studies of signaling pathways, gene regulation and the structure of elements. In panel A: mRNA for the luciferase reporter protein is transcribed downstream from the activated promoter and the reporter luciferase protein is translated. The activity of a promoter is correlated into the amount of luciferase produced in the cells. In panel B: to measure the activity of promoter, transfect the cells with a control plasmid (Renilla luciferase) to perform dual-luciferase assay to control lusiferase activity.

4.2.2 Real time quantitative RT-PCR

The cellular RNA was extracted using Trizol/RNeasy hybrid RNA extraction protocol (Jahn et al., 2008). Immortalized RIG-I$^{+/+}$/MDA5$^{+/+}$, RIG-I$^{-/-}$/MDA5$^{+/+}$, RIG-I$^{+/+}$/ MDA5$^{-/-}$ and RIG-I$^{-/-}$/MDA5$^{-/-}$ MEF cells were harvested in 6- well plates after overnight stimulation either with synthetic EBOV ssRNA mimic or poly I:C.

One µg of cellular RNA was transcripted to cDNA using TaqMan Reverse Transcriptase kit (Applied Biosystems) with random hexamers as primers. The obtained cDNA was then amplified by PCR using TaqMan Universal PCR Mastermix and Gene Expression Assays (Applied Biosystems). The expression

levels of endogenous mouse IFN-ß mRNA was analyzed with quantitave RT-PCR and results were normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels using ΔΔCt-method.

4.3 Protein expression studies

4.3.1 SDS-PAGE and Western blotting (Studies II–III)

For immunoblotting HEK293 cells were grown on 12-well plates to 80–90% of confluency. Cells were transfected with 500ng of expression plasmid (wtRIG-I, ΔRIG-I or MDA5) together with 250ng of IRF3 expression plasmid. Filovirus VP24 expression plasmids were also transfected into these 12-well plates in increasing dosage (200–2000 ng/well). The cells were left for overnight incubation and harvested the next day. Cells were lysed on ice with either Passive lysis buffer provided in Dual Luciferase Assay Kit (Promega) (studies II and III) or for native gels with native lysis buffer as described before (Song et al., 2016). Lysis buffers were supplemented with Complete Protease Inhibitor Cocktail (Roche) and PhosStop Phosphatase Inhibitor Cocktail (Roche).

Proteins were separated on in house 10% or 4–12% or Any kD SDS-PAGEs or for analysis of IRF3 dimers on 12.5% Criterion Tris- HCl Gels (without SDS; BioRad) and transferred onto an Amersham Protran 0.2 mm nitrocellulose (GE Healthcare) or PVDF blotting membranes (Millipore). Immunoblotting was carried out by using in house-produced or commercially available antibodies: mouse anti-HA1.1 epitope tag (1:1000 dilution, BioLegend), mouse anti-GAPDH (1:700, 6C5, Santa Cruz Biotechnology), rabbit anti-IRF3 (1:200) (Österlund et al., 2007) and rabbit anti-RIG-I (Lin et al., 2006) (1:200). The production of anti-MDA5 antibodies were executed by immunizing rabbits 4 times with 50mg of E. coli produced MDA5 CARD domain (provided by Dr. J. Hiscott).

Secondary antibodies used in immunoblotting were IRDye 800CW goat anti-rabbit IgG and IRDye 680RD goat anti-mouse IgG (LI-COR Biosciences) according to manufacturer's guidelines. The quantitation and visualization of bands in membranes was performed with Odyssey Fc Imaging System (LICOR Biosciences).

4.3.2 Importin binding assays (Studies I and II)

GST and chimeric human GST-importins (α1, α3, α5, α6 and α7) were produced in *E. coli* BL21 cells and human GST-importin α4 was produced in *Sf9* cells as described before (Fagerlund et al., 2005; Melén et al., 2003). To summarize, the production of GST and GST-importin α fusion proteins were done at +30 °C under 0.2 mM isopropyl-beta-thiogalactopyranoside (IPTG) induction for 3 h in *E. coli*

BL21 cells. The cells were later collected by centrifugation and lysed in 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100 (binding buffer) followed by sonication and centrifugation at 13,000 rpm for 5–20 min in order to be clarified. Chimeric GST-importin α4 was produced in Sf9 cells at +28 °C according to previous study (Summers & Smith, 1989).

One mg of soluble cellular protein samples were bound to 25 µl of Glutathione Sepharose 4B beads (GE Healthcare) at +4 °C in IP buffer for 60 min followed by washing twice with the binding buffer in order to perform the pull-down experiment. In order to verify the purity and quantity of each importin α isoforms, we used Coomassie Blue staining on 12% SDS-PAGE (compared to known standard protein, bovine serum albumin).

Sf9 cells were infected for 72 h with GST-NP gene expressing baculoviruses in order to produce GST influenza A virus NP fusion protein (Fagerlund et al., 2002). Cells were harvested and prepared and GST- NP fusion protein was bound to Glutathione Sepharose 4B beads as described above. HEK293 cells on 6-well plates, were transiently transfected with pEBB-HA-BOMV VP24, pEBB-HA-LLOV VP24, pEBB-HA-RESTV VP24, pEBB-HA-SUDV VP24, pEBB- HA-TAFV VP24, or pcDNA3.1(+)/myc-His-EBOV VP24 expressing gene constructs using TransIT-LT1 transfection reagent (Mirus Bio LCC) according to the manufacturer's instructions in order to produce VP24 proteins for importin binding assays. After 24h of transfection, the cells were harvested and lysed in IP buffer. The lysate was sucked through a 25 G needle followed by centrifugation (10,000 x g, +4 ^{o}C, 5 min). To perform the pull-down experiment, protein samples were bound to 25 µL of Glutathione Sepharose-immobilized GST and GST- fusion proteins at +4°C for 1 h and washed three times with IP buffer.

Some filovirus VP24 proteins were not able to be produced or extracted from transfected HEK293 cells so the cells were in vitro translated as radioactively labeled proteins (TnT® Quick Coupled Transcription/Translation Systems; Promega, Madison) BDBV and MLAV VP24 cDNAs in pcDNA3.1+ plasmid (Thermo Fisher Scientific) and MARV VP24 cDNA in pcDNA3.1+/myc- His B plasmid and wtVP24 and mutVP24 were [^{35}S]-Met/Cys-labeled (Easy Tag^{TM} Express Protein Labeling Mix, PerkinElmer) and allowed to bind to Sepharose-immobilized GST and GST-fusion proteins as described above. Sepharose beads were dissolved in Laemmli sample buffer, and the proteins were separated on 12% SDS-PAGE for the analysis of GST or GST-fusion protein- bound proteins. Samples were transferred onto PVDF membranes (Millipore,) followed by staining with primary anti-HA1.1 and secondary anti-mouse HRP antibodies (Dako). Gels with [^{35}S]-labeled proteins were fixed and treated with Amplify reagent (Amersham Biosciences). Autoradiography was performed using HyperMax films (Amersham Biosciences).

4.3.3 Immunofluorescence and imaging (Studies I–III)

In Study I, Huh7 cells were grown on glass coverslips for 24h before transfection with EBOV VP30, VP35, VP40, GP and NP coding genes in the eukaryotic expression vector pcDNA3.1(−)A-myc-His and with EBOV VP24 coding gene in the eukaryotic expression vector pEBB-HA for indirect IF analysis and confocal laser microscopy. As a transfection reagent we used either FuGENE6 (Roche Diagnostics, Indiapolis, IN, USA) or Lipofectamine 2000 transfection reagents (Mirus Bio LCC, Madison, WI, USA) according to the manufacturer's instructions. 48h post-transfection cells were fixed with 3% paraformaldehyde at RT for 20 min, permeabilized with 0.1% Triton X-100 for 5 min and processed for immunofluorescence microscopy. The images were acquired using Leica TCS NT confocal microscope. Vero cells were either mock infected or infected with EBOV strain Mayinga at MOI 1 and incubated at +37 ºC for 24 h. After incubation the cells were trypsinized and mixed with uninfected cells at a ratio of 1:3 (infected cells: mock infected cells). The mix was then grown on glass coverslips for 6–7 h and fixed with 80% acetone followed by staining with commercial polyclonal anti-EBOV NP antibodies to verify EBOV infection. Immune serum that was collected from an EBOV -infected patient (diluted 1:20 in PBS containing 0.2% BSA and 0.1% Triton-X-100) was mixed and incubated with the fixed cells. Rabbit antibodies against EBOV VP24, VP30, VP35, VP40, NP and GP (diluted 1:100 in PBS containing 0.2% BSA and 0.1% Triton-X-100) was added and incubated at +37 ºC for 1 h. After washing the slides twice, cells were incubated with Alexa fluor 599-conjugated anti-rabbit (Thermo Fisher Scientific, Inc.) and anti-human FITC-conjugated immunoglobulins (Jackson ImmunoResearch Inc.). For staining of the cell nuclei, DAPI (Sigma-Aldrich) was added. IF and confocal microscopy was used to analyze slides. The procedures with live EBOV took place in a BSL-4 facility (Solna, Sweden).

To study the intracellular localization of filovirus VP24 proteins (Study III), Huh7 cells were grown on coverslips for 20h before transfection with expression plasmids for N-terminally HA-tagged VP24 gene. 24h after transfection, cells were fixed with 4% paraformaldehyde at room temperature (RT) for 15 min, permeabilized with 0.1% Triton- X 100 in PBS for 5min and incubated for 1h at RT with primary mouse antibodies (anti-HA, Cell Signaling Technology) diluted in 3% BSA/PBS. The cells were then washed with 0.5% BSA/PBS the samples were labeled for 1h at RT with fluorescent Alexa Fluor 488 secondary anti-mouse antibodies (Thermo Fisher Scientific) diluted in 3% BSA/PBS. The coverslips were washed with 0.5% BSA/PBS and mounted with Moviol® 4-88ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) in PBS on objective slides. The imaging was performed using Leica DFC7000 T fluorescent microscope with a 63x objective. Mitotracker staining of TAFV VP24 gene transfected cells was

performed according to manufacturer's instructions (MitoTracker® Red CMXRos, Cell Signaling Technology) followed by detection of VP24 protein as described above.

To analyze the nuclear import of IRF3, Huh7 cells were grown on coverslips in 12-well plates. Cells were transfected with EBOV and NLS mutated EBOV VP24 expression plasmid for 24h. The transfected cells were then either left unstimulated or stimulated by transfecting 10 mg of poly(I:C) per well with Lipofectamine 2000 according to manufacturer's instructions. After 18h the cells were fixed as above and stained with mouse anti- HA and rabbit anti-IRF3 antibodies followed by staining with Alexa-anti-mouse-568 and Alexa-anti-rabbit-488 and mounting as described above. The visualization was performed using Zeiss Axioimager microscope with 63x oil objective. Images were manually analyzed with Image J software and statistical differences between the groups were analyzed using Chi- square test.

In Study II, the cells were grown and transfected using glass coverslips. Cells were grown to 70–80% confluency and fixed with 3% paraformaldehyde in PBS for 15–30 min at RT. The fixed cells were then blocked and permeabilized in 1% BSA, with 0.1% Triton X-100 in PBS for 30 min at RT. Primary antibody (anti-HA, BioLegend) were diluted 1:500 into 1% BSA, with 0.1% Triton X-100 in PBS or 1% BSA in PBS and incubated for 60 min at RT. Secondary antibodies were Rhodamine Red-X-labeled goat anti-rabbit immunoglobulins (1:100; Jackson ImmunoResearch Laboratories) and were diluted in 1% BSA, with 0.1% Triton X-100 in PBS and incubated for 60 min at RT. After washing coverslips were mounted on ProLong Gold Antifade Mountant (Life Technologies). For imaging we used Leica TCS NT confocal microscope.

4.3.4 Adjuvants and immunization (Study I)

Freund's complete (FCA) and incomplete adjuvants were purchased from Difco laboratories (Detroit, Michigan, USA; lot no 4129875 and 4287998) and used as according to manufacturer's guidelines. AS03 (in separate vials) was from GSK A/H1N1 pandemic influenza vaccine Pandemrix (GlaxoSmithKline Biologicals, SA, Rixensart, Belgium; lot no A81CA540A). To summarize the procedure, we mixed the virus antigen in PBS with an equal volume of FCA or AS03 followed by thorough mixing of the adjuvant and the antigen. New Zealand White (NZW) rabbits between two to three kg and five- to eight-month-old − were immunized subcutaneously (FCA) or intramuscularly (AS03) with one of the six chimeric E. coli or Sf9 cell-produced EBOV proteins (GST-VP24, −VP30, −VP35, −VP40, −NP and GP-His). Immunization occurred four times in two-week intervals, using either FCA or AS03 as an adjuvant. Also, three HSD:PO guinea pigs that varied in weight between 300-350g and aging between five to eight months old were immunized subcutaneously

with GST-VP24 using FCA. Total amount of purified *E coli*-produced EBOV GST-fusion protein (GST-VP24, −VP30, −VP35, −VP40 and −NP) was 50 µg and baculovirus-produced His-tagged GP inoculated at two separate sites was 25 µg/site in 250 µl. Blood samples from rabbits were collected before the first immunization and before each booster immunization. Rabbits were bled 10 days after the last immunization. Blood samples from guinea pigs were collected before the first immunization and 10 days after the fourth immunization. To collect the serum, blood was allowed to clot at +4 ºC. Ethical approval for animal immunization was provided by the Ethics committee of animal experimentation in Southern Finland (permission no: ESLH ESAVI/11411/04.10.07/2014 to DVM Anna Meller)

4.4 Statistics

4.4.1 Statistical analyses

Statistical analyses in Study II and III were performed with Student's independent t-test and calculated with SPSS Statistics 20 (IBM) and by an ordinary one-way ANOVA Dunnett's multiple comparisons test with a single pooled variance. IFA images and the statistical differences between different groups were analyzed using Chi-square test.

4.4.2 Phylogenetical analyses

Complete filovirus genomes and filovirus VP24 sequences were analyzed using Multiple Sequence Comparison by Log Expectation (MUSCLE). Best model for the description of the phylogenetic relationships was estimated with Molecular Evolutionary Genetics Analysis Computing Platform 7 (MEGA 7). Phylogenetic trees were constructed using MEGA 7 by maximum likelihood approach using General Time Reversible model with gamma distribution and possibility of evolutionary invariability for some sites.

5 Results

5.1 Cloning and expression of filovirus genes and production of recombinant proteins (Studies I–III)

5.1.1 Expression and purification of EBOV proteins

EBOV protein encoding genes (VP24, VP30, VP35, VP40, NP and GP) were synthesized from the West African Ebola virus strain (Gire et al., 2014). cDNAs were subcloned into a prokaryotic pGEX-2T expression plasmid and expressed as GST-tagged fusion proteins under IPTG induction in *E. coli* BL21 cells. The purification was performed from soluble *E. coli* extracts with Glutathione Sepharose affinity chromatography. The resulted semi-purified protein extracts were then purified by SDS-PAGE. The final purified proteins were verified by Coomassie Brilliant Blue staining (Fig. 4.). Immunization was carried out using the purest protein fractions. The cell extracts obtained from IPTG-induced E. Coli (25 mg of bacterial cell protein) resulted in two to five mg of highly purified recombinant protein

EBOV coding genes (VP24, VP30, VP35, VP40, NP and GP) with BamHI ends were subcloned to eukaryotic pBV- boost expression plasmid and expressed in *Sf9* cells for baculoviral expression. The cell extracts were used in the Western blot analyses of rabbit and guinea pig immune sera. EBOV GP gene with BamHI ends was subcloned into the pBVboost-6xHis expression plasmid, but due to the instability of the His-tag, we were unable to purify the baculovirus-produced chimeric GP-His fusion protein by affinity chromatography. Therefore, we used SDS-PAGE purification, which resulted in purified protein fractions for immunizations as verified by Coomassie Brilliant Blue staining. *Sf9* cell extract, containing 10 mg of total cell protein, resulted in 250–500 µg of purified GP protein.

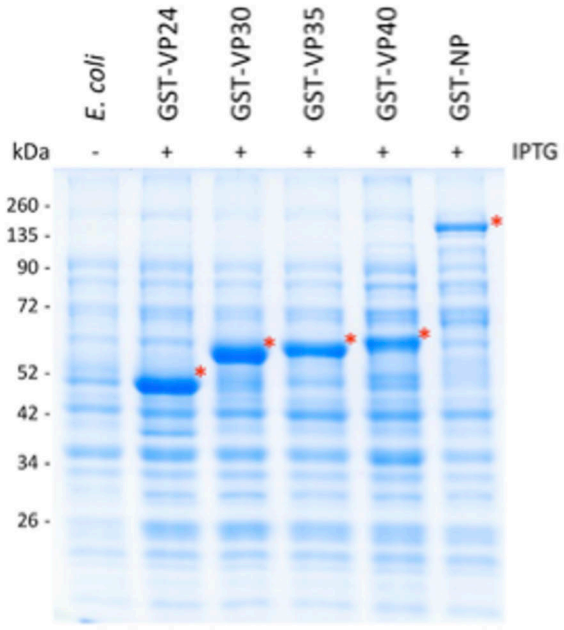

Figure 4. Purification of EBOV proteins. Five µg of total cellular protein of each sample was loaded and separated on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue. Bands, indicating recombinant EBOV structural proteins, are marked with red asterisks.

5.1.2 Immunizations to elicit maximal antibody responses

Four immunizations with each EBOV protein using FCA in six rabbits and AS03 in another six rabbits was carried out in order to compare the adjuvant capacity of FCA and AS03. Each rabbit and guinea pig immune serum were diluted from 1:200 to 1:125000 and antigen binding was detected by immunoblotting. All of the rabbits with the exception of VP24-immunized rabbits, showed high and specific antibody titers even at 1:125000 dilutions. The FCA and AS03 adjuvant effect was comparable to each other. Immune sera from VP24-immunized rabbits, using FCA and AS03, showed positive antigen staining at 1:5000 and 1:25000 dilutions after the fourth immunization, respectively. Three guinea pigs were immunized with GST-VP24 using FCA in order to obtain anti-VP24 immune sera with higher antibody titers. All guinea pigs showed very high and specific antibody titers giving positive staining at 1:125000 dilutions.

The immune sera against individual EBOV proteins were verified by Western blotting. The specificity of final bleeds of rabbit and guinea pig immune sera after four immunizations was analyzed. The analyzed dilution of rabbit and guinea pig antisera was 1:200. Rabbit and guinea pig immune sera showed very high specificity

to EBOV structural proteins and no cross-reactivity of immune sera against a heterologous protein was seen in any animal.

Confocal laser microscopy imaging of EBOV gene transfected HuH7 cells showed that all immune sera, except the rabbit anti-VP24, showed good specificity and immunoreactivity at 1:1250 dilutions or even at 1:6250 dilutions. Instead, the rabbit anti-VP24 immune sera showed immunoreactivity ranging from 1:250 to 1:1250 dilutions. VP24, VP30, VP35 and VP40 showed both nuclear and cytoplasmic staining pattern while NP and GP showed granular intracellular staining patterns.

In order to analyse whether anti-EBOV antibodies could recognize native EBOV proteins in virus-infected cells and whether the EBOV genes would be expressed during the infection, we carried out IFA analysis with rabbit anti-EBOV antisera against different viral proteins. Vero cells were infected with EBOV (MOI 1, 24h) and stained with human immune serum obtained from an EBOV-infected patient and rabbit polyclonal antibodies against different EBOV proteins. All EBOV proteins showed cytoplasmic staining patterns with rabbit polyclonal antibodies and cells stained with human and rabbit antisera showed colocalization.

5.1.3 Expression of cloned filovirus genes (Studies II–III)

EBOV genes (VP24, VP30, VP35, VP40, NP and GP) and produced proteins were verified by *in vitro* translation, immunofluorescence and immunoblotting. The pcDNA3.1/myc-His(-)- plasmid derived expression of EBOV proteins were confirmed by Western blotting. In addition, immunofluorescence confirmed that all six genes were expressed properly, and the subcellular localization differed from one protein to another. To show the expression levels of these six EBOV proteins immunoblotting was performed with anti-myc and rabbit-EBOV GP antibodies and result showed that all of the six proteins were expressed successfully. VP24 gene was also cloned to another expression vector (pEBB-HA-N) in order to obtain better expression of the protein. VP24 expression was confirmed using anti-HA antibody.

The expression of all the available filovirus VP24 genes were cloned into pEBB-HA-N expression vector. The conformation of proper expression levels was confirmed by immunofluorescence. To increase the protein expression of BDBV and MARV, VP24 constructs were also codon optimized. All nine filovirus VP24 proteins were successfully expressed and detected in Huh7 cells. Interestingly, the intracellular localization of different filovirus VP24s varied, but the majority of the proteins were expressed both in the nucleus and the cytoplasm (expression in cytoplasm and nucleus: EBOV 95%, MLAV 53%, BOMV 96%, LLOV 96%, TAFV 71%, RESTV 56%, SUDV 92%, BDBV 50% and codon optimized BDBV 92%) (Fig. 5.). MARV VP24 was expressed mainly in cytoplasm (91%) but codon

optimized expression construct directed higher MARV VP24 expression and thus the proteins was found both in the nucleus and the cytoplasm (93%).

Unlike other VP24s, RESTV formed granule-like structures in the cytoplasm resembling mitochondrial structures. Colocalization analysis of RESTV VP24 was performed using MitoTracker staining in order to study whether localization is closely related to mitochondria. The distinct granule structures were separated from mitochondria.

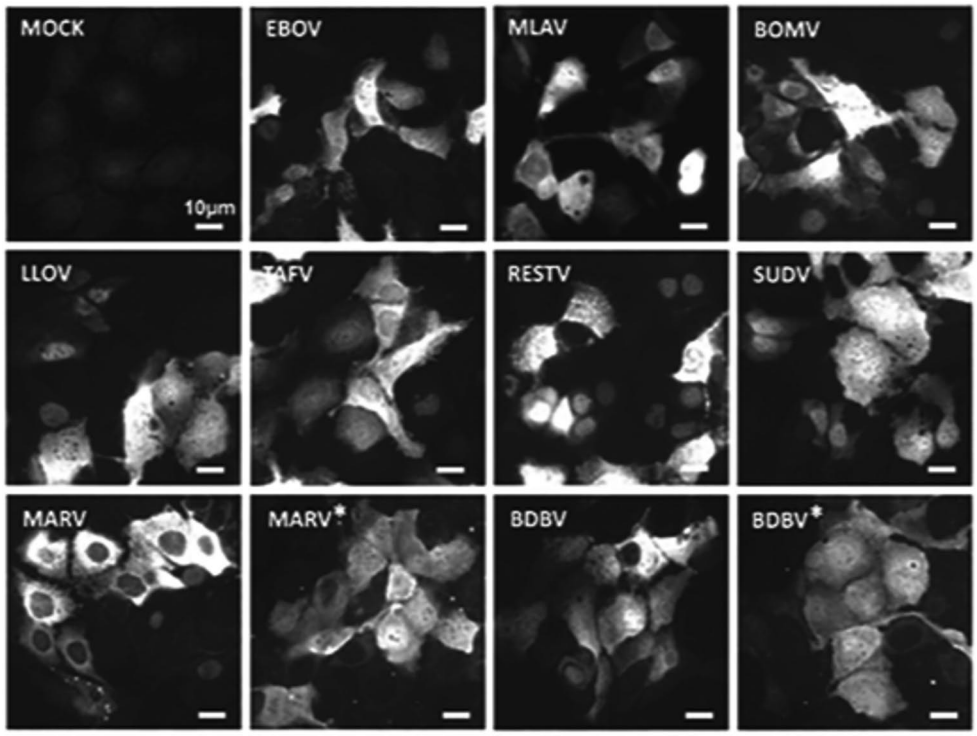

Figure 5. Representative images of filovirus VP24 expressing Huh7 cells. BDBV* and MARV* refer to codon optimized expression constructs of the respective genes due to the low or undetectable expression of the original gene sequences in Western blotting.

5.2 Inhibition of innate immune responses (Studies II–III)

5.2.1 EBOV genomic RNA mimic activates both RIG-I and MDA5 pathways

As many studies have indicated, the main PRRs in early innate immune responses are RIG-I and MDA5 (Randall & Goodbourn, 2008). In order to determine their role

in regulating type I IFN gene expression we constructed an EBOV ssRNA mimic and used this RNA to stimulate mouse embryonic fibroblasts defective in RIG-I or MDA5 or both (IFN-λ1 gene is defective in mice). The poly I:C was used as a positive control. The expression of endogenous IFN-ß mRNA was measured with RT-qPCR and the signal was normalized using endogenous GAPDH mRNA.

Both EBOV ssRNA mimic and Poly I:C stimulated RIG-$I^{+/+}$/$MDA5^{+/+}$ MEF cells to increase IFN-ß mRNA expression in a dose-dependent fashion. However, there were clear differences in RIG-$I^{-/-}$/ $MDA5^{+/+}$ and RIG-$I^{+/+}$/$MDA5^{-/-}$ MEF cells. Cells with a functional RIG-I molecule showed a better response to EBOV ssRNA mimic stimulation as compared to RIG-I defective, MDA5-positive cells. These results show that both RIG-I and MDA5 molecules contribute to cell stimulation by EBOV RNA but IFN-ß mRNA expression is somewhat stronger by RIG-I pathway compared to MDA5 pathway. As a negative control for the experiment MEF cell lines that were defective in both RIG-I and MDA5, showed no increased IFN-ß gene expression (Fig. 6.).

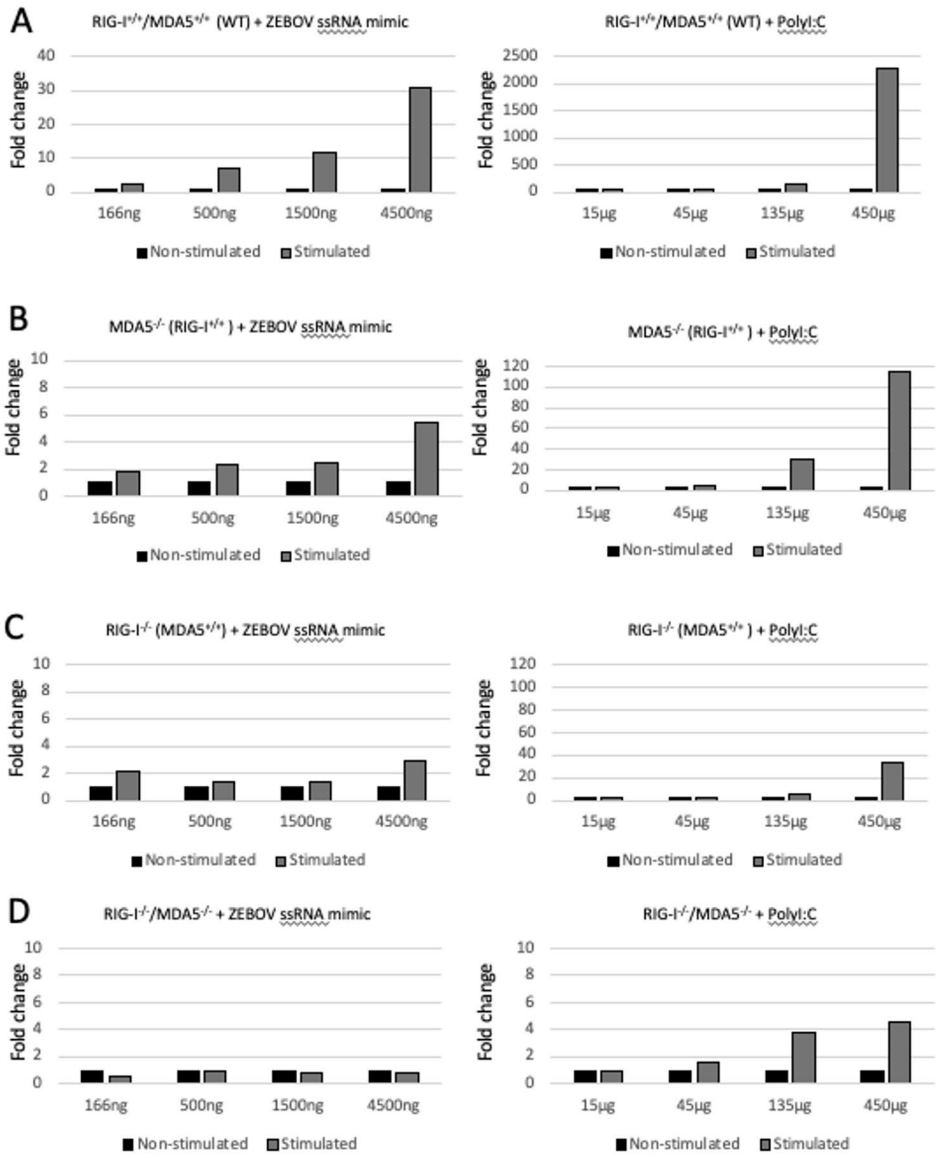

Figure 6. Regulation of IFN-ß gene expression in RIG-I and MDA5 defective mouse cell lines by EBOV ssRNA mimic.

5.2.1.1.1 RIG-I pathway is more strongly activated than MDA5 pathway

From previous studies we know that RIG-I and MDA5 differ from each other by being activated by different types of RNA molecules (Brisse & Ly, 2019). Small 5'-phosphorylated ss/dsRNA molecules are recognized by RIG-I and longer non-5'-phosphorylated RNA molecules by MDA5. For our studies, we compared the

relative stimulatory activity of RIG-I and MDA5 as activators of type I and III interferon promoters. We transfected HEK293 cells with increasing amounts of wtRIG-I, ΔRIG-I, (constitutively active form of RIG-I) and MDA5 expression plasmids together with IFN-β or IFN-λ1- promoter-luciferase reporter plasmids. To activate wtRIG-I, polyI:C stimulation was required since wtRIG-I is not constitutively active. The experiments showed that an increase in IFN-β and IFN-λ1 promoter activation is dose-dependently following the increasing amounts of wtRIG-I+polyI:C, MDA5 and ΔRIG-I. Our findings suggested that 30 ng of expression plasmids is an optimal amount for further studies to conduct.

5.2.1.1.2 The effects of different Ebola virus proteins in interferon gene expression

The dominant role of RIG-I pathway in enhancing IFN-α/β and IFN- λ1 gene expression is important in host innate immune responses against RNA viruses. Previously VP35 is known to inhibit IFN-α/β production (Cárdenas et al., 2006; Chang et al., 2009b; Feng, Cerveny, Yan, & He, 2007; Luthra et al., 2013; Prins et al., 2009). We investigated systematically the effects of EBOV proteins in RIG-I pathway. HEK293 cells were transfected with EBOV expression constructs, IFN-λ1-promoter-luciferase construct and the construct expressing the ΔRIG-I. Hepatitis C virus (HCV) NS3/4, which is a strong inhibitor of IFN-λ1 activation due to its capability of degrading MAVS, was used as a control (Kaukinen et al., 2013). The main findings in this experimental setup were that VP30, VP35, VP40, NP and GP expression showed no inhibition, whereas VP24 and HCV NS3/4A inhibited IFN-λ1 promoter activation. We followed this result by repeating the experiment by adding IFN-α and IFN-β promoters to the experiments. Cells were co-tranfected with expression plasmids for VP24 and VP40. The results indicated that IFN-α promoter was not markedly activated and positive control showed minimum activation but IFN-β promoter was clearly activated. VP24 showed a dose-dependent inhibitory effect suggesting that it inhibits the activation of type I (IFN-β) and type III (IFN-λ1) interferon gene expression.

5.2.2 Filovirus VP24 inhibits IFN- λ1 and IFN- β promoter activation

Previous studies have shown that EBOV VP24 inhibits IFN signaling and the expression of IFN-induced genes by blocking the nuclear import of phosphorylated STAT1 (Reid et al., 2006). There is also evidence that amino acids (aa) 42 and 142–146 of VP24 are crucial for the inhibition of STAT binding to importins α (Mateo et al., 2010). In our studies EBOV VP24 gene was compared to the representative

strains from other outbreaks (sequences for strains from years 1976, 1977, 1994, 1995, 1996, 2002, 2007, 2008 and 2014). Sequence comparison revealed five amino acid changes: N50H from years 2007/2008, Q58H from the year 1995, V102I from the year 2002, R163K from the year 1995 and Q163R from the year 2002, and M212K from years 1976/1977 indicating very conserved structure of VP24 genes. VP24 clone made by us was identical to other strains seen in the 2014 viral sequences (Gire et al., 2014). We performed an experiment to confirm the functionality of our EBOV VP24 gene which proved that the gene construct was fully functional and biologically active. HEK293 cells were transfected with MxA-promoter-luciferase construct together with EBOV VP24 and VP40 in pcDNA3.1/myc-His(-)A expression plasmids. At 4h after transfection the cells were stimulated with IFN-α for 16 h. VP40 had no inhibitory effect on MxA-promoter activation while VP24 of the 2014 EBOV strain inhibited strongly and dose-dependently the activation of MxA promoter.

RIG-I and MDA5 pathway use downstream signaling molecules MAVS and TBK1 that regulate the phosphorylation and activation of IRF3 and other transcription factors. To determine the possible target for VP24, HEK293 cells were transfected with expression plasmids for VP24 or VP40 or Zika virus (ZIKV) NS3 together with expression plasmids for ΔRIG-I, MDA5, MAVS, TBK1 or constitutively active form of IRF3 (IRF3-5D) and IFN-λ1-promoter-reporter or IFN-β promoter. The results indicated that VP24 can inhibit IFN promoter activation stimulated by all the above molecules including the constitutively active form of IRF3. This suggests that VP24 inhibitory mechanism acts at the same level or downstream of IRF3. An interesting finding in these experiments was that EBOV VP24 almost completely inhibited MDA5 induced IFN promoter activity compared to the inhibitory effect of VP24 on RIG-I pathway with same amounts of expression plasmids.

Since there was a clear dose-dependent inhibitory effect of EBOV VP24 on IFN promoter activity we further studied the possible effect of other filovirus VP24s. All the available mammalian filovirus VP24 proteins were analyzed for their potential inhibitory effect on RLR stimulated IFN-β or IFN-λ1promoter activation. HEK293 cells were transfected together with expression plasmids for ΔRIG-I, IFN-β or IFN-λ1-promoter- luciferase reporter, RSV-Renilla and increasing amounts of different filovirus VP24 expression constructs. The values were normalized with Renilla luciferase values, negative control was HCV NS3/4. VP24 proteins of EBOV, LLOV, TAFV, RESTV, SUDV, MARV and BDBV dose-dependently inhibited the activation of the RIG-I pathway. The level of inhibition appeared to be more pronounced for IFN-λ1 promoter. In contrast, MLAV and BOMV VP24 proteins appeared to show no inhibition of ΔRIG-I induced IFN-β or IFN-λ1 promoter activation, not even with the highest amounts of VP24 expression plasmids (Fig. 7.).

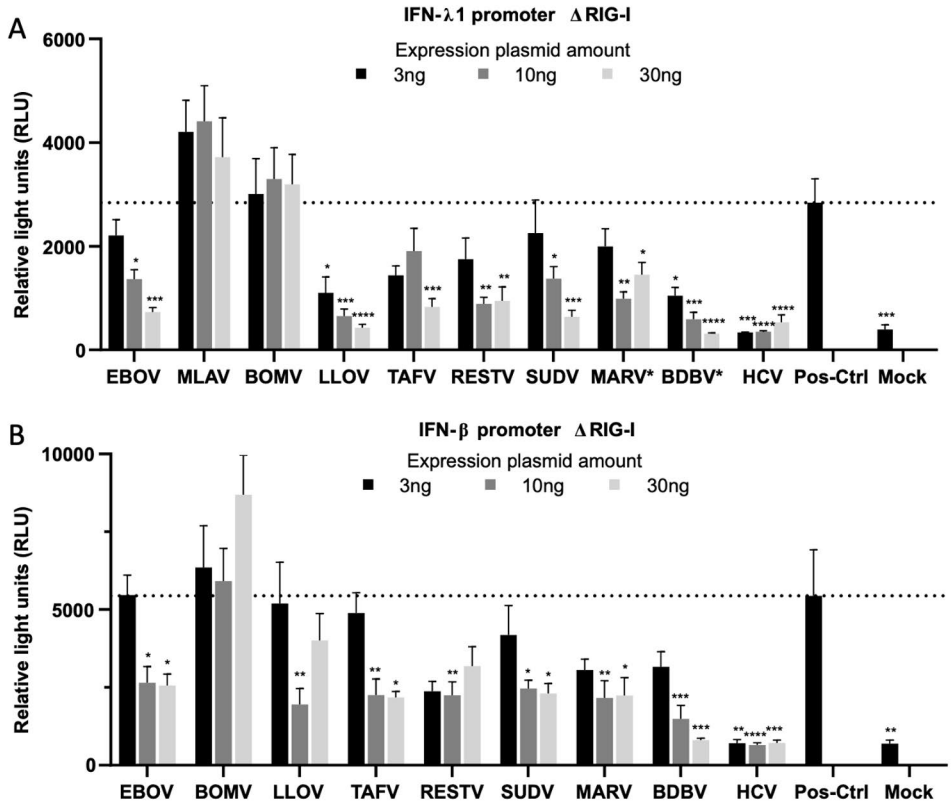

Figure 7. Inhibitory effects of filovirus VP24 proteins on RIG-I induced interferon gene expression.

5.2.3 Filovirus VP24 does not inhibit the phosphorylation, dimerization or nuclear import of activated IRF3

Since many filovirus VP24 proteins were able to inhibit the transcriptional activity of type I and III IFN promoter activation and EBOV VP24 also inhibited the constitutively active form of IRF3, we analyzed whether VP24 inhibited the phosphorylation or dimerization of IRF3. For the experiment, HEK293 cells were co-transfected with expression plasmids for wtIRF3 and wtRIG-I, ΔRIG-I or MDA5, together with expression plasmids for RESTV, SUDV, ZEBOV and LLOV VP24 expression plasmids. In some experiments we also included as controls EBOV VP40 or HCV NS3/4 expression plasmids. ΔRIG-I and MDA5 were able to directly stimulate IRF3 phosphorylation, whereas with wtRIG-I we used poly I:C to stimulate IRF3 phosphorylation. Interestingly, none of the filovirus VP24 proteins that were able to inhibit the transcriptional activity of interferon gene expression were able to efficiently inhibit IRF3 phosphorylation (Fig. 8A.).

After phosphorylation IRF3 dimerizes, which in turn triggers its nuclear translocation. The dimerization analysis of EBOV VP24 showed that in the presence of EBOV VP24 protein IRF3 dimers were formed (Fig. 8B.). To further clarify the immunoblotting results, we analyzed whether either wtVP24 or NLS-mutated VP24 (mutVP24; nuclear localization site mutated) can interfere with the nuclear import of activated IRF3. EBOV VP24 expressing cells, stimulated with poly I:C, showed that IRF3 accumulation in the nucleus occurred equally well in control cells and in wtVP24 and mutVP24 expressing cells, thus indicating that VP24 does not inhibit the nuclear import of activated IRF3.

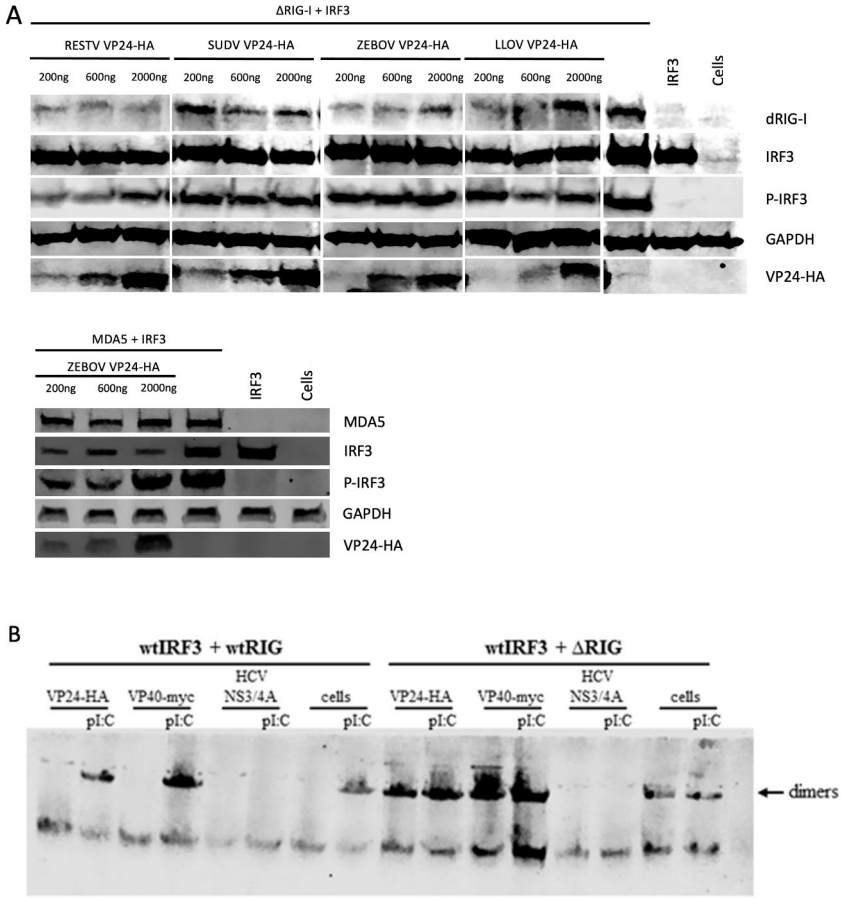

Figure 8. Panel A illustrates the effect of RESTV, SUDV, EBOV and LLOV VP24 on RIG-I and MDA5 activated IRF3 phosphorylation. Panel B shows that EBOV VP24 does not inhibit IRF3 dimerization.

5.2.4 Binding of EBOV VP24 to importin α

EBOV VP24 is known to inhibit the binding of STAT-dimers to importin α molecules (Reid et al., 2006). Since one of the main findings in our experiments was that VP24 protein of filoviruses inhibit the IRF3-mediated activation of IFN-λ1 gene expression, we addressed the question whether the importin-binding function of VP24 is needed for this inhibition. Five amino acids L201A, E203A, P204A, D205A and S207A have been shown to efficiently abolish the binding of VP24 importin α molecules. EBOV VP24 expression construct with mutations in importin-binding site was created for these experiments as the required amino acids of VP24 were mutated into alanine to create a mutant VP24. We used both N-terminally HA-tagged and C- terminally His/myc-tagged expression constructs for these mutated VP24 genes. The expression levels of HA-tagged VP24 constructs were analyzed by immunoblotting resulting in slightly higher levels of expression levels for mutated VP24s (mutVP24) compared to that of the wild type VP24 (wtVP24). mutVP24 localized primarily in the cytoplasm in transfected Huh7 cells, compared to the nuclear localization of wtVP24 as nuclear localization for mutVP24 was 0% compared to wtVP24 localization of 35%. The binding of wtVP24 and mutVP24 differed from each other and the importin binding experiment showed that while wtVP24 efficiently bound to importins α5, α6 and α7, mutation of the five amino acids of VP24 reduced the binding to the background level. The biological activity of mutVP24 was then verified by transfecting HEK293 cells with either wtVP24 or mutVP24 together with MxA-promoter-luciferase construct. Four hours post transfection, the cells were stimulated with IFN-α for 16 h followed by measurement of MxA-promoter-luciferase activity. The results were in line with other studies as mutVP24 was not able to inhibit MxA gene expression (Mateo et al., 2010).

In order to determine the possible effect of NLS mutation on VP24 regulated IFN-λ1 gene expression, HEK293 cells were co-transfected with the expression plasmids for wtVP24 or mutVP24, and ΔRIG-I and IFN-λ1-promoter-luciferase construct. The results showed that while wtVP24 inhibited the IFN- λ1 gene expression dose-dependently, mutVP24 was not able to do so. We then followed this experiment by transfecting HEK293 cells with either MDA5 or ΔRIG-I together with IFN-β or IFN-λ1-promoter-luciferase reporter and with wtVP24 or mutVP24. Increasing amounts of wtVP24 efficiently inhibited RIG-I and MDA5 induced IFN promoter activation, while the mutVP24 was almost devoid of this inhibitory activity. Mixing wtVP24 together with mutVP24, however, showed an efficient inhibition in RIG-I and MDA5 induced IFN promoter activation suggesting that wtVP24 - mutVP24 dimers likely enter the nucleus in sufficient amounts to inhibit the activation of IFN promoters (Fig. 9). These findings suggest that the binding of VP24 to importins and its location in the nucleus is necessary to efficiently inhibit RIG-I and MDA5 regulated IFN gene expression.

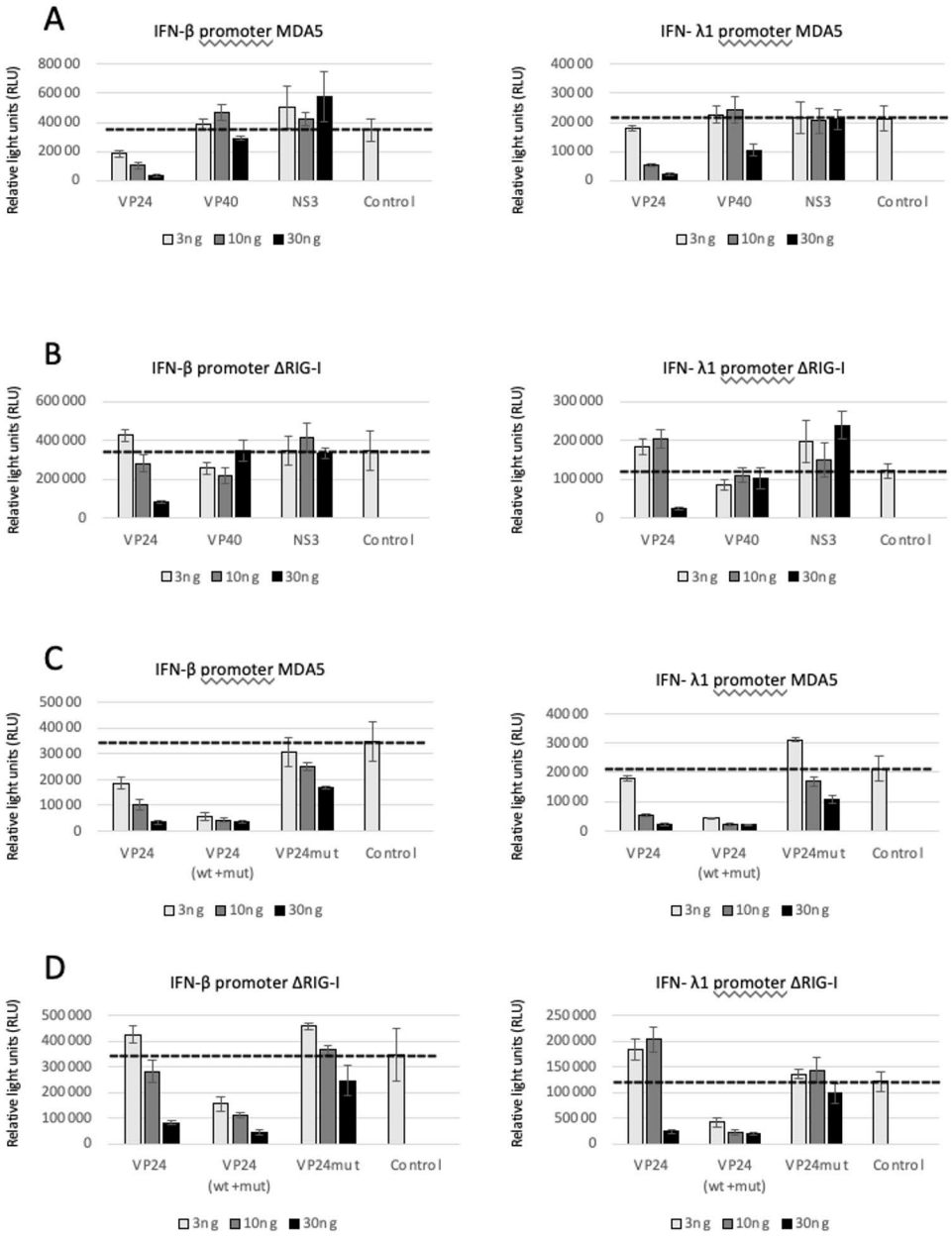

Figure 9. Inhibition of MDA5 and RIG-I induced interferon gene expression by wild type and NLS-mutated EBOV VP24.

6 Discussion

6.1 Not all filoviruses are the same

The majority of filovirus research has focused on EBOV due to several past EBOV outbreaks and especially its recent outbreak in West Africa between 2013 to 2016 that led to over 28 000 cases and over 11 300 deaths. The most recent and still ongoing epidemics in the Democratic Republic of Congo further justifies research of pathogenesis of filoviruses. Filovirus disease is commonly characterized by rapid and uncontrolled viral replication and suppression of host innate immune antiviral responses, mainly the production of type I and III interferon (Basler, 2015). In order for virus to replicate in host cells, it must surpass the activation of antiviral and cytokine gene expression. The most recent modeling shows that filovirus infection leads to efficient virus replication accompanied by which leads to a burst of immunological mediators better known as cytokine storm causing the distinct symptoms of EVD and MVD.

According to the most recent knowledge, six of the currently known twelve filoviruses can cause disease in humans. These include BDBV, EBOV, SUDV, TAFV, MARV and RAVV. At present the remaining 6 filoviruses are considered as animal pathogens. In our studies, we compared the possible differences in filovirus genomes covering four genera (Ebolavirus, Marburgvirus, Cuevavirus and Dianlovirus) and their VP24 proteins. Interestingly, we observed quite large differences in the sequences of different VP24s. For instance, the differences in EBOV and MARV, which are known to cause lethal outbreaks with similar symptoms, showed only 34% similarity in amino acid sequence. Recently, two more filovirus species were added to the filovirus family indicating that the whole filovirus family has drifted even further from each other and in the future the dispersion of the characteristics of filoviruses and their genome and protein effect might differ even further from each other (Shi et al., 2018).

The intracellular localization of VP24 in different filoviruses is somewhat different. Most of the investigated VP24s were found both in the cell nucleus and cytoplasm. However, MARV VP24 was cytoplasmic at low expression level and nucleo-cytoplasmic when codon optimized MARV VP24 construct was used. The nuclear accumulation of different filovirus VP24s in the nucleus correlated very well

with their interaction with importin α5. The genetically distant MARV and MLAV VP24s were also weakly bound to importin α5.

Based on the most recent COVID-19 pandemic, the question remains, whether there is also a risk for filoviruses to mutate even further and become transmitted efficiently from person to person via aerosols for instance. Evolution of pandemic virus requires a virus which is easily transmitted from one person to another in an immunologically naive population. Secondly, reproduction number (R_0) should be greater than 1, meaning that every person that is infected commonly infect more than one new patient. The current state of transmission of filoviruses is mainly dependent on direct contact to bodily fluids or blood (Burk et al., 2016). Filoviruses are unlikely to be transmitted during the incubation period and transmissibility is generally the highest in the late phases of the infection. To summarize, studies have shown that filoviruses tend to evolve slowly. However, relatively little is known about the cumulative effects of mutations on the virulence of filoviruses (Martina & Osterhaus, 2009; Morikawa et al., 2007). Notably, comparison of the genomic differences between filoviruses causing severe outbreaks showed that the mutations could not explain the pathogenesis of the virus. Further studies are needed to understand the pandemic threat posed by filoviruses and inevitable mutations which is part of evolution.

6.2 Efficient protein production, purification and antibody production

In our studies we expressed recombinant EBOV VP24, VP30, VP35, VP40 and NP as GST fusion genes in E. coli BL21 cells under the induction by IPTG. The end result was relative high protein production levels when a two-step purification procedure was applied: Glutatione Sepharose affinity chromatography followed by preparative SDS-PAGE. However, due to the cytotoxicity or interfering elements in the full-length GP protein, GP was produced by baculovirus expression system in eukaryotic Sf9 insect cells as a His-tagged fusion protein.

In order to enable efficient purification proteins were expressed with GST- and 6xHis- tags. Rabbits and guinea pigs were immunized with tagged proteins as it is known that removing the GST-tag by protein cleavage is very difficult. Our studies showed low backgrounds in Western blot experiments and IF, which is most likely due to the fact that in most cases, antibodies against GST and 6xHis do not hamper the use of EBOV protein-specific immune sera. Humans seldom have cross-reactive antibodies against Sf9 cell proteins unlike E. coli proteins and this was evident for rabbit and guinea pigs' sera which showed no antibodies against Sf9 cell proteins.

Immunization with rabbits using four doses of EBOV proteins either with FCA or AS03 adjuvants resulted in high antibody titers with EBOV VP35 but VP24 and

VP40 however showed poor antibody titers which gradually increased with subsequent immunizations. Most cases showed maximal antibody levels after three immunizations. In order to improve efficacy of VP24 antibody production, guinea pigs were immunized with GST-VP24 using FCA. The finding shows that different animal species, including humans, might show clear differences in their immune responses against viral antigens. The sera from final bleeds showed very high specificity against EBOV proteins indicating that these proteins unlikely have any common or cross-reactive antigenic epitopes.

Rabbit and guinea pig antibodies in Western blot were compared to IF results in EBOV gene transfected HuH7 cells to investigate the specificity and quantity of antibodies. Although we cannot verify the expression level of transiently transfected gene products, all sera after four immunizations showed parallel results in IF experiments compared to Western blot analyses, indicating that polyclonal antibodies could recognize EBOV structural protein antigen epitopes both in a native cellular form as well as in a denatured form in Western blotting. All EBOV proteins produced in infected cells were picked up by our polyclonal antibodies, however it is not known which of the EBOV proteins are immunogenic in humans and how immune response against different proteins contribute to immunity or cure against EBOV disease as cellular effects might arise from the production of all the filovirus proteins.

6.3 Role of RIG-I-Like receptors in filovirus infections

Innate immune responses are vital cellular defense mechanisms against pathogenic microbes, including viruses. In order to infect cells, viruses have developed mechanisms to avoid activation of antiviral and cytokine gene expression and also interfere with IFN-signaling and functions of IFN-induced antiviral proteins. RIG-I-like receptors, especially RIG-I and MDA5, play a dominant role in filovirus infections. Previous studies have shown that despite these PRR recognize different sections of the same viral genome due to their differing RNA binding preferences, they can act both independently and in synergy resulting in much more efficient innate immune response (Sanchez David et al., 2016). Other studies have also verified that even if RIG-I and MDA5 have their own specific functions, they function synergistically and are cross regulating IRF and NFκB signaling pathways and both pathways are vital for innate immunity (Czerkies et al., 2018; Kasumba & Grandvaux, 2019).

In this study, the functions of RIG-I and MDA5 were compared. Synthetic genomic EBOV RNA mimic was produced and used to activate MEF cells that were either RIG-I or MDA5 defective and compared them to the wild type cells and RIG-

I/MDA5 double knock-out cells. The results showed that after stimulating the cells with ssRNA mimic, both RIG-I and MDA5 were involved in IFN gene expression, while the RIG-I pathway appeared to be the dominating one. When analyzed separately the overall and combined activity of individual pathway was much weaker compared to wild type cells where both RIG-I and MDA5 were activated at the same time in same system. This further verifies the relationship between these RLRs and shows that either pathway is active in host cell defense but suggests that synergistic relationship between RIG-I and MDA5 is vital in filovirus infections. Our findings based on synthetic genomic EBOV ssRNA mimic suggests that both pathways are likely activated by filovirus RNA and MDA5 pathway is even more sensitive than the RIG-I pathway in the presence of filovirus RNA.

6.4 The ability of filovirus VP24 to downregulate IFN stimulated genes

To our best knowledge, there is no previous data on the possible ability of SUDV, LLOV, BUDV, BOMV, TAFV and MARV VP24 to regulate the IFN gene expression. VP24 of different filoviruses varied up to 26% indicating that there may be differences in their ability to mediate interferon antagonism and downregulating IFN stimulated genes. Previous studies have indicated that EBOV VP24 inhibits type I and III interferon gene expression, and also the nuclear import of IFN-induced phosphorylated and dimerized STAT1 and STAT2 (Guito et al., 2017; Xu et al., 2014). It is commonly known that host innate immune responses to filovirus infection is largely dependent on both type I and III IFNs. In our study we further examined how different filovirus VP24 proteins interact with type I and III interferon gene expression. Seven studied filovirus VP24, with exception of MLAV and BOMV, clearly inhibited IFN promoter activation. The only non-human pathogenic filoviruses that inhibited IFN promoter activation were RESTV and LLOV. EBOV VP24 did not inhibit IRF3 phosphorylation and nuclear import of IRF3 when studied downstream of ΔRIG-I and MDA5. Both RIG-I and MDA5 pathways were inhibited by the same filovirus VP24 molecules and MDA5 seemed to be more strongly inhibited compared to RIG-I pathway but this is likely due to the fact that RIG-I seems to be a stronger activator IFN promoters in general.

Previous studies have also shown that some filovirus proteins inhibit interferon gene expression. MLAV VP24 has been shown not to be able to inhibit IFN-induced gene expression (Messaoudi et al., 2015; Williams et al., 2020). RESTV showed clear inhibition on IFN-β and IFN-λ1 promoter activation, which is in line with previous studies (Anthony & Bradfute, 2015). One difference in the findings compared to our studies was that MARV VP24 was able to inhibit IFN promoter activation. This may be explained by differential experimental conditions and

MARV VP24 protein expression level. Our study provides a systematic comparison of all available mammalian-infecting filovirus VP24 proteins on IFN-β and IFN-λ1 promoter activation. Our studies demonstrate that seven out of nine filovirus VP24s are efficiently inhibiting IFN promoter activation.

When further proceeding downstream of RIG-I and MDA5 pathway to identify possible targets of VP24 we found that the ectopic expression of ΔRIG-I, MAVS, TBK1 or constitutively active form of IRF3 (IRF3-5D) were effectively inhibited by EBOV VP24. We thus identified the inhibitory effect of EBOV VP24 on the RIG-I pathway to take place downstream of IRF3. VP24 did not seem to inhibit the phosphorylation or dimerization of IRF3.

VP24 has been under an active investigation in filovirus research. Presently, there is evidence that EBOV VP35 and possibly VP24 are both interfering with IFN-λ production in EBOV-infected dendritic cells (Ilinykh et al., 2015). Consistent with our studies it was shown that VP24 of another virus strain of EBOV VP24 can inhibit IFN-β and IFN-λ production, though the EBOV VP24 originated from another virus strain that we used (Guito et al., 2017). Previous findings and our results show that VP24 is a multifunctional protein that has essential functions in filovirus lifecycle. It associates with host cell membranes, regulates the assembly of viral ribonucleoprotein complexes, inhibits type I and III IFN production by so far undefined mechanism, inhibits IFN- induced antiviral response by binding to importin α which leads to impaired nuclear translocation of STATs, and inhibits IFN-induced phosphorylation of p38 MAP kinase, and interferes with the nuclear transport of host mRNA transcription complex hnRNP C1/C2 (Guito et al., 2017; Halfmann, Neumann, & Kawaoka, 2011; Han et al., 2003; Mateo et al., 2010; Shabman, Gulcicek, Stone, & Basler, 2011; Xu et al., 2014).

6.5 Role of importin binding in filovirus infection model

VP24 has been shown to bind to importin α. Previous study suggested that the binding of VP24 to importins increased its half-life compared to the mutant VP24 which had an impaired importin binding capacity. The mutant VP24 were degraded more rapidly (Schwarz et al., 2017). Our studies showed higher expression levels of mutVP24 protein compared to wtVP24 in transfected cells. This indicates that the reduced inhibitory effect on IFN-λ1 activation by mutVP24 was not due to a reduced stability of the protein but is likely dependent on VP24-importin α binding properties. wtVP24 and mutVP24 were compared, and they had weak binding activity also to other importin α isoforms. Possible explanation for this phenomenon could be that VP24 has an additional weak binding capacity, although not functionally significant, to other armadillo repeats or areas of the banana-shaped

importin α molecules apart from the very C-terminal armadillo domains 8–10 that have been described to function as the EBOV VP24 binding domain (Xu et al., 2014)

Previous studies indicate that VP24 inhibits IFN signaling and IFN-induced gene expression (such as MxA gene) by sterically blocking the binding of STAT1 transcription factors onto importins and thus preventing STAT1 translocation into the nucleus (Xu et al., 2014). Also, VP24 binding to importins have been proven to be strongest to importins α1, α5 and α6 (Reid et al., 2006). Our own experiments supported the previous observations, since we found the strongest binding of EBOV VP24 to importin α5 and α6 as well as to importin α7. Importin α7 is genetically closer to the α5 and α6 isoforms (Fagerlund et al., 2005). Importins α3 and α4 play an important role in the transport of phosphorylated IRF3 dimers into the nucleus. These importins also recognize the bipartite nuclear localization signal on IRF3 (Kumar et al., 2000; Zhu et al., 2015). These findings indicate that the mechanism for VP24 for inhibiting IFN-λ1 production is not similar to the mechanism of inhibition on IFN-induced response (STAT nuclear translocation). There are few explanations and models of the possible mechanism for VP24 to inhibit IFN gene expression. On the contrary, another study suggested a more general block in nuclear transport of proteins, since VP24 also prevented the nuclear transport of a cellular protein hnRNP C1/C2 (Shabman et al., 2011). The third possible mechanism for VP24, besides blocking the nuclear import machinery, is its inhibitory functions in the formation of transcription initiation complex in the nucleus. Our current results indicate that the nuclear localization of VP24 is essential for its inhibitory activity on RLR signaling since both RIG-I and MDA5 pathways were inhibited by EBOV wtVP24 while the inhibitory activity of NLS mutated VP24 was very weak. Furthermore, when NLS mutant VP24 plasmid was mixed with low amounts of wtVP24 plasmid it most likely leads to import of wt-mutVP24 heterodimers into nucleus and inhibition of IFN promoter activation. These studies suggest that nuclear import of VP24 is needed for its inhibitory activity on IFN activation. Further studies are needed in order to determine the exact mechanism of VP24 - mediated inhibition of RLR signaling.

6.6 Future directions

Research on filoviruses have been very active for quite some time, the growing knowledge on the mechanisms by which EBOV and MARV counteract host defenses are becoming increasingly clearer. The main filovirus proteins that counteract with host immune systems are VP24, VP35 and VP40. Our studies have been focusing mainly on VP24 and these findings provide knowledge for future filovirus studies and further understanding in filovirus pathogenesis. The most relevant question remains to be answered: what is the exact mechanism of VP24 protein inhibition of

type I and III interferon gene expression. Other fundamental questions that also need to be addressed are: how the results of immunological studies relate to the specific mechanism that drive the disease such as the severity of symptoms, regulation of inflammatory responses, coagulopathy and multiorgan failure. Also, how much of the data and results from innate immune studies can be exploited in effective therapeutic development in drug development and other medical interventions. One possible target from the current findings from our studies in therapeutical manner would be drug(s) which counteracts VP24 IFN-antagonist functions and therefore enhances the benefits of naturally produced IFNs.

7 Conclusions

This thesis aimed to provide further understanding of filovirus life cycle, pathogenesis, and better understanding of virus-host interactions at a molecular level. The main objectives were to determine possible new functions of filovirus proteins and to further investigate new mechanisms for interferon signaling in infected cells.

Based on the results of the studies the following conclusions can be made:

1. RIG-I-like receptors play an important role of filovirus infections and RIG-I and MDA5 are both required in adequate interferon response as they work synergistically.
2. We have identified a new innate immune inhibitory function for VP24 by showing that EBOV VP24 can interfere with RLR signaling and IFN gene expression.
3. Although the genome of filoviruses varies, most filovirus family VP24 proteins can inhibit interferon gene expression.
4. Nuclear localization of VP24 is essential for its inhibitory activity on RLR-dependent type I and type IFN gene expression.

Together these results provide important knowledge on filovirus pathogenesis and biology and can be utilized in future studies and make an excellent baseline for designing possible novel drugs or new modalities of treatment of filovirus infections.

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