INTERACTION OF MOUSE NOROVIRUS (MNV) WITH THE CELLULAR IMMUNE RESPONSE OF HOST CELLS

Svenja Fritzlar

ORCID ID: 0000-0003-3407-1758

A thesis submitted for the degree of Doctor of Philosophy

August 2017

Department of Microbiology and Immunology
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne

This thesis is submitted in total fulfilment of the degree.

Abstract

Human noroviruses (HuNoV) cause the majority of non-bacterial gastroenteritis cases worldwide and generate an economic burden of 60 billion USD every year. Noroviruses are highly infectious and predominantly cause issues in closed environments such as cruise ships, hospitals and nursing homes. Due to the lack of a tissue culture or small animal model, HuNoV research has been impaired and so far no drug treatment or vaccine is available. Despite recent advances in the field and the successful replication of HuNoV in B cells and human intestinal organoids, models of HuNoV replication in vitro still remain to be established. Fortunately, murine norovirus (MNV) was discovered in 2003 and has since been used as a model system to investigate NoV infections.

In this study we show that MNV infection reduces the surface expression of MHC class I proteins. The reduction in MHC class I levels on the cell surface is based on reduced intracellular levels of the protein. We reveal that MHC class I transcription is not reduced during MNV infection, implying that either MHC class I translation is affected or MHC class I proteins are degraded during MNV infections. We were able to partially rescue the surface expression of MHC class I proteins on MNV infected cells with MG132, a proteasome inhibitor. These findings indicate that MNV interferes with the MHC class I pathway in either directly degrading the protein or targeting it for the degradation pathway within the cell. Furthermore, we identified MNV NS3 as the viral protein which is essential and sufficient for the MHC class I surface reduction when separately expressed in cells.

Additionally, we investigated the effect of MNV on cytokine secretion. The secretion of the cytokines IFNβ and TNFα is significantly reduced in MNV infected cells, which is not

due to a down regulation of cytokine mRNA transcription. Analysis of the intracellular expression of cytokines and host cell translation in general, revealed a continuous decrease in global host cell translation in MNV infected cells. The translational shutdown seems to be induced by the dsRNA-sensitive regulator PKR. PKR becomes phosphorylated and phosphorylates the translation initiation factor eIF2α, impeding host cell translation. Whilst the translation of host proteins is stalled, viral proteins are still able to be translated due to a cap-independent mechanism.

Furthermore, we interrogated the interaction of MNV with the microtubules and the microtubule-associated protein GEF-H1. We discovered an interaction of GEF-H1 with the viral protein MNV NS3, which leads to changes in the expression levels and location of GEF-H1 within the cell and prevents the formation of GEF-H1 induced microtubule fibres. This indicates a potential interference of MNV NS3 with GEF-H1, which has been proposed to play a major role in the immune detection of viral replication. Despite various approaches to identify a similar role of GEF-H1 during MNV infection, we have so far not been able to support the proposed function of the protein. Considering the multiple roles of GEFs like GEF-H1, it is possible that MNV and specifically NS3 acts on a different GEF-H1-regulated pathway during MNV infection.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree of diploma.

This thesis comprises only original work towards the Doctor of Philosophy. No other person's work has been used without due acknowledgement in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

This thesis is fewer than the maximum word length as approved by the Research Higher Degree Committee.

_____

Svenja Fritzlar, August 2017

Statement of Contributions by others to this thesis

A/Prof Jason Mackenzie

Published works incorporated into the thesis

Sarvestani, S. T., Cotton, B., Fritzlar, S., O'Donnell, T. B., & Mackenzie, J. M. (2016). Norovirus infection: replication, manipulation of host, and interaction with the host immune response. Journal of Interferon & Cytokine Research, 36(4), 215-225.

This PhD project has been financially supported by a Victorian International Research Scholarship (VIRS) and a Melbourne International Fee Remission Scholarship (MIFRS) from the Victorian Government and the University of Melbourne. Research contained within this thesis was financed by grants from the National Health and Medical Research Council of Australia

Acknowledgements

There are a few people who deserve a special mention, because they have supported this project or have played a big role in keeping my spirits high throughout my PhD. Therefore I would like to thank the following people in no specific order.

My supervisor Jason Mackenzie for not only giving me the opportunity to do my PhD with him, but also agreeing to host me as a Masters student a few years ago, which initiated this PhD. Thank you for sharing your invaluable knowledge about virology and giving me endless advice and guidance throughout my PhD. Our meetings were a place of new ideas and I always enjoyed our discussion. Thank you for taking the time for all the 'quick' chats that ended up in an hour long conversation.

Members of the Mackenzie Lab past and present: Beccie for her incredible knowledge and technical skills and being a great mentor. Tanya for being my PhD buddy and letting me rant about science or discuss the latest TV shows on coffee breaks. Turgut for being my cloning hero and keeping me updated about Melbourne foodie news. Adam and Ben for all their advice and taking me to the pub after bad science days. Suz for making sure I don't lose my German skills and sharing her science experiences. Paulina, Josh, Jake and Nathan for making science more fun and dealing with 'stressed me' in the last 6 months.

My PhD committee for providing insight and feedback and being a great source of inspiration. Our meetings have always pushed my projects further.

My collaborators the Purcell Lab, Simmons Lab, Hartland Lab, Brooks Lab and Villadangos Lab for helping my projects along by providing feedback, advice and reagents.

Karena Waller for giving me the opportunity to teach and demonstrate during my PhD. It has been a lot of work, but an invaluable experience which is much appreciated.

My family, my parents and my sister, who have always been providing me with a great supply of encouragement and support, despite being on the other side of the world. Thank you for always being there for me and keeping me sane over the last 3.5 years. Kenneth for patiently dealing with me after frustrating days in the lab and celebrating the good ones with G&Ts. Thank you for being my voice of reason and cheer squad at the same time. Bec and Sam for being amazing housemates and friends, discussing anything and nothing and for not judging my choice of TV programs. And all my friends for making sure that there is more in life than science.

Table of Contents

Table of Figures

List of Abbreviations

A	
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
APC	Antigen presenting cell
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
B	
BFA	Brefeldin A
BSA	Bovine serum albumin
C	
CBP	CREB-binding protein
cDNA	Complementary DNA
CIITA	Class II major histocompatibility complex transactivator
CLIP	Class II-associated invariant chain peptide
CMV	Cytomegalovirus
CTL	Cytotoxic T cell
CV	Calicivirus
D	
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double-stranded RNA
E	
EDTA	Ethylenediamine tetraacetic acid
eIF	Eukaryotic initiation factor
eIF2α	Eukaryotic initiation factor 2α
EBV	Epstein Barr Virus
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERK	Extracellular signal regulated kinase
F	
FADD	Fas-associated protein with death domain
FCS/FBS	Fetal calf serum/Fetal bovine serum
FCV	Feline Calicivirus
FUT	Fucosyltransferase
G	
GADD34	Growth arrest and DNA damage-inducible protein
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GEF-H1	Guanine nucleotide exchange factor H1
GFP	Green fluorescent protein
H	
6xHis	Hexahistidine
HBGA	Human blood group antigen
HCMV	Human cytomegalovirus
HHV-7	Human herpes virus 7
HIV	Human immunodeficiency virus
h.p.i..	hours post infection
h.p.t.	hours post transfection
HSV	Herpes Simplex Virus
HuNoV	Human norovirus
I	
IAV	Influenza A Virus
IF	Immunofluorescence
IFN	Interferon
IFNα	Interferon alpha
IFNβ	Interferon beta
IKK	IκB kinase

IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
IRF3	Interferon regulatory factor 3
ISRE	IFN-stimulated responsive element
K	
KO	Knock-out
M	
MARCH	Membrane-Associated Ring-CH
MAVS	Mitochondrial antiviral signalling protein
MDA-5	Melanoma differentiation-associated gene 5
*Met*Luc	*Metridia* luciferase
MFI	Median fluorescence intensity
MG132	N-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
MLC	Myosin regulatory light chain
MNV	Mouse norovirus
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTOC	Microtubule organizing centre
MyD88	Myeloid differentiation factor 88
N	
NFκB	Nuclear factor κ B
NK	Natural killer cell
NOD	Nucleotide-binding oligomerization domain-containing protein
NoV	Norovirus
NS	Non-structural
NTP	Nucleotide triphosphate
NTPase	Nucleoside triphosphatase
NV	Norwalk virus
O	
ORF	Open reading frame
P	
PABP	Poly(A)-binding protein
PAK	p21-activated kinase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PFA	Paraformaldehyde
Pfu	Plaque forming units
p-eIF2α	Phosphorylated eIF2α
p-PKR	Phosphorylated Protein kinase R
PKR	Protein kinase R
PLC	Peptide loading complex
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
PV	Poliovirus
Q	
qRT-PCR	Quantitative reverse-transcription PCR
R	
RC	Replication complex
RdRp	RNA dependent RNA polymerase
RHDV	Rabbit haemorrhagic disease virus
RIG-I	Receptor interacting protein
RIP	Retinoic acid inducible gene
RLR	RIG-I like receptor
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
RT-PCR	Reverse transcription PCR
S	
SaV	Sapovirus
sgRNA	Subgenomic RNA
siRNA	Small interfering RNA
SODD	Silencer of death domains
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription

T		U	
TAP	Transporter associated with antigen processing	UPR	Unfolded protein response
		UTR	Untranslated region
TBK1	TANK-binding kinase 1		
TBS	Tris-buffered saline	V	
TCR	Trans Golgi network	VESV	Vesicular exanthema of swine virus
TGN	T cell receptor	VPg	Viral protein genome-linked
TLR	Toll-like receptor	VLP	Virus-like particle
TNFα	Tumor necrosis factor α		
TRADD	TNF receptor-associated death domain protein	W	
		WHO	World Health Organisation
TRAF	TNF receptor-associated factor	WNV	West Nile Virus

Chapter 1

Introduction

1.1 Norovirus

1.1.1 Structure and classification

Noroviruses (NoVs) are members of the *Caliciviridae* family, which also includes the genera Vesivirus, Lagovirus, Sapovirus and Nebovirus. Vesiviruses, Nebovirus and Lagoviruses mainly include animal viruses like Vesicular exanthema of swine virus (VESV), Feline calicivirus (FCV) (both Vesiviruses), Rabbit haemorrhagic disease virus (RHDV) (Lagovirus) and Newbury-1 virus (Nebovirus) (Thiel and König 1999, Oliver, Asobayire *et al*. 2006). NoVs and Sapoviruses contain human infectious viruses. All members of the *Caliciviridae* family share the characteristics of a positive sense single-stranded polyadenylated RNA (ssRNA) genome and an icosahedral capsid with a T=3 symmetry (Prasad, Matson *et al*. 1994). The virus capsid is made of 180 capsid proteins which interact and form 90 dimers. The NoV capsid is made of two viral proteins, the major and the minor capsid proteins, VP1 and VP2 respectively. The major capsid protein VP1 provides the structure and stability to the capsid, whereas VP2 is suggested to associate with the S domain of VP1 inside the capsid and play a role in RNA genome binding and protection (Bertolotti-Ciarlet, Crawford *et al*. 2003, Vongpunsawad, Venkataram Prasad *et al*. 2013). VP1 can be structurally divided into two domains, the shell (S) domain and protruding (P) domain. The S domain is the most conserved region of VP1 and is essential for the formation of the icosahedral capsid. The P domain is important for the formation of dimers and can be further separated into the fairly conserved P1 and the highly variable P2 domain (Prasad, Hardy *et al*. 1999). The P2 domain is the most exposed domain to the surrounding environment including host factors and receptors making it the most likely antigen within the host.

Because of its conserved S and variable P domains, VP1 has been the most important protein used for strain specific analysis, identification and classification of NoVs (Zheng, Ando *et*

al. 2006). Based on the VP1 sequence, NoVs can be classified into 5 groups, termed genogroups (GI-GV) (Figure 1) and several subclusters, termed genotypes (Vinjé, Green *et al*. 2000). GI and GII viruses are responsible for the majority of human NoV (HuNoV) outbreaks including Norwalk strain (GI), Snow Mountain strain (GII) and Sydney strain (GII) (Lochridge and Hardy 2003, Koo, Ajami *et al*. 2010, Matthews, Dickey *et al*. 2012, Control and Prevention 2013). They are also the most diverse genogroups with 8 and 17 genotypes, respectively (Zheng, Ando *et al*. 2006). GIII includes bovine NoVs, GIV contains human and canine NoVs and GV forms the group of murine NoVs.

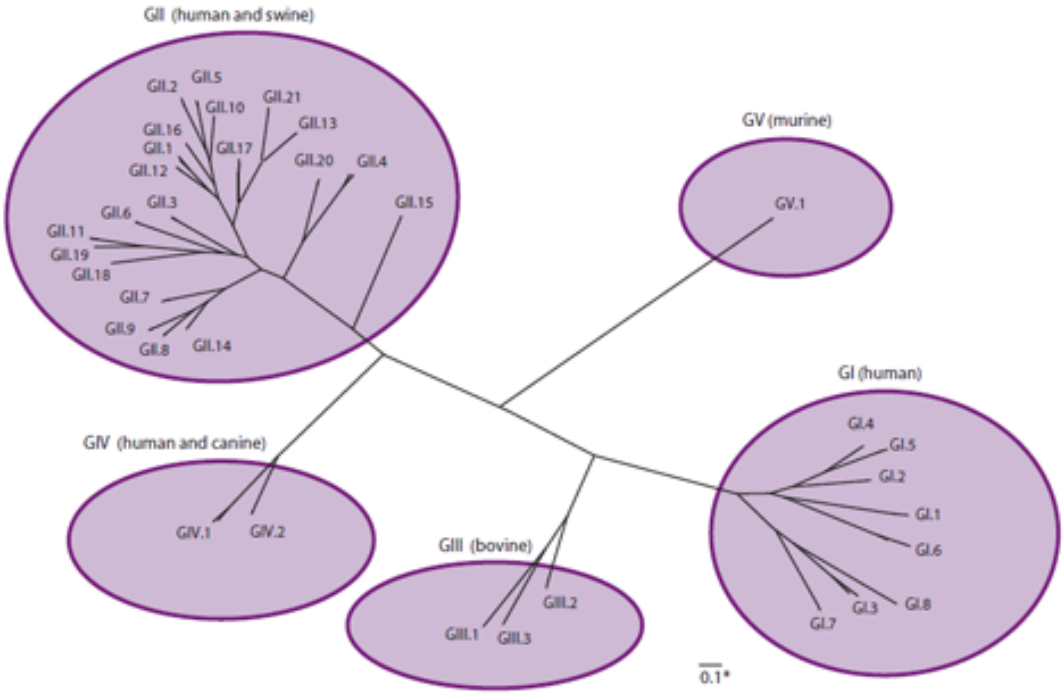


Figure 1 ***Overview of the current NoV genogroups (Hall, Vinjé et al. 2011)****. NoVs can be divided into 5 genogroups (GI-GV), with GI, GII and GIV including HuNoVs. Genogroups can further be subdivided into genotypes, based on their sequence homology.*

1.1.2 History, epidemiology and prevalence

NoV symptoms were first described as 'winter vomiting disease' in 1929 (Zahorsky 1929) and were not immediately associated with a viral infection. In 1968, on outbreak of HuNoV at a primary school in Norwalk (Ohio) caused disease in the majority of staff and children and was used as a model to study 'Norwalk-like' symptoms. A bacterial infection was ruled out as the cause of illness, but it was not until a few years later, that Kapikian *et al* demonstrated via electron microscopy that the observed disease was caused by a virus (Kapikian, Wyatt *et al*. 1972). Insights into HuNoV infection were largely inhibited by the lack of an animal or tissue culture model and were mainly investigated with human volunteer studies.

HuNoVs are to date difficult to cultivate, but recent studies show that HuNoV might be able to infect and replicate in B cells (Jones, Watanabe *et al*. 2014) as well as in human enteroid cultures (Ettayebi, Crawford *et al*. 2016). B cells have been shown to be permissive to HuNoV infection in the presence of *Enterobacter cloacae* bacteria which are able to produce the H-type Histo-blood group antigen (HBGA) (Jones, Watanabe *et al*. 2014). This successful co-infection is likely due to the ability of *E. cloacae* to produce H-type HBGA, because HuNoV infection of B cells could also be achieved in the absence of *E. cloacae* and the addition of purified H-type HBGA. HBGAs play an important role in the resistance to HuNoVs and will be discussed later. Human intestinal enteroid cultures have been successfully infected with different strains of HuNoV, although some strains needed the addition of bile to be able to infect the organoids (Ettayebi, Crawford *et al*. 2016). Monolayers of these organoids contain several different cell types and enterocytes were found to be the major target for HuNoVs. However, these cell culture systems were only recently implemented, lack practicality in the laboratory setting and remain to be

established. Fortunately, MNV was discovered in 2003 and has led to a vast increase in the understanding of NoV replication and interaction with its host (Karst, Wobus *et al*. 2003).

HuNoVs cause the majority of nonbacterial gastroenteritis cases, affecting around 685 million people worldwide every year (Control and Prevention 2013). In the U.S. it causes approximately 23 million infections per year (Mead, Slutsker *et al*. 1999) and in Australia the number of cases is estimated at 1.8 million per year (Angulo, Kirk *et al*. 2008). The symptoms firstly described in 1929 as well as during the 1968 Norwalk outbreak have not changed over time. The onset of disease is usually quick and occurs in the first 24h after exposure to the virus and lasts for 2-3 days (Graham, Jiang *et al*. 1994). Symptoms include diarrhoea, vomiting, abdominal pain, abdominal cramps, nausea and fever (Rockx, de Wit *et al*. 2002). To date there is no antiviral treatment or vaccine available and infections are in most cases treated with electrolytes and rehydrating substances. Although the infection is usually non-lethal, children and the elderly, in particular, can develop severe infections that lead to hospitalisation and death, mainly due to dehydration. In the developing countries at least 220,000 deaths per year are associated with HuNoV infection (Mead, Slutsker *et al*. 1999, Patel, Widdowson *et al*. 2008).

Most HuNoV infections occur in either restaurants and catered meal settings or closed environments such as nursing homes, hospitals, schools and cruise ships (Fankhauser, Monroe *et al*. 2002). The source of primary infections is mostly contaminated food or water, whereas secondary infections are caused by person to person contact, lack of hygiene and contaminated surfaces (Fankhauser, Monroe *et al*. 2002, Matthews, Dickey *et al*. 2012). High risk foods are fresh fruit and vegetables that either have been exposed to contaminated water or sewage or have been handled by infected individuals neglecting preventative actions (DeWaal and Bhuiya 2007).

Another source of HuNoV infection is shell fish from contaminated waters which accumulate the virus through filtering of the water (Morse, Guzewich *et al*. 1986). HuNoV infections occur in all age groups and throughout the year with higher case numbers in winter (Mounts, Ando *et al*. 2000). They can cause sporadic infections as well as major outbreaks and epidemics (Eden, Bull *et al*. 2010). Three characteristics of HuNoVs allow for the rapid spread of the virus to a large group of people, especially in closed environments, as it is rapidly resolved in healthy individuals. Firstly, less than 10 viral particles are needed to cause an infection in healthy individuals (Teunis, Moe *et al*. 2008) posing a great danger of transmission to people in close contact with infected individuals. Secondly, those infected with HuNoV can shed virus for up to three weeks after the onset of symptoms even though symptoms tend to last for no more than 2-3 days (Rockx, de Wit *et al*. 2002). This is not only valid for symptomatic, but also for asymptomatic individuals who might not even be aware of the infection, but are potential spreaders of disease (Graham, Jiang *et al*. 1994). Lastly, NoVs are resistant to a variety of decontamination substances and can persist in the environment for a long time (Barker, Vipond *et al*. 2004). Viral particles stay stable and infectious for at least 61 days in ground water and can be detected in water for up to 3 years after contamination with HuNoV (Seitz, Leon *et al*. 2011).

1.1.3 Innate and adaptive protection

Studies have shown that HuNoV infection causes an adaptive immune response with the production of antibodies against HuNoV 5 days after infection (Graham, Jiang *et al*. 1994, Hutson, Atmar *et al*. 2004). Individuals who have been exposed and mounted an antibody response to the virus are potentially protected from a secondary infection, but this is not valid for every case (Parrino , Schreiber *et al*. 1977, Graham, Jiang *et al*. 1994). This could be due to the huge strain

variability and change in circulating strains (Lindesmith, Donaldson *et al*. 2011) as well as the fact that adaptive immunity protection seems to be rather short lived (Parrino , Schreiber *et al*. 1977). Volunteers who were reinfected with the same HuNoV strain 2-3 years after their first exposure still became infected (Parrino , Schreiber et al. 1977). The strain variety as well as the lack of long term memory to HuNoV infection pose great challenges for the development of a vaccine.

Human volunteer studies revealed that some people are inherently resistant to a HuNoV infection, even after exposure to high doses (Lindesmith, Moe *et al*. 2003). It has been shown that this resistance is mainly based on HBGAs. HuNoVs have been shown to strongly bind to H type I and Le^b HBGAs and that the P domain of VP1 is responsible for binding to HBGAs (Hutson, Atmar *et al*. 2003, Tan, Hegde *et al*. 2004). There are three different types of HBGAs, H, A and Lewis (Shanker, Hu et al. 2017). All HBGAs are carbohydrates which are synthetically related and either tri-or tetrasaccharide functional groups. They are located at the distal end of carbohydrate chains on glycolipids or glycoproteins on the cell surface (Hutson, Atmar *et al*. 2004). The synthesis of HBGAs is dependent on the expression of the fucosyltransferases (FUT) 1-3 which mediate the transfer of fucose to the HBGA. FUT2 expression allows the synthesis of the H type I HBGA and individuals expressing FUT2 and H type I are characterised as Se^+ (Hutson, Atmar *et al*. 2004). Norwalk virus virus-like particles (VLPs) have been shown to bind to saliva from Se^+ individuals, but not to saliva from Se^- volunteers (Lindesmith, Moe *et al*. 2003). Similar observations were made when Se^- volunteers were infected with HuNoV as none of the tested Se^- volunteers became infected (Lindesmith, Moe *et al*. 2003). This indicates that the lack of FUT2 expression In Se^- individuals might protect from HuNoV infection, whereas the expression of FUT2 might lead to higher susceptibility.

1.1.4 Vaccine development and antivirals

As previously mentioned, there are currently no antiviral treatments or vaccines available against HuNoV. Even though a HuNoV infection in healthy adults is a rather self-limiting disease, it can cause serious complications in infants, the elderly and immunocompromised people (Bok and Green 2012, Krones and Högenauer 2012). Considering that a majority of HuNoV outbreaks occur in schools, nursing homes and hospitals (Fankhauser, Monroe *et al*. 2002), prevention of outbreaks in these setting through a vaccine would reduce the exposure of HuNoV to individuals prone to high risk infections. Vaccination of healthcare staff as well as food handlers would also decrease the risk of a HuNoV outbreak and spread.

So far, the development of a HuNoV vaccine has been hindered by the lack of a tissue culture model for HuNoV replication. It not only impairs the knowledge about HuNoV and its interaction with the host, it can also be challenging in assessing the efficiency of potential vaccines. Usually, sera from vaccinated volunteers would be tested in a neutralisation assay, but this is not feasible with HuNoV. As a substitute, a VLP HBGA blockade assay has been utilised so far, which analyses the ability of VLPs to bind to HBGAs and seems to correlate with human protection (Johnson, Mathewson *et al*. 1990, Reeck, Kavanagh *et al*. 2010). Apart from technical issues, there is also the problem of the great strain diversity of HuNoVs. Two genogroups, GI and GII, cause the majority of outbreaks with 73% and 26%, respectively (Fankhauser, Monroe *et al*. 2002). Taken together both genogroups contain 25 genotypes, highlighting the diversity of HuNoVs. Based on cross-protection studies on GI and GII HuNoVs, it seems unlikely that a GI vaccine may infer cross protection against GII strains (Wyatt, Dolin *et al*. 1974). However, a recent study showed that antibodies raised against HuNoV VLPs can be cross reactive to other genotypes (Lindesmith, Ferris *et al*. 2015). Another aspect to consider is the so far poorly understood long

term protection after exposure to HuNoV. Even though 50% of all children will have been exposed to a HuNoV within the first 5 years of their lives reaching almost 100% in adults (Hinkula, Ball *et al*. 1995), protection from a secondary infection seems to be limited. Rechallenge studies with volunteers have shown that an adaptive immune response naturally lasts for 2-3 years (Parrino , Schreiber *et al*. 1977), raising questions about how long a HuNoV vaccine might provide protection. Pre-exposure to a HuNoV might also influence the protectiveness of a vaccine (Atmar and Estes 2012).

Most potential HuNoV vaccines that have been developed in the last decade are based on the fact that antibodies against HuNoV are successful in preventing an infection by blocking the interaction of HuNoV and HBGAs (Reeck, Kavanagh *et al*. 2010, Atmar, Bernstein *et al*. 2011). The majority of vaccines consists of HuNoV VLPs, but there have been other developments like 24-mer P-particles (P-domain of VP1 only) as well (Fang, Tan *et al*. 2013). Many current vaccine candidates contain VLPs from GI and GII to increase the protectiveness of the vaccine to both genogroups (El-Kamary, Pasetti *et al*. 2010, Atmar, Bernstein *et al*. 2011, Sundararajan, Sangster *et al*. 2015). They all consist of two doses administered 1-3 weeks apart and are either given intranasal or intramuscular. The intranasal route has been utilised to create a tissue specific immune response in mucosal tissues, the target for HuNoVs (El-Kamary, Pasetti *et al*. 2010). The protectiveness of the vaccine candidates varied and so far only one vaccine has advanced to Phase IIb trials (Bargatze, Mendelman *et al*. 2016, Limited 2016).

There has been less emphasis on identifying a potential antiviral against HuNoV, but a few candidates are being tested. T-705 is an antiviral that is hypothesised to act against the RNA-dependent RNA polymerase (RdRp) of target viruses and is currently being tested for influenza A

treatment (Sidwell, Barnard *et al*. 2007). It has also been shown to act against MNV in moderate doses and is a potential antiviral treatment for HuNoV (Rocha-Pereira, Jochmans *et al*. 2012). A different set of compounds acting against the RdRp has been identified for HuNoV. They were shown to vary in their ability to specifically inhibit a certain strain or genogroup or to broadly act against different genogroup strains (Eltahla, Lim *et al*. 2014). Other potential antivirals include 3C and 3C-like protease inhibitors (Kim, Lovell *et al*. 2012), human IFNα and ribavirin (Chang and George 2007) an antiviral previously been shown to be active against Hepatitis C (Rocha-Pereira, Jochmans *et al*. 2013).

1.1.5 Viral Replication

The open reading frames (ORFs) 1-3 in HuNoV and ORFs 1-4 in MNV are encoded within the viral single stranded, positive sense genomic RNA. On its 5' end, the genomic RNA is covalently linked to the viral non-structural protein NS5 or Viral Protein genome-linked (VPg), whereas the 3' end is poly-adenylated (Figure 2). In addition to the coding regions of the ORFs, NoVs share the characteristics of conserved untranslated regions (UTRs) which possess distinct secondary structures (Gutiérrez-Escolano, Brito *et al*. 2000, Simmonds, Karakasiliotis *et al*. 2008). These structures have been shown to extend into the coding regions (Simmonds, Karakasiliotis *et al*. 2008) and play a role in replication, translation and pathogenesis during NoV infection (Simmonds, Karakasiliotis *et al*. 2008, Bailey, Karakasiliotis *et al*. 2010, McFadden, Bailey *et al*. 2011).

For successful viral genome replication, the viral proteins, especially the RNA-dependent RNA polymerase (RdRp), have to be translated with the aid of the host cell translation complex. The genomic RNA serves as a template for the initial translation of the non-structural proteins,

whereas the structural proteins VP1 and VP2 or VF1, respectively, are translated from the subgenomic RNA. Studies depleting or inhibiting Vpg/NS5 revealed its major role in the recruitment of the translation complex as well as the initiation of translation (Chaudhry, Nayak *et al*. 2006, Guix, Asanaka *et al*. 2007). Leen *et al* showed that VPg forms a helical core with flexible N-/C-terminal regions, important for the diverse function of the protein (Leen, Kwok *et al*. 2013). HuNoV VPg as well as MNV NS5 have been confirmed to interact with components of the translation complex, namely eIF4F (Goodfellow, Chaudhry *et al*. 2005, Chaudhry, Nayak *et al*. 2006), eIF3 and cap-binding protein (Daughenbaugh, Fraser *et al*. 2003) and eIF4A (Goodfellow, Chaudhry *et al*. 2005), highlighting its purpose in initiating translation. Chung *et al* recently showed that an interaction between the C-terminus of VPg and eIF4G is required for efficient translation and a direct association between VPg and the core components of the eIF4F complex as well as the Poly(A)-binding protein (PABP) can be observed (Chung, Bailey *et al*. 2014). Furthermore, the UTRs, flanking the coding regions of the ORFs, seem to be essential for the genomic RNA translation by interacting with host proteins that are associated with the regulation of translation such as La, PTB and DDX3 (Simmonds, Karakasiliotis *et al*. 2008, Vashist, Urena *et al*. 2012). RNA interference studies with the interacting host cell proteins as well as studies using viral RNA depleted of UTRs observed a reduction in viral replication when the interaction of the UTRs with the host cell proteins was inhibited (Vashist, Urena *et al*. 2012). This interaction is thought to support viral replication by promoting viral RNA circularisation which in turn induces the binding of complementary sequences on the 5' and 3' end UTRs (Sandoval-Jaime and Gutiérrez-Escolano 2009, López-Manríquez, Vashist *et al*. 2013).

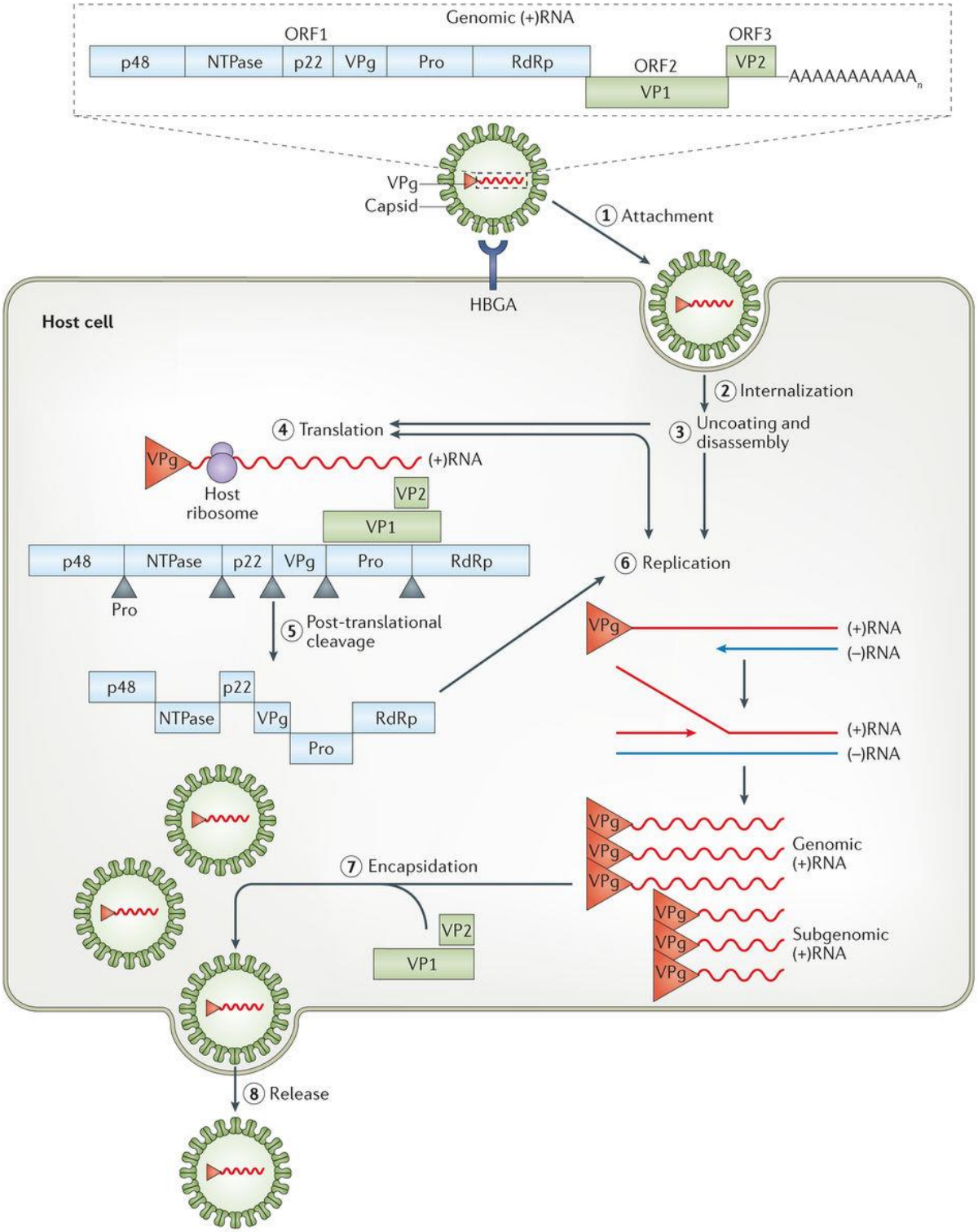


Figure 2 ***Schematic overview of NoV replication (de Graaf, van Beek et al. 2016)****. NoVs are (+) sense, single stranded RNA viruses which rely on the generation of a (-) sense RNA template for genome replication. VPg/NS5 is bound to the (+) sense RNA to mediate viral translation by the host cell translation machinery.*

Once the host cell translation complex has been recruited, the non-structural proteins of HuNoV, as well as MNV, are translated as a polyprotein, which is cleaved into the single viral proteins by the viral protease NS6/Pro (Sosnovtsev, Belliot *et al*. 2006). The protease cleaves the precursor at the following sites: ^{341}E/G^{342} (NS1/2-3), ^{705}Q/N^{706} (NS3-4), ^{870}E/G^{871} (NS4-5), ^{994}E/A^{995} (NS5-6) and ^{1177}Q/G^{1178} (NS6-7) (Sosnovtsev, Belliot *et al*. 2006) (Figure 3). The proteases of HuNoV and MNV share a sequence identity of about 60 % (Leen, Kwok *et al*. 2013) and the conserved residues H30 and C139, which have been proven to be essential for the protease activity. These residues together with E54 (HuNoV) and D54 (MNV) form the catalytic triad of the enzyme (Zeitler, Estes *et al*. 2006, Someya, Takeda *et al*. 2008). May *et al* discovered that the efficiency of NS6/Pro differs for different cleavage sites, leading to the cleavage of NS2-3 and NS3-4 at a higher rate than NS4-5, NS5-6 and NS6-7 (May, Korba *et al*. 2013, May, Viswanathan *et al*. 2014). They recently identified the residues P4-P2' upstream of the cleavage site to be an important cleavage signal for Pro and responsible for controlling the enzyme efficiency, leading to a biased cleavage (May, Viswanathan *et al*. 2014).

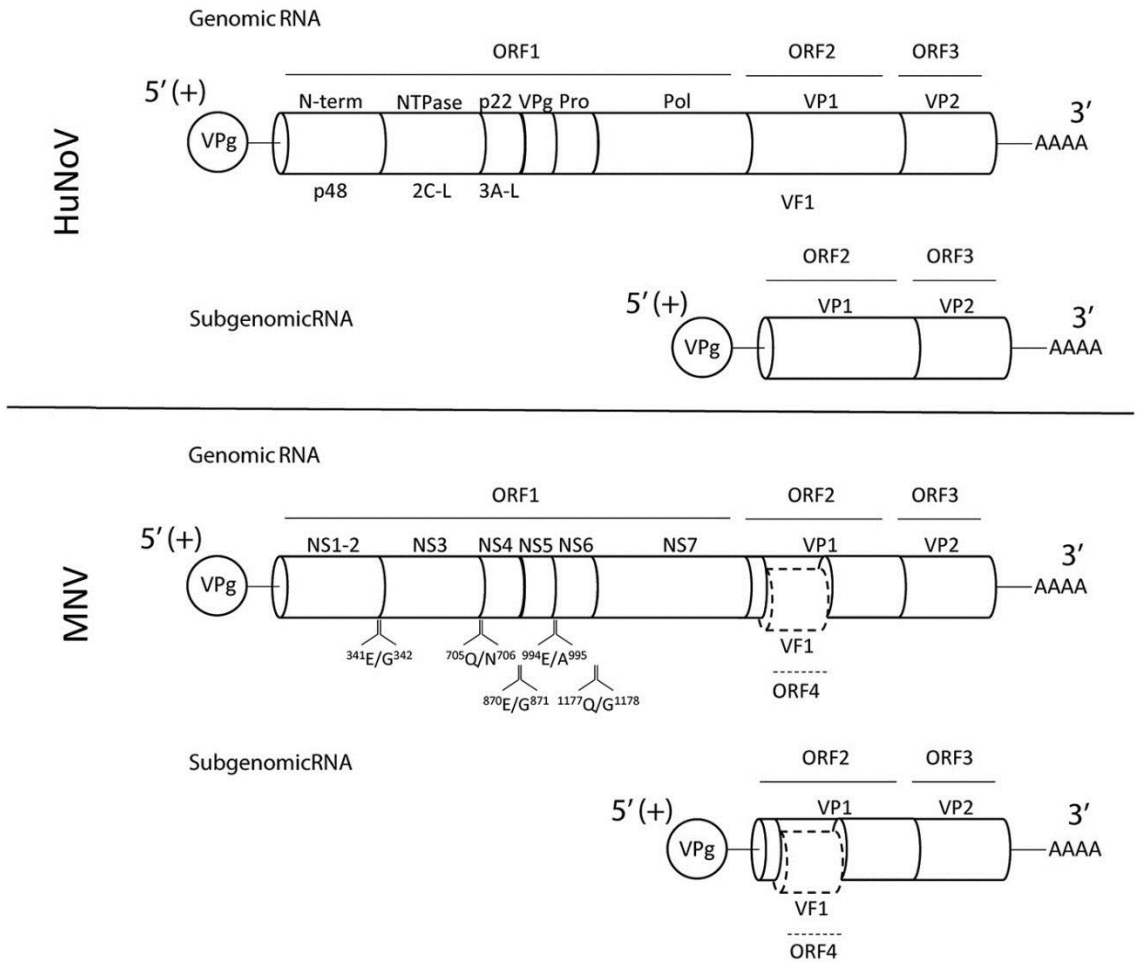


Figure 3 ***Genomic structure of HuNoV and MNV (Sarvestani, Cotton et al. 2016)****. HuNoV and MNV are similar in the structural organisation of their genomes. ORF1 encodes the NS proteins, whereas ORF2 and ORF3 encode the structural proteins VP1 and VP2. The major difference between both genomes is the additional ORF in MNV (ORF4), which encodes the virulence factor VF1.*

After the successful translation and processing of the non-structural proteins including the viral RdRp, the viral replication complex (RC) is formed in the cytoplasm of the host cell, containing all NS proteins as well as VP1 and VP2 (Hyde and Mackenzie 2010, Thorne, Bailey *et al*. 2012). The complex can be observed as punctate foci via immunofluorescence analysis, localising close to the microtubule organising centre (MTOC) (Hyde, Gillespie *et al*. 2012). The major structural protein VP1 has been shown to co-localise to acetylated tubulin indicating a potential role of the cytoskeleton in positioning of the RC within the cell (Hyde, Gillespie *et al*. 2012). The RC is

associated with host cell membrane complexes (Wobus, Karst *et al.* 2004, Belov and van Kuppeveld 2012) and virus-induced membranous vesicles, which are formed during infection and are redistributed to the RC (Hyde, Sosnovtsev *et al*. 2009). Theses membranes are derived from the ER, Golgi and endosomes of host cells through a mechanism that is so far unknown (Hyde, Sosnovtsev *et al*. 2009). The NS proteins NS1-2 and NS4 of HuNoV and MNV have been shown to affect the Golgi apparatus and protein secretion and co-localise to the ER, Golgi and endosomes when over-expressed in cells (Hyde and Mackenzie 2010), indicating a potential role in the redistribution of host membranes.

Like all (+)ssRNA viruses, NoV genome replication is dependent on generating a negative sense intermediate, which serves as a template for the positive sense genomic RNA. The positive as well as the negative strand of the genomic and subgenomic RNA are synthesised by the viral RdRp (NS7/Pol), which is initiated *de novo* or VPg-dependent (Rohayem, Robel *et al*. 2006, Subba-Reddy, Goodfellow *et al*. 2011). *De novo* replication occurs during the generation of the negative sense template and is supported by the interaction of the RdRp with the S-domain of VP1 (Subba-Reddy, Yunus *et al*. 2012). The connection between VP1 levels and replication initiation led to the hypothesis that capsid assembly in later stages of infection and a reduction in VP1 abundance would lead to a reduction in RNA replication (Subba-Reddy, Yunus *et al*. 2012). The positive sense genomic RNA replication is initiated via VPg, which serves as a protein primer at the 3' end of the negative sense template (Rohayem, Robel *et al*. 2006). The RdRp covalently binds VPg to the initial nucleotide of the genomic and subgenomic RNA via a tyrosine residue that is conserved in HuNoV and MNV (Y27 and Y26, respectively) (Subba-Reddy, Goodfellow *et al*. 2011). In addition to the genomic RNA, a subgenomic RNA containing the ORFs 2 and 3 is generated by the RdRp from the

negative sense template (Herbert, Brierley *et al*. 1997). The subgenomic RNA is identical with the last 2.4 kb of the genomic RNA and is also linked to VPg and poly-adenylated. Two mechanisms by which the subgenomic RNA replication is initiated have been proposed: premature termination and internal initiation (Thorne and Goodfellow 2014). The latter has been supported by findings of conserved secondary structures upstream of VP1 (Simmonds, Karakasiliotis *et al*. 2008). These structures were recently identified as the promotor for the subgenomic RNA. It consists of a highly conserved stem loop structure and is located 6 nucleotides upstream of VP1 in the coding region of NS7 (Yunus, Lin *et al*. 2015). Lin *et al* discovered that this promotor as well as a short template sequence are preferentially recognized by the RdRp of HuNoV and MNV and ensure stable binding of the RdRp (Lin, Thorne *et al*. 2015). It is assumed that the separate generation of the subgenomic RNA leads to the production of higher levels of the structural proteins, which are needed to form the viral capsid consisting of 180 copies of VP1 (Prasad, Rothnagel *et al*. 1994).

1.1.6 Murine Norovirus

In 2003 an unknown NoV was discovered in mice and was allocated to genogroup V (GV) (Karst, Wobus *et al*. 2003). This murine norovirus (MNV) shares characteristics with the HuNoV strains, and is cultivable in mice. Mice infected with MNV have detectable levels of viral RNA in the liver, spleen and proximal intestine 1 day after infection, but they clear the infection by day 3 post infection (Karst, Wobus *et al*. 2003). MNV specifically infects macrophages and dendritic cells in the previously mentioned tissues and can be cultivated in cell lines such as the murine macrophage cell line RAW264.7 (Wobus, Karst *et al*. 2004). Only recently, the receptors for MNV infection have been identified as CD300ld and CD300lf and expression of these receptors is sufficient to make most cell types susceptible to MNV infection (Haga, Fujimoto *et al*. 2016, Orchard, Wilen *et al*. 2016). Studies on knockout mice revealed a strong correlation between the

innate immune response and MNV infection. STAT1$^{-/-}$ as well as IFN$\alpha\beta\gamma$R$^{-/-}$ mice succumb to MNV infection, whereas RAG$^{-/-}$ mice do not (Karst, Wobus *et al*. 2003). Because of its similarity to the human strains, MNV is used as a model to further our knowledge of NoVs (Wobus, Thackray *et al*. 2006). It is still unclear to what extent findings in MNV research are transferable to HuNoVs, but the viruses do share molecular characteristics. They exhibit the same size (28-35 nm), shape (icosahedral) and buoyant density (1.36 ± 0.04 g/m³), as well as the organization of their genomes (Wobus, Thackray *et al*. 2006). For MNV, the positive sense ssRNA genome consists of four known ORFs, which encode the non-structural proteins (ORF 1), the major (VP1) and the minor (VP2) capsid protein (ORF 2, ORF 3, resp.) and the newly discovered ORF 4 overlapping with ORF 2 and encoding the protein VF1 (McFadden, Bailey *et al*. 2011) (Figure 3). VF1 has been shown to antagonize the innate immune response by blocking type I IFN production. It is suspected to have a role in controlling apoptosis, because RAW264.7 cells infected with a virus strain lacking ORF4 showed higher levels of apoptosis (McFadden, Bailey et al. 2011). It might also impact viral persistence, because STAT-/- mice infected with a virus strain not expressing ORF4 displayed a delayed onset of symptoms (McFadden, Bailey *et al*. 2011). The functions of all NS proteins are not fully understood yet, but some of the interactions and cellular localisations are known and described below.

1.1.6.1 MNV NS proteins

NS1-2 (Nterm, 38 kDa) contains several predicted protein motifs. A histidine region (H-box) common in histidine protein kinases, a motif containing asparagines and cysteine (NC-motif) as well as transmembrane (TM) α helices, known to act as membrane anchors can be found at the sequence level (Fernandez-Vega, Sosnovtsev *et al*. 2004, Sigrist, de Castro *et al*. 2013).

Localisation studies in Vero C1008 cells show that NS1-2 exhibits a reticular localisation and surrounds the nuclear envelope (Hyde and Mackenzie 2010). In addition, dense punctate foci are also observed throughout the cell. These foci co-localise with endoplasmic reticulum (ER) markers, but in contrast to the human NS1-2, they do not co-localise with the Golgi apparatus (Fernandez-Vega, Sosnovtsev *et al*. 2004, Hyde and Mackenzie 2010). NS3 is a nucleoside triphosphatase (NTPase) and possesses a molecular weight of 39 kDa. In NS3 transfected cells both distinct vesicle-like structures and disperse cytoplasmic staining can be observed for the viral protein, but it does not co-localise with any known organelle markers (Hyde and Mackenzie 2010). A recent study discovered an association of MNV NS3 and HuNoV NS3 with the microtubule network, specifically β-tubulin. They also showed that movement of NS3 within the cell was dependent on a functional microtubule network (Cotton, Hyde *et al*. 2016). NS4 (p20) with a molecular weight of 18 kDa, forms dense cytoplasmic foci in the cell, and co-localises with the Golgi as well as with endosomes. Expression of NS4 is connected with a partial dispersion of the Golgi apparatus, which has also been observed in poliovirus infections. During poliovirus infection the dispersion of the Golgi is caused by the 3A protein, which is comparable to MNV NS4 (formerly named 3A-like protein) (Hyde and Mackenzie 2010). NS5 or VPg is covalently linked to the 5' end of the viral RNA. NoVs, like all members of the *Caliciviridae* family, lack a cap at the 5' end of their RNA. As the cap is usually associated with the initiation of translation, VPg has been suggested to replace the function of the cap. Findings, including binding of VPg to elongation initiation factors (eIFs) and loss of infectivity after removal of VPg from the viral RNA, indicate an association of VPg with the initiation of translation (Daughenbaugh, Fraser *et al*. 2003, Daughenbaugh, Wobus *et al*. 2006). NS5 shows a dispersed cytoplasmic distribution, when expressed on its own. NS6 (19 kDa) is a protease with a chymotrypsin-like fold, that co-localises with mitochondria and cleaves

the precursor polyprotein. NS7 (57 kDa) is the RNA-dependent RNA polymerase, which ensures replication of the viral genome and presents a diffuse staining within the cell, including both the cytoplasm and the nucleus during transfection (Fernandez-Vega, Sosnovtsev *et al*. 2004, Hyde and Mackenzie 2010).

1.2 Innate Immunity

1.2.1 Innate immune response to viruses

Although the cellular responses to viral infections are as diverse as the viruses themselves, some general mechanisms are known. The most common reactions following virus infection is the induction of cytokine secretion, especially interferon type I (IFN-I) and apoptosis to combat viral replication, alert surrounding cells and to reduce the number of infected cells and viral spread. These reactions are often induced by the recognition of dsRNA, a replicative intermediate typical of viral replication (Vilcek 1996). dsRNA is a pathogen-associated molecular pattern (PAMP), which is a shared characteristic of replication for many viruses. This PAMP can be recognised by pattern recognition receptors (PRRs), which can be cytosolic like melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I) or attached to a cellular membrane like the endosome-associated Toll-like receptor 3 (TLR3) (Figure 4).

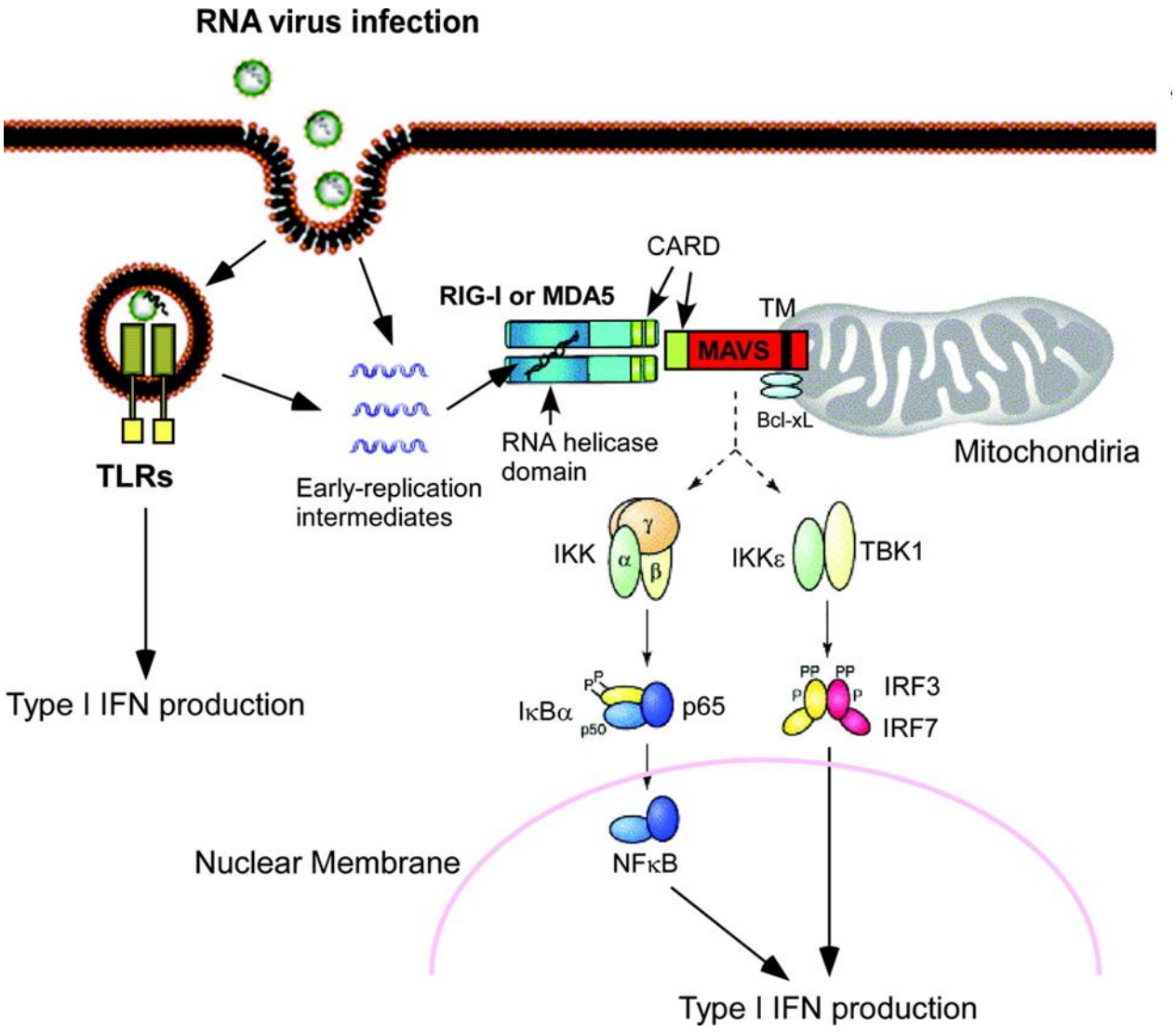


Figure 4 ***IFN induction through dsRNA recognition in virus infected cells (Yajima and Knowlton 2009).*** *During NoV replication dsRNA is formed, which can be recognised as a PAMP by MDA5. Via signalling through MAVS and TBK1, IRF-3/IRF-7 get translocated into the nucleus and mediate the transcription of type I IFN.*

When dsRNA is recognised by the cell, several antiviral signals, including the production of cytokines, like IFNα/β, can be triggered. The cytokine response can differ depending on the pathway that has been activated by dsRNA. Activation of MDA5 as well as TLR3 leads to the phosphorylation, dimerization and translocation of IRF-3 into the nucleus (Sato, Tanaka *et al*. 1998). The phosphorylation is mediated through the IκB kinase (IKK) related kinases IKKε and

TANK binding kinase 1 (TBK-1) and allows the translocation of IRF-3 into the nucleus where it associates with the coactivators CREB binding protein (CBP) and p300 (Hiscott, Pitha *et al*. 1999, Kumar, McBride *et al*. 2000, Fitzgerald, McWhirter *et al*. 2003). IRF-3 is not only an important transcription factor for type I IFNs, but also regulates the expression of the chemokine RANTES and specific ISGs (ISG54, ISG56, ISG60) (Génin, Algarté *et al*. 2000, Grandvaux, Servant *et al*. 2002). It has been shown to bind to the IFN-stimulated response element (ISRE) which can be found in the promotor region of many IFN associated genes (Au, Moore *et al*. 1995).

Another important regulator of cytokine transcription and specifically type I IFN activation is NF-κB, which is mainly activated through the TLR3 pathway during viral infections (Alexopoulou, Holt *et al*. 2001). Through the adaptor protein MyD88, the activation signal of TLR3 is conferred to the kinase IRAK which has been suggested to interact with TNFR-associated factor 6 (TRAF6) and leads to the downstream translocation of NF-κB into the nucleus via degradation of the inhibitory complex IκB in the cytosol (Muzio, Ni *et al*. 1997, Kopp and Medzhitov 1999). Once NF-κB is present in the nucleus it can act as a transcription factor for a wide variety of cytokines, like IL-6, TNFα and IFNβ (Lenardo, Fan *et al*. 1989, Collart, Baeuerle *et al*. 1990, Libermann and Baltimore 1990, Pahl 1999).

1.2.2 The role of cytokines during viral infections

Levels of cytokines such as type I IFN are low in uninfected cells and an up-regulation of cytokine expression is considered to be a vital part of the host immune response to viral infections. The induction of type I IFN in MNV infected cells is critical for controlling the acute infection, as it has been shown that the lack of IFNα/β leads to lethal MNV infections in IFNαβγ$^{-/-}$ mice (Wobus, Karst *et al*. 2004). Type I IFN expression is mainly activated through IRF-3 and IRF-7, but can also be

activated by other transcription factors like NF-κB (Lenardo, Fan *et al*. 1989, Karst, Wobus *et al*. 2003, Changotra, Jia *et al*. 2009, Thackray, Duan *et al*. 2012). To inhibit virus replication, IFNα/β produced from infected cells can induce the expression of antiviral effectors such as RNAse L and Protein kinase R (PKR), in an autocrine and paracrine manner (Thomson and Lotze 2003). For example PKR, the double-stranded RNA-activated protein kinase, disturbs replication by inhibiting viral as well as host protein synthesis (Kalvakolanu and Borden 1996, Samuel 1998). Translation of proteins is inhibited through the phosphorylation of eIF2α, a major part of the translation initiation complex. eIF2α is phosphorylated by PKR and can no longer bind to the complex and global protein translation is stalled. Additionally, PKR has recently been shown to enhance the induction of IFNβ via the MDA5/MAVS pathway of dsRNA detection (Pham, Santa Maria *et al*. 2016). In contrast to that, 2'5' oligoadenylate synthetase-induced RNAse L degrades viral RNA and thus limits replication (Silverman 1994, Dong and Silverman 1999, Terenzi, Ying *et al*. 1999). In addition to promoting the immediate cellular response, IFNs can also modulate the adaptive and innate immune response. They have the ability to inhibit cell division, recruit macrophages, natural killer (NK) cells and cytotoxic T-cells (CTLs), and upregulate the expression of major histocompatibility complex (MHC) class I and II proteins. They also enhance the processing and transport of viral peptides to be presented by MHC proteins at the cell surface (Thomson and Lotze 2003).

Another important cytokine which is induced during many viral infections is the pro-inflammatory cytokine TNFα. TNFα expression is mainly induced through the TLR3/NF-κB pathway and can have several effects on the infected and surrounding cells (Goldfeld, Doyle *et al*. 1990, Idriss and Naismith 2000). There are two forms of TNFα which can be synthesised, a soluble and

a membrane bound form (Kriegler, Perez *et al*. 1988, Luettig, Decker *et al*. 1989). Membrane bound TNFα is 26 kDa in size, bigger than soluble TNF, and exhibits cytotoxic activity (Kriegler, Perez *et al*. 1988). It can induce apoptosis through binding of TNF receptor (TNF-R) 1 and TNF-R2, but has so far not been shown to regulate inflammatory processes (Ruuls, Hoek *et al*. 2001). The membrane bound form is expressed on macrophages and acts in a cytotoxic way on TNFα sensitive cells via direct contact with the target cell (Decker, Lohmann-Matthes *et al*. 1987). In the context of viral infections it has been demonstrated to play a role in the IL-10 dependent expression of HIV in latently infected cells (Barcellini, Rizzardi *et al*. 1996).

Soluble TNFα can bind to TNFα receptors such as TNF-R1 and activate the transcription of TNFα regulated genes (Figure 5), the production of reactive oxygen species (ROS) and caspase activation (Chen and Goeddel 2002, Wang, Du *et al*. 2008, Kim, Lee *et al*. 2010). Once TNFα binds to TNF-R1, the silencer of death domains (SODD) gets released from the intracellular domain of TNF-R1 and the adaptor protein TNF receptor-associated death domain (TRADD) can bind instead. The binding of TRADD allows the recruitment of further adaptor proteins such as receptor interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) (McWhirter, Pullen *et al*. 1999, Chen and Goeddel 2002). FADD has the ability to recruit and activate caspase-8, which leads to the induction of apoptosis. TRAF and RIP interact with IKKα and IKKβ which are important kinases for the activation of the transcription factor NF-κB. In addition to NF-κB another crucial transcription factor c-Jun can be activated by TRAF2 interaction with the JNK signalling pathway (Chen and Goeddel 2002). The activation of NF-κB leads to the inhibition of the apoptotic pathways induced by TNFα, which is caused by the induction of apoptosis inhibitors such as c-IAPs, caspase-8-c-FLIP and A1 via NF-κB (Karin and Lin 2002).

Furthermore, TNFα dependent NF-κB activation leads to the induction of inflammation associated proteins (GRO-1, IL-6, MCP-1 COX-2 and PGES), apoptosis or decreased cell growth factors (JunB and TIEG) and survival or cell growth factors (A20, syndecan-4, SOD-2 and COX-2) (Zhou, Scoggin *et al*. 2003). Only the expression of the regulatory proteins Mitogen-activated protein kinase phosphatase-1 (MKP-1) and Early growth response protein 1 (Egr-1) have been shown to be inducible by TNFα, but independent of NF-κB (Zhou, Scoggin *et al*. 2003).

In summary, IFNβ and TNFα can have different effects on the infected cell, because IFNβ mainly acts to induce an antiviral state, promoting cell survival whereas TNFα can induce apoptosis and inflammation and therefore their interaction during infections plays a crucial part in the infection outcome.

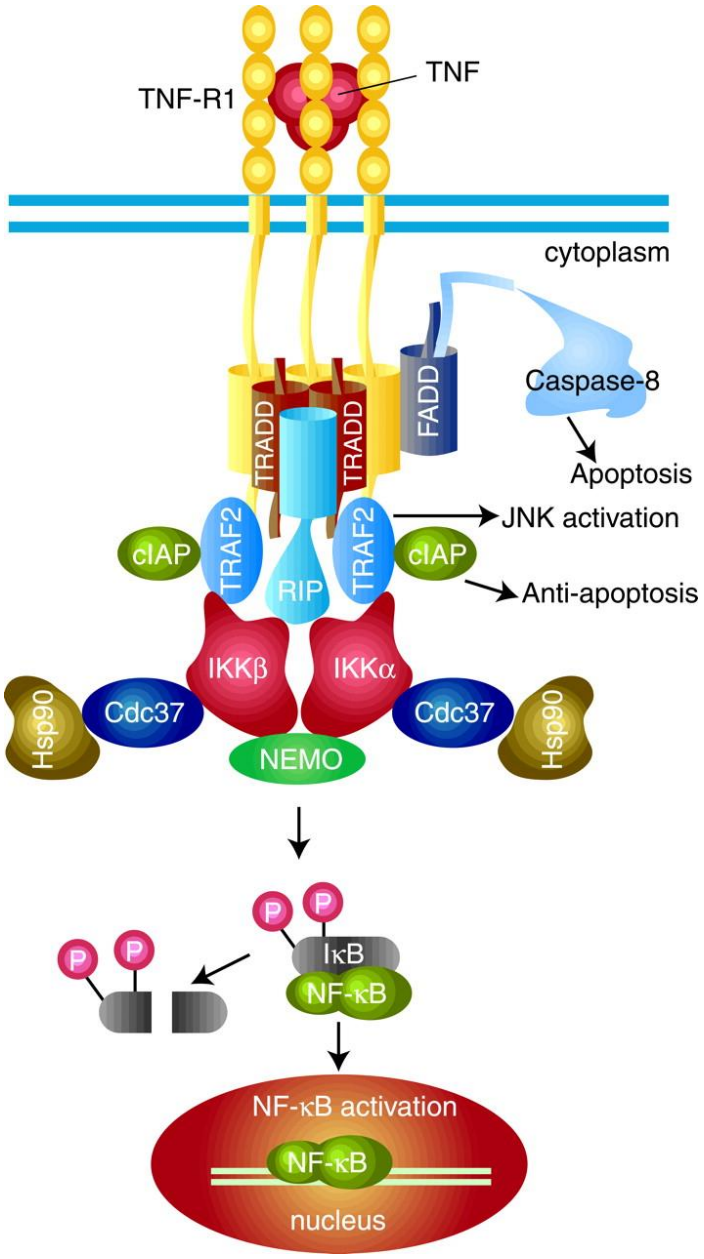


Figure 5 ***Overview of the TNFα signalling pathway (Chen and Goeddel 2002)***.

1.2.3 MHC proteins

MHC proteins are a class of polymorphic proteins which mediate the recognition of self-antigens and foreign antigens. They are important components of both the innate and adaptive immune response and act by presenting antigens on the cell surface of infected cells to CD8+ (MHC class I)

and CD4+ (MHC class II) T cells. The interaction between the MHC surface proteins and the T-cells is mediated by T-cell receptors (TCR) specific for MHC class I and II proteins (Corr, Slanetz *et al*. 1994, Sakihama, Smolyar *et al*. 1995). When a TCR binds to a foreign antigen-MHC complex, different signalling cascades are activated including the proximal signalling through CD3 phosphorylation, calcium-mediated signalling through Ca^{2+} influx and GTPase Ras-MAPK signalling (Cahalan and Chandy 2009, Smith-Garvin, Koretzky *et al*. 2009, Morris and Allen 2012). This results in the activation of the T cell and the immune response to the infection. Apart from having the same function of presenting antigens, MHC class I and II proteins share other characteristics like genetic location and a vast polymorphism (Neefjes, Jongsma *et al*. 2011). In humans, MHC class I and II proteins are encoded by the HLA gene complex HLA A, B, C, E, F and G and HLA DP, DM, DOA, DQ and DR, respectively (Strachan 1987, Trowsdale 1987).

1.2.3.1 MHC class I

MHC class I proteins mainly present peptides of endogenous origin, but also have the ability to present exogenous peptides that have been endocytosed by the cell (cross-presentation) (Kurts, Robinson *et al*. 2010). MHC class I proteins are widely expressed in many cell types and can be upregulated during infection, e.g. via IFNβ stimulation (Israël, Le Bail *et al*. 1989). Peptides presented by MHC class I proteins on the cell surface are derived from proteins that have been degraded by the either cytosolic or nuclear proteasomes (Figure 6). Degraded proteins, which have been truncated into peptides are transported into the ER lumen by the ATP-binding cassette (ABC) transporter TAP (*t*ransporter associated with *a*ntigen-*p*rocessing) (Vyas, Van der Veen *et al*. 2008). Within the ER a number of MHC class I heterodimer molecules are present, consisting of the polymorphic heavy (α) and light (β_2-microglobulin) chain. The otherwise unstable MHC class I

molecules are stabilized by chaperone proteins like calreticulin, ERp57, protein disulphide isomerase (PDI) and tapasin. The interaction between MHC class I proteins and antigens is supported by the peptide loading complex (PLC) comprising TAP, tapasin, MHC class I, ERp57 and calreticulin, leading to the incorporation of the antigen peptide into the binding groove of MHC class I (Park, Lee *et al*. 2006, Wearsch and Cresswell 2008, Wearsch, Peaper *et al*. 2011). Once the MHC class I and peptide complex is formed, the chaperone proteins detach from MHC class I proteins and induce the further translocation to the plasma membrane via the Golgi apparatus. Complexes of antigen peptides and MHC class I proteins at the cell surface are relatively stable, but are eventually degraded through an interaction with ubiquitin ligases of the Membrane-Associated Ring-CH (MARCH) family and the endosomal pathway (Bartee, Mansouri *et al*. 2004).

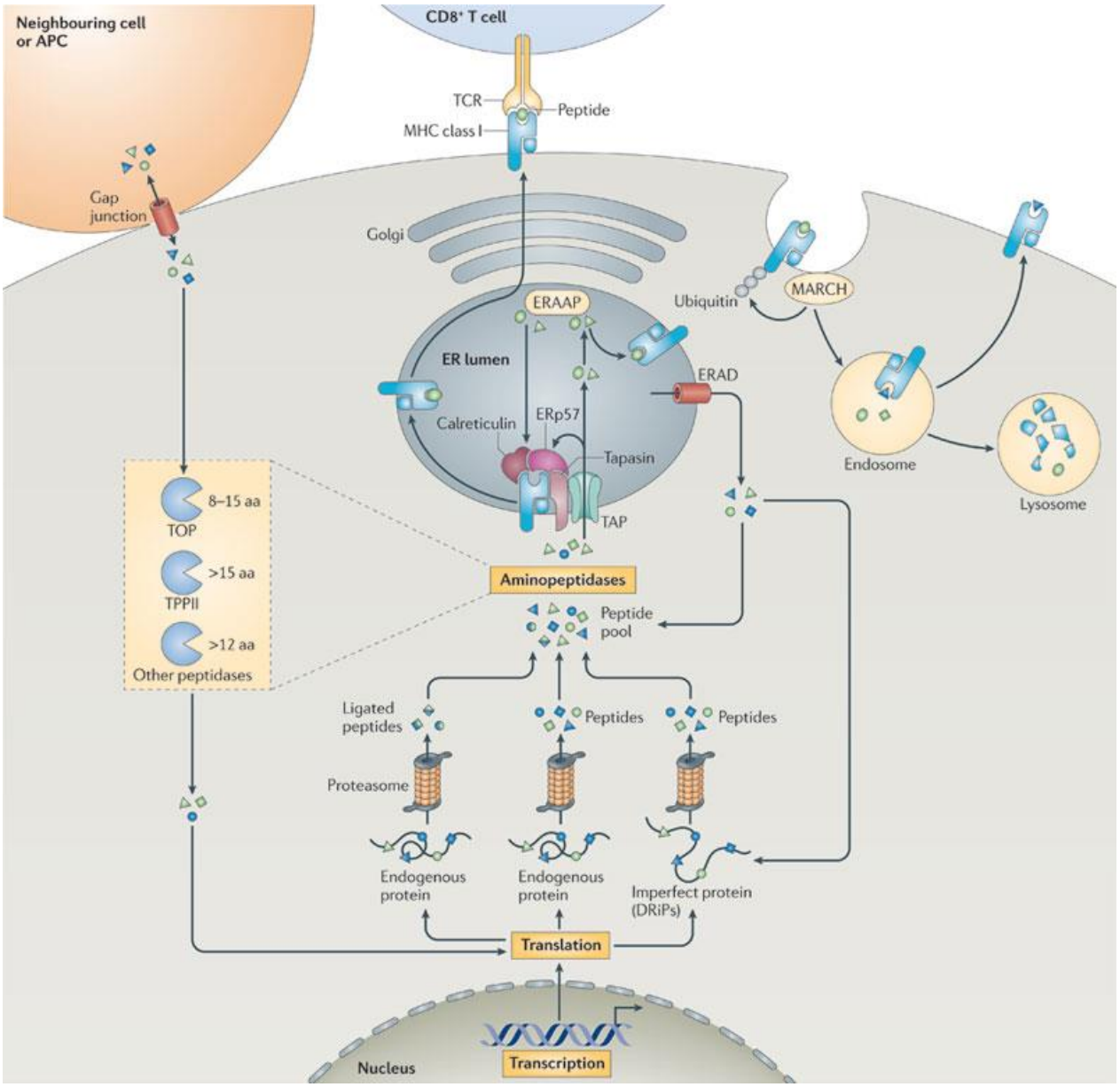


Figure 6 ***Schematic overview of the MHC class I synthesis pathway and the formation of MHC class I and peptide complexes (Neefjes, Jongsma et al. 2011)***

1.2.3.2 MHC class II

In contrast to MHC class I proteins, MHC class II proteins are not ubiquitously expressed, but are mainly restricted to professional antigen-presenting cells (APCs). Their expression is controlled by IFN-γ, which induces the co-activator class II major histocompatibility complex transactivator

(CIITA) (Steimle, Siegrist *et al*. 1994). CIITA in turn aids to activate the transcription factor RFX5, which leads to the transcription of MHC class II genes (Scholl, Mahanta *et al*. 1997). MHC class II proteins consist of two transmembrane chains (α and β) forming a heterodimer that assembles within the ER. There, the protein binds to the invariant chain (Ii) preventing the association with peptides in the ER (Figure 7). The Ii ensures MHC class II translocation to the MHC class II compartment (MIIC) via a sorting motif for late endosomal compartments (Bertolino and Rabourdin-Combe 1995, Landsverk, Bakke *et al*. 2009). After successful translocation, the Ii is partially digested leaving the Class II-associated invariant chain peptide (CLIP) that is in turn replaced by the antigen peptide. This process is mediated by the HLA-DM protein in humans or H2-DM protein in mice (Kropshofer, Vogt *et al*. 1996). Peptides which are loaded onto MHC class II proteins originate from the endosomal pathway and have been degraded by proteases associated with the endosomes (Neefjes, Jongsma *et al*. 2011). When the antigen peptide is successfully bound to MHC class II, the complex is transported via association with the cytoskeleton (myosin (microfilaments) and kinesin (microtubules)) to the plasma membrane for interaction with CD4+ T cells (Rocha, Kuijl *et al*. 2009). Activated CD4+ T cells recruit proteins to regulate inflammation and interact with B cells to induce B cell memory to an antigen (Mitchison 2004, Yuseff, Lankar *et al*. 2009). Similar to MHC class I, MHC class II gets ubiquitylated by a ubiquitin ligase, MARCH1, leading to the degradation of MHC class II proteins (Shin, Ebersold *et al*. 2006, De Gassart, Camosseto *et al*. 2008).

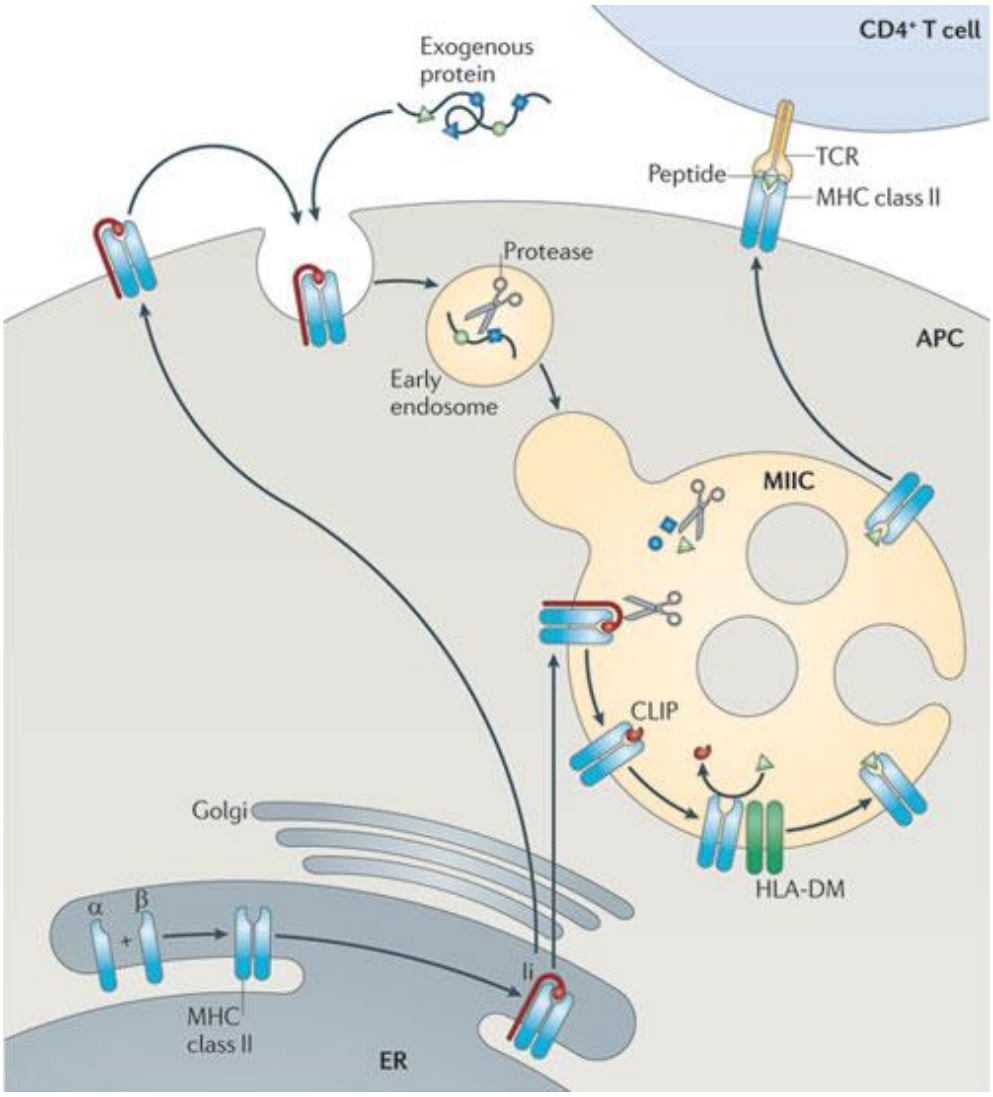


Figure 7 ***Schematic overview of the MHC class II and peptide formation and the presentation of the complex (Neefjes, Jongsma et al. 2011)***

1.2.3.3 Viral immune evasion through MHC class I targeting

When cells get infected by viruses, they do not only contain the viral genome but also considerable amounts of viral proteins. These viral proteins, as any other proteins in the cell, are susceptible to degradation and further presentation by MHC class I proteins. To prevent the presentation of viral antigen peptides several viruses have developed evasion strategies interfering with different stages of MHC class I antigen presentation. Some viruses such as

Epstein-Barr virus (EBV) inhibit the degradation of viral proteins via the proteasome, preventing the translocation of these peptides into the ER and the formation of viral antigen and MHC class I complexes (Levitskaya, Sharipo *et al*. 1997, Sharipo, Imreh *et al*. 1998). Once viral proteins have been degraded, they are translocated into the ER via TAP. At this stage, viruses such as herpes simplex virus-1 (HSV-1) and human cytomegalovirus (HCMV) interfere with antigen presentation by inhibiting peptide binding to TAP (HSV-1) (Hill, Jugovic *et al*. 1995, Ahn, Meyer *et al*. 1996) or preventing ATP-binding and hydrolysis which is needed for the transport via TAP (HCMV) (Ahn, Gruhler *et al*. 1997, Hewitt, Gupta *et al*. 2001). Viral peptides which have been transported into the ER usually form a complex with MHC class I proteins with the aid of the PLC. Viruses have been shown to avoid this formation of MHC class I and peptide complexes by degrading MHC class I proteins in the ER (e.g. HCMV) (Stevenson, Efstathiou *et al*. 2000, Boname and Stevenson 2001). The poliovirus 3A protein has been shown to interfere with MHC class I proteins by inhibiting the ER to Golgi transport, leading to a reduced surface expression and protection from CD8+ T cells (Deitz, Dodd *et al*. 2000). Human herpes virus-7 (HHV-7) disturbs the next step in the MHC class I antigen presentation pathway, relocating the peptide and MHC class I complexes to lysosomes and thus inhibiting the transport from the ER to the plasma membrane (Hudson, Howley *et al*. 2001). Even MHC class I proteins presenting peptides at the cell surface can be targeted by viral proteins. The Nef protein of the human immunodeficiency virus-1 (HIV-1) is known to interact with the cytoplasmic tail of MHC class I proteins at the plasma membrane, causing the internalisation of the complex, accumulation of MHC class I in the trans-Golgi network (TGN) and finally degradation via the endosomal pathway (Schwartz, Maréchal *et al*. 1996, Le Gall, Erdtmann *et al*. 1998). Although inhibiting or reducing the presentation of viral antigens by MHC class I proteins avoids the recognition of viruses by $CD8^+$ T cells, decreased levels of MHC class I

surface proteins can also lead to the stimulation of NK cells. Therefore some viruses additionally express MHC class I homologues to mimic a normal level of MHC class I surface expression and avoid the detection by NK cells (Farrell, Degli-Esposti *et al*. 2000).

In conclusion, viruses have developed very different strategies to avoid immune detection, immune signalling and the subsequent activation of an immune response. Interfering with innate immune pathways and antigen presentation via MHC class I proteins provides an advantage to the virus by increasing replication and protecting them from the host immune response.

1.3 MNV and the cytoskeleton

The cytoskeleton plays an important role for the stability, function and movement of the cell. It consists of three different fibres, microfilaments (actin), microtubules (tubulin) and intermediate filaments (e.g. vimentin), which have different functions within the cell. Microfilaments are important for cell motility, vesicle and organelle transport, cell signalling and cell shape (Landry and Huot 1995, Valderrama, M Durán *et al*. 2001, Pollard and Borisy 2003). Microtubules play a major role in mitosis and the formation of the spindle apparatus and form a network for intracellular transport (Sharp, Rogers *et al*. 2000, Welte 2004). Unlike microfilaments and microtubules, intermediate filaments are not associated with cell motility. They are the most stable fibres of the cytoskeleton and provide mechanical support for the plasma membrane. They have also been shown to be in contact with the extracellular matrix and aid in cell-cell interactions (Lodish H 2000). The interaction of MNV with the different fibres of the cytoskeleton has recently been investigated (Hyde, Gillespie *et al*. 2012). Vimentin, a component of the intermediate filaments showed some co-localisation with MNV, but its appearance was not affected during infection. In contrast, actin, a component of the microfilaments, did not co-localise with the virus

and was only affected in its morphology at later stages of infection, when a cytopathic effect of the virus was visible.

1.3.1 MNV and the microtubules

As described in previous sections, the viral RC of MNV was observed to localise proximal to the MTOC, which forms the origin of microtubule formation and the core of the network. The microtubule network emerges from the MTOC, which contains a specific form of tubulin, γ-tubulin (Zheng, Wong *et al*. 1995). Studies with Nocodazole, a drug preventing the polymerization of microtubules, showed that efficient MNV replication was dependent on functional microtubules. Nocodazole treatment resulted in an accumulation of viral RNA at the periphery and a reduction in viral protein synthesis. Investigating the interaction partners of the MNV RC revealed an association with acetylated tubulin. In contrast to unmodified tubulin, acetylated tubulin has an increased stability due to its posttranslational modification and is likely to form filamentous bundles within the cell. Acetylated tubulin highly co-localised with the structural protein VP1, leading to its redistribution and the lack of filamentous fibres (Hyde, Gillespie *et al*. 2012). A recent study also revealed that the non-structural protein NS3 of MNV and Norwalk virus was associated with β-tubulin, a major component of the microtubule network (Cotton, Hyde *et al*. 2016). Overexpression of HuNoV and MNV NS3 formed vesicle-like structures within the cell which were highly motile. Inhibiting microtubule activity using Nocodazole showed that the ability of NS3 vesicles to move within the cell is dependent on a functional microtubule network (Cotton, Hyde *et al*. 2016). NS3 induced vesicles were less motile in cells treated with Nocodazole and appeared to lack the ability of fusing together with other NS3 vesicles. It has been proposed that NS3 is using the microtubule network to transport host components required for viral replication

to the RC located close to the MTOC. This is supported by previous findings which revealed that the impairment of the microtubule network with Nocodazole leads to the dispersion of the otherwise focused RC, indicating a crucial role of the microtubules for viral replication (Hyde, Gillespie *et al*. 2012).

Additionally, MNV induces the formation of microtubule bundles at the cell periphery during later stages of infection (12-18 h.p.i.). A similar observation has been made in cells infected with rotavirus, a virus causing the majority of gastroenteritis infections in children (Bernstein 2009). Microtubules in rotavirus infected cells formed ring-like structures at the cell periphery, comparable to the bundling of microtubules in MNV infected cells. The aggregation of microtubules in the periphery was accompanied with the disassembly of microtubules in the centre (Yang and McCrae 2012). Recently, GEF-H1, a guanine nucleotide exchange factor (GEF), has been shown to have a similar effect on microtubules, inducing microtubule bundles in GEF-H1 transfected cells (Chiang, Zhao *et al*. 2014). GEF-H1 has been proposed to play a role in the innate immune response against viral infections, marking it as a potential candidate which connects the observed phenotypic effect on the microtubule network during MNV infection and the host response to MNV (Chiang, Zhao et al. 2014).

1.3.2 GEF-H1 as a viral sensor

Guanine nucleotide exchange factors (GEFs) are regulators of GTPases. They activate GTPases by mediating the exchange from guanosine diphosphate (GDP) to guanosine triphosphate (GTP) (Figure 8). GTPases can in turn be deactivated through GTPase activating proteins (GAPs). GAPs transform GTP which is bound to the GTPase to GDP by facilitating the hydrolysis of GTP. GEF-H1 is a GEF which regulates Rho and Rac GTPases including RhoA and RhoB, supporting multiple

processes in the cell like vesicle transport, cytoskeleton morphology and pathogen recognition (Ren, Li *et al*. 1998, Zhao, Alonso *et al*. 2012, Nagae, Meng *et al*. 2013, Pathak and DerMardirossian 2013).

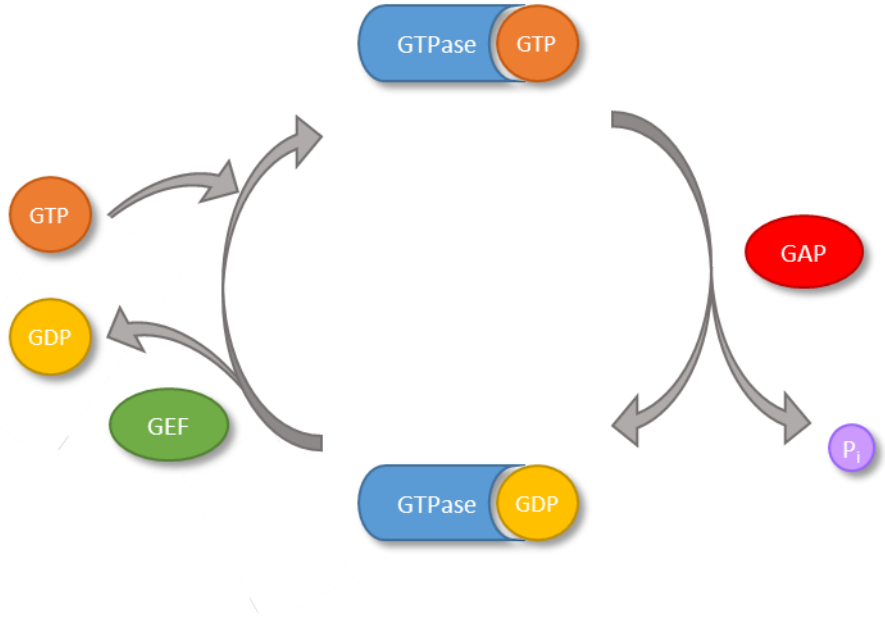


Figure 8 ***Schematic model of the GDP to GTP exchange on GTPases facilitated by guanine nucleotide exchange factors (GEFs).*** *The reverse change from GTP to GDP is mediated by GTPase activating proteins (GAPs).*

GEF-H1 is a 110 kDa protein which consists of two major domains which are typical for the Dbl family of GEFs (Ren, Li *et al*. 1998). The Dbl homology (DH) domain is responsible for the catalytic activity of GEF-H1, whereas the pleckstrin (PH) domain determines the location of most GEFs within the cell (Hart, Eva *et al*. 1994, Zheng, Zangrilli *et al*. 1996). Unlike the majority of GEFs, GEF-H1 locates to the microtubules. It can bind to the microtubules via its N-terminal C1 domain, which regulates the activity of GEF-H1 and mediates the binding to the dynein motor complex (Krendel, Zenke *et al*. 2002, Meiri, Marshall *et al*. 2012). Microtubule-bound GEF-H1 is in an inactive state, but can be activated upon stimulation via pathogen infections or the detachment

from the microtubule network (Krendel, Zenke *et al*. 2002, Chiang, Zhao *et al*. 2014). The interaction of GEF-H1 with the microtubules can be inhibited by several proteins such as Par1b or p21-activated kinase (PAK) 4 as well as the depolymerisation of microtubules (Callow, Zozulya *et al*. 2005, Chang, Nalbant *et al*. 2008, Yoshimura and Miki 2011). Non-microtubule bound GEF-H1 becomes active and regulates important processes within the cell. In connection with extracellular signal regulated kinase (ERK) it activates RhoA in response to mechanical force and leads to the reinforcement of the plasma membrane (Guilluy, Swaminathan *et al*. 2011). GEF-H1 can also form a connection between the microtubule and actin network through RhoA. Activated RhoA leads to the activity of Rho-associated, coiled-coil containing protein kinase (ROCK) which can phosphorylate the myosin regulatory light chain (MLC). MLC regulates the interaction between myosin and actin and causes the induction of stress fibres (actin) in cells treated with the microtubule depolymerising drug Nocodazole (Chang, Nalbant *et al*. 2008).

Another important function of GEF-H1 is the regulation of vesicle trafficking. It has been shown to interact with Sec5, a component of the exocyst complex, which is involved in trafficking and exocytosis (Pathak, Delorme-Walker *et al*. 2012). Depletion of GEF-H1 in vitro leads to the accumulation of Rab11 positive vesicles (recycling vesicles), highlighting its importance in cell trafficking. Apart from its function in cell structure and intracellular transport, GEF-H1 has also been shown to be involved in the recognition and defence against microbial infections (Fukazawa, Alonso *et al*. 2008, Zhao, Alonso *et al*. 2012, Chiang, Zhao *et al*. 2014). During infection with *Shigella*, GEF-H1 plays a role in regulating the tight junctions between the cells of the epithelial barrier as well as mediating the activation of NF-κB through RhoA and Nucleotide-binding oligomerization domain-containing protein (NOD) 1 (Fukazawa, Alonso *et al*. 2008). Additionally,

GEF-H1 can regulate receptor-interacting serine/threonine-protein kinase (Rip) 2, which is an activator of the crucial transcription factor NF-κB (Zhao, Alonso *et al*. 2012). During Influenza A virus (IAV) infection GEF-H1 is released from the microtubules and becomes active to support the recognition of viral RNA via RIG-I and MDA5 (Chiang, Zhao *et al*. 2014). In the proposed model, GEF-H1 is suggested to be activated and released from the microtubules upon viral infection (Figure 9). After recognition of viral RNA through RIG-I or MDA5, mitochondrial antiviral-signalling protein (MAVS) is activated and induces the activation of TBK1 and IKKε. GEF-H1 interacts with TBK1 and appears to connect the activation of MAVS with the induction of TBK1 (Chiang, Zhao *et al*. 2014). GEF-H1 depletion leads to a reduced phosphorylation of interferon regulatory factor 3 (IRF3) and transcription of IFNβ, a downstream effect of TBK1 activation during IAV infection.

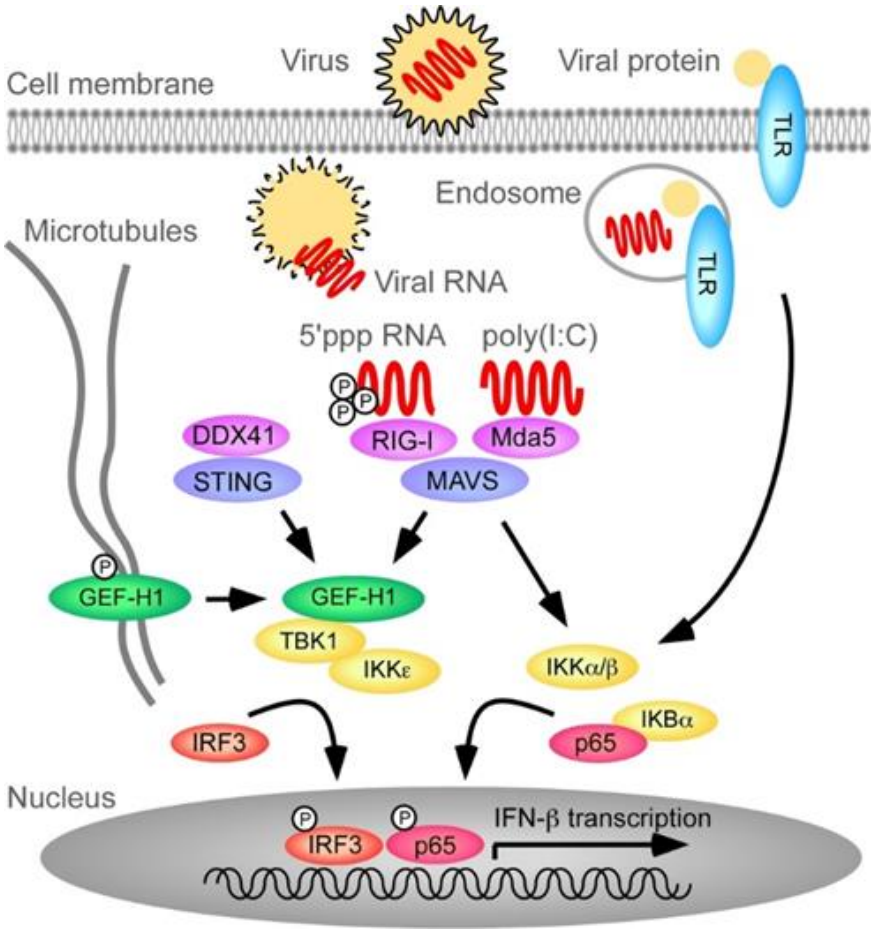


Figure 9 ***Proposed model of GEF-H1 in supporting the viral sensing pathway (Chiang, Zhao et al. 2014)****. In the context of viral infections, GEF-H1 becomes dephosphorylated and is released from the microtubules to aid in the immune response to viral infections and promote IFNβ transcription.*

1.4 Aims

The aim of this study was to uncover the role of the host immune system in response to MNV infection. MNV is able to replicate quickly and efficiently, despite various defence mechanisms in place to detect, fight and clear the infection. We were therefore interested in investigating aspects of the host immune signalling pathways including the synthesis and transport of major antiviral proteins as well as the interaction of MNV with the important transport network of microtubules.

To address the first aim of this study, we analysed the expression of immunity-related proteins during MNV infection. MHC class I proteins display peptides on the cell surface, which can be either host or pathogen derived. They are important for the presentation of foreign antigens to surrounding cells and immune cells to activate the immune response against the infection. As MHC class I proteins are crucial immune response regulators, they are often targeted by viruses to dampen the antiviral immune response. We therefore examined the effect of MNV on the surface expression of MHC class I proteins and the MHC class I synthesis pathway.

The second aim of this study was to characterise the effect of MNV infection on the host protein secretion and in particular cytokine secretion. Cytokines play a major role in the innate immune response by signalling to immune cells, alerting neighbouring cells and amplifying the intracellular antiviral response. IFNβ and TNFα were chosen as representatives of two antiviral signalling pathways and used throughout the study. To identify the inhibitory step in the synthesis and secretion of these cytokines, the transcription, translation and secretion of the cytokines was analysed.

The third aim was to uncover the role of the cytoskeleton during MNV infection. Specifically, the focus was intended on the microtubules and GEF-H1, a protein associated with the microtubule network and reported to play a role in viral sensing. Effects of GEF-H1 on MNV infection and the virus on GEF-H1 were analysed via co-expression studies of GEF-H1 and viral proteins, GEF-H1 knockdowns and expression of GEF-H1 mutants.

Chapter 2

Materials and Methods

2.1 Cell lines

RAW264.7, a murine macrophage cell line derived from the Balb/c (H-2d) mouse strain was used for most MNV infection experiments. DC 2.4 cells are a murine dendritic cell line which has been developed from C57BL/6 (H-2b) mice has been used for MNV infections requiring a different MHC class I background. Both cell lines were kept in Dulbecco's modified Eagle's medium (DMEM) with 10 % Foetal bovine serum (FBS) and 2 mM GlutaMAX™ (Gibco, Life Technologies). RAW264.7 and DC2.4 cells were detached from the culture vessel using a 18G x 1 ½'' needle to harvest cells. Cells were centrifuged at 1500rpm for 3 min and resuspended in media or appropriate buffer. 293T, HeLa and Vero1008 cells were used in protein expression experiments and maintained in DMEM with 10 % FBS, 2 mM GlutaMAX™ and 1mM Sodium Pyruvate (all Gibco, Life Technologies). The semi adherent 293T cells were removed from the culture flask by brisk pipetting. For all other cell lines trypsin EDTA (0.05 %) was used to detach cells from the culture vessel and cells were resuspended in FBS containing media to inactivate the enzyme. All cell lines were grown at 37 °C and 5 % CO_2.

2.2 Plasmids and Antibodies

2.2.1 Plasmids

Plasmids encoding the 6xHis tagged viral proteins MNV NS1-2, 3 4, 6, 7 and VP1 were generated by Jennifer Hyde (Hyde and Mackenzie 2010) from the pSPORT MNV vector and introduced into a pcDNA3.1(+) background (Invitrogen). pcDNA3.1(+)-MNV-NS5-HIS was generated using the following primers to amplify the MNV NS5 sequence via PCR (Forward primer: VPG.EcoRV-F 5'-GATATCACCACCATGGGAAAGAAGGGCAAG-3' and reverse primer VPG.SpeI-R (5'-ACTAGTTCAATGATGATGATGATGCTCAAAGTTGATCTT-3'). The PCR product was cut with the

restriction enzymes *Spe*I and *Eco*RV, whose restriction sites had been incorporated into the forward and reverse primer, respectively. The pcDNA3.1(+) vector was digested with *Xba*I (generates the same DNA fragment as *Spe*I) and *Eco*RV, before ligation of the PCR product and the vector with the T4 DNA ligase (Promega). RG204546 vectors expressing GEF-H1-WT-GFP, C53R-GFP, ΔDH-GFP and S885A-GFP were kindly provided by HC Reinecker (Harvard Medical School, Boston). The pBI-CMV5 vector containing the secreted *Metridia* Luciferase sequence was purchased from Clontech. Bidirectional pBI-CMV5 secreted Luciferase and viral protein expressing vectors were generated by amplifying the NS gene sequences from the pSPORT-MNV plasmid via PCR using the following primer sets (Geneworks) (Table 1).

Table 1 Primer sequences used for amplifying the MNV NS protein sequences from the pSPORT-MNV vector. Sequences are displayed in 5'-3' orientation.

Name	Sequence
N-term.NotI.F	5' ATAGCGGCCGCGCCACCATGAGGATGGCAACGCC 3'
N-term.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGTTCGGCCTGCCATTC 3'
NTPase.NotI.F	5' ATAGCGGCCGCGCCACCATGGGGCCCTTCGACCTTGC 3'
NTPase.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGCTGGAGGCCGAAATC 3'
p20.NotI.F	5' ATAGCGGCCGCGCCACCATGAACAAGGTCTATGAC 3'
p20.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGCTCAGAGTGGTACCA 3'
VpG.NotI.F	5' ATAGCGGCCGCGCCACCATGGGAAAGAAGGGC 3'
VpG.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGCTCAAAGTTGATCTT 3'
Pro.NotI.F	5' ATAGCGGCCGCGCCACCATGGCCCCAGTCTCC 3'
Pro.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGCTGGAACTCCAGAGC 3'
Pol.NotI.F	5' ATAGCGGCCGCGCCACCATGGGACCCCCCATGC 3'
Pol.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGCTCATCCTCATTCAC 3'
VP1.NotI.F	5' ATAGCGGCCGCGCCACCATGAGGATGAGTGATGGC 3'
VP1.SpeI.R	5' ACTAGTTCAATGATGATGATGATGATGTTGTTTGAGCATTCG 3'

The pBI-CMV5 vector and the PCR products were cut with the restriction enzymes *Eco*RV and *Not*I and ligated with T4 DNA Ligase (Promega). The pBI-CMV5-mCherry vector was generated using the following primer set to amplify mCherry via PCR and integrate *Bam*HI and *Kpn*I restriction

sites. (BamHI-mCherry-F: 5' CTAGTGGATCCCATGGTGAGCAAGGGCGAGG 3', mCherry-KpnI-R: 5' AGCTTGGTACCTGCTTGTACAGCTCGTCCATGCC 3'). The PCR product was digested with *Bam*HI only leaving the end of the construct blunt while the vector was then cut with *Bam*HI and *Eco*RV (blunt digest). The cut vector and insert were combined through ligation with T4 DNA ligase.

Lentivirus constructs for GEF-H1 and its mutants were generated using a pLenti6-mCherry vector (provided by the Purcell lab, DMI, University of Melbourne). mCherry was cut out of the multiple cloning site using the restriction enzymes *Eco*RI and *Sac*II. The DNA sequences for GEF-H1 WT and the mutants C53R, DH and S885A were amplified via PCR and *Eco*RI and *Sac*II restriction site were introduced using the following primer sets (Table 2). Amplified PCR products were cut with *Eco*RI and *Sac*II and were ligated with the cut pLenti6 vector using the T4 DNA ligase. Successful introduction of GEF-H1 and its mutants was verified via digests and sequencing. Due to unsuccessful introduction of C53R into the pLenti6 vector, site directed mutagenesis was used to introduce the C53R mutation into the existing pLenti6-GEF-H1-GFP construct with the primer set listed in Table 2.

Table 2 Primer sets used for the generation of GEF-H1 Lentivirus constructs

Name	Sequence
GEF_EcoRI_Fwd	5' CCGGGAATTCGTCGACTGGATCCGG 3'
GEF_SacII_Rev	5' TATGACCGCGGCCGGCCGTTTAAACTC 3'
C53R_Fwd_Mut	5' GGCATGACCATGCGCTATGCCTGTAAC 3'
C53R_Rev_Mut	5' GTTACAGGCATAGCGCATGGTCATGCC 3'
C53R_Fwd_Seq	5' GACTGGATCCGGTACCGAGGAG 3'

2.2.2 Primary Antibodies

Name	Supplier	Species origin	Dilution IF	Dilution Western blot	Dilution FACS
Actin	Sigma	Rabbit	-	1:1000	-
dsRNA	SCICONS (Hungary)	Mouse	1:250	-	1:500
eIF2α	Invitrogen	Rabbit	-	1:1000	-
GEF-H1	Pierce	Rabbit	1:500	1:2000	-
GM130	BD Biosciences	Mouse	1:250	-	-
GM130	Calbiochem	Rabbit	1:250	-	-
6xHIS	Abcam	Rabbit	1:250	1:1000	1:500
IFNβ	Abcam	Rabbit	1:200	1:1000	-
HLA-ABC		Mouse, APC	-	-	1:100
MHC class I (X^d)	Provided by J Mintern	Mouse, biotinylated	-	-	1:200
MHC class I ($H2\text{-}K^d/D^d$)	Biogems, Lonza	Mouse, FITC	1:100	1:1000	1:500
NS4 (MNV)	Provided by K Green	Guinea pig	1:250	-	-
NS6 (MNV)	Provided by K Green	Guinea pig	1:250	-	-
NS7 (MNV)	Invitrogen	Rabbit	-	1:8000	-
p-eIF2α (Ser52)	Life Technologies	Rabbit	-	1:1000	-
p-PKR	Abcam	Rabbit	-	1:1000	-
PKR	Abcam	Rabbit	-	1:1000	-
Puromycin	Kerafast	Mouse	1:250	1:2000	-
TNF-α	Chemicon	Rabbit	1:250	1:1000	-
α-Tubulin	Invitrogen	Mouse	1:250	-	-
β-Tubulin	Molecular Probes	Mouse	1:250	-	-
VPg (NS5)	Invitrogen	Rabbit	1:500	-	1:1000

2.2.3 Secondary Antibodies

Name	Supplier	Species origin	Dilution IF	Dilution Western blot	Dilution FACS
Alexa488 Ms	Life Technologies	Donkey	1:500	1:1000	1:3000
Alexa488 Rb	Life Technologies	Donkey	1:500	1:1000	1:3000
Alexa594 Gp	Life Technologies	Goat	1:500	1:1000	-
Alexa594 Ms	Life Technologies	Donkey	1:500	1:1000	-
Alexa594 Rb	Life Technologies	Donkey	1:500	1:1000	-
Alexa647 Ms	Life Technologies	Donkey	1:500	1:1000	-
Alexa647 Rb	Life Technologies	Donkey	1:500	1:1000	1:3000
APC (SAV)	Provided by J Mintern	-	-	-	1:500

2.3 Transfections

2.3.1 DNA transfections

293T, HeLa and Vero1008 cells were transfected using Lipofectamine®2000. RAW264.7 cells were transfected using Lipofectamine®LTX or Fugene®6. Cells were seeded 24 h before transfection and transfected at a cell confluency of 70-80 %. Transfections were performed according to manufacturer's protocol. In brief, for Lipofectamine® transfections in a 24-well plate, 0.5 µg plasmid DNA in serum free Opti-MEM® media and 1.5 µL Lipofectamine® in serum free Opti-MEM® were mixed, incubated for 5 mins and added drop wise to cells. If Lipofectamine®LTX was used, 0.5 µL Plus Reagent was added to diluted plasmid DNA before combining it with the diluted

Lipofectamine®. For Fugene®6 transfections in 24-well plates 0.5-0.75 µg plasmid DNA were diluted in Opti-MEM® and left at RT for 5 min. 1-1.5 µL Fugene®6 were diluted in Opti-MEM® and incubated at RT for 5 min. Diluted plasmid DNA and Fugene®6 were mixed together and incubated at RT for 15 min. The cell culture media was replaced and the DNA and Fugene®6 mixture was added drop wise to the cells. Cells were incubated at 37 °C for 18-24 h depending on experimental design and toxicity of expressed proteins.

2.3.2 siRNA transfections

Three siRNAs (siRNA1-3) against mouse GEF-H1 were purchased from Bioneer Pacific and tested for their efficiency to knock down GEF-H1 separately as well as in combination. Only siRNA3 showed a significant reduction in GEF-H1 expression and was used in all following experiments. Lipofectamine®RNAiMAX was used to transfect RAW264.7 cells with siRNA according to manufacturer's protocol. In brief, 40 pmole siRNA was diluted in Opti-MEM® as well as Lipofectamine®RNAiMAX. The diluted Lipofectamine® and siRNA were mixed and incubated for 5 min at room temperature. The cell culture media was exchanged and the siRNA and Lipofectamine® mixture was added drop wise to cells (12-well). Cells were incubated at 37 °C for 24 h before they were treated again with 40 pmole siRNA. If cells were infected after siRNA knock down, virus was added to the cells 12 h after the second siRNA treatment. For cells were treated twice with siRNA 24 h apart, cells were seeded at a lower confluency of 30-50% at the time of the first siRNA treatment to avoid overgrowth.

2.3.3 Lentivirus transductions

Lentiviral particles were generated by transfecting 293T cells with the following constructs: RSV-G (10%), pMDG (10%), pMDLg (50%) and the pLenti6 plasmid of choice (30%). A total amount of

2.5 µg DNA (6-well) was transfected using Lipofectamine®2000 as described above. 4 h after transfection the media was changed to DMEM with 2% FBS to remove residual Lipofectamine® and any supplements interfering with the Lentivirus transduction. Cell supernatants were harvested at 48h post transfection and centrifuged at 1500 rpm for 3 min to pellet any cells and cell debris. Viral particle containing supernatants were filtered (0.2 µm filter) and were stored at -80 °C. For efficient transduction, Lentiviral particles were titrated and used in a dose dependent manner. Cells were incubated with viral particles in serum-free media for 2 h to ensure binding and entry before the media was changed to DMEM containing 2% FBS. To support Lentivirus infection, cells were additionally supplied with DEAE Dextran (10 µg/mL) during the infection. Transduced cells were incubated for 24-48 h to ensure successful transduction and synthesis of the proteins.

2.4 Viral infection

RAW264.7 macrophages were infected with multiplicity of infection (MOI) 5, unless indicated differently. The culture medium was replaced with serum-free medium without antibiotics or GlutaMAX™ prior to infection. Virus was added to the media and culture dishes were gently rocked to ensure even spread of virus. If not indicated differently, cells were harvested or fixed 12 h after infection with MNV, 18 h after infection with X-31 (mouse IAV) and 21 h after infection with West Nile Virus (WNV).

2.5 Drug treatments

Brefeldin A (BFA, Sigma), a protein transport inhibitor was used as a control treatment in protein secretion studies. BFA was used in a concentration of 1 µg/mL and added to the cell specific culture medium. Nocodazole (Merck Calbiochem) interferes with the cytoskeleton by inhibiting

the polymerization of microtubules. 10 µM Nocodazole was used as control in microtubule-associated experiments. Polyinosinic:polycytidylic acid (Poly(I:C)) is a double stranded RNA analogue and was used as a positive control for immune activation. Poly(I:C) was used in a concentration of 20 µg/mL and added to the cell culture medium in combination with Lipofectamine2000®. MG132 is an inhibitor of the proteasome and was used in MHC class I experiments to inhibit protein degradation (Lee and Goldberg 1998). MG132 was used in a concentration of 0.5 µM and added to the culture media. Trypsin was used to remove cell surface proteins from cells. Undiluted trypsin was applied for 5 min or until cells lifted off the cell culture dish. Cells were collected, spun down and trypsin was removed. Cells were washed in PBS twice before adding the appropriate lysis buffer for further analysis. Puromycin interferes with host cell translation and was used to identify translation levels within the cells (Yarmolinsky and Gabriel 1959). Puromycin was used at 10 µg/mL and was added to the cells 20 min before harvesting/fixation.

2.6 Secreted Luciferase reporter assay

The Ready-To-Glow™ Secreted Luciferase Reporter System assay (Clontech) was performed according to the manufacturer's protocol. Briefly, cells were transfected with the pBI-CMV5 vector containing the secreted *Metridia* Luciferase sequence alone or an additional sequence encoding one of the MNV proteins or mCherry. 24 h after transfection, 50 µL of the culture supernatants (secreted Luciferase) or the lysates (intracellular Luciferase) were transferred into white bottom 96-well microtest plates (BD Biosciences), before 5 µL of the Luciferase reagent was added. The luminescence was measured directly with the FLUOstar® Omega or CLARIOstar® plate

reader (BMG Labtech) and the ratio of the intracellular to extracellular amount of *Metridia* Luciferase activity was calculated.

2.7 Immunofluorescence staining and confocal imaging

Cells intended for confocal imaging analysis were grown on 10 mm round coverslips and treated according to the experiment. Cells were fixed in 4 % (w/v) paraformaldehyde (PFA) (Electron Microscopy Sciences) (in PBS) for 10 min and permeabilised in 0.1 % (w/v) Triton™ X-100 (Sigma) and 4 % PFA (10 min). After a wash step with PBS, 0.2 M glycine (Sigma) was added to reduce autofluorescence of formaldehyde groups. Samples used for staining with the GEF-H1 antibody were fixed in ice-cold methanol for 3 min followed by a 30 sec incubation with ice-cold acetone. Fixed cells were stained with relevant primary antibodies in 1 % (w/v) BSA for 45-60 min, washed in 0.1 % BSA in PBS and incubated again with the secondary antibodies for 30-45 min. Coverslips were then washed in PBS only and DAPI (0.5 µg/mL) was added for 5 min. Final washing steps were performed with Milli-Q water (Millipore) and coverslips were dried and mounted on cover slides with Ultramount #4 (Fronine). Samples were kept cool and dark until imaging with confocal microscopes. Confocal pictures were collected either with the LSM 700 or LSM 710 confocal microscope using the ZEN® software (Zeiss).

2.8 Immunoprecipitation

293T cells transfected with either pcDNA3.1-NS3-HIS, RG204546-GEF-H1-GFP or co-transfected with both plasmids were lysed in NP40 buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1 % NP-40). Cell debris was discarded and lysates were incubated with cOmplete His-Tag Purification Resin (Roche) for 1 h at RT or at 4 °C overnight. Lysate and resin mix was processed according to

manufacturer's protocol, resulting in fractionation of His resin-bound proteins and residual cellular proteins.

2.9 Immunoblotting

Samples were lysed in NP40 or SDS lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1 % SDS) and centrifuged at 21,000 rcf for 10 min to sediment cell debris. All protein samples were handled at 4 °C or on ice and supplied with Protease Inhibitor Cocktail III (Astral Scientific). If phosphorylated proteins were to be analysed the lysis buffer was additionally supplied with PhosSTOP™ (Roche). Protein samples were boiled at 95 °C in SDS loading buffer (125 mM Tris-HCl, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromophenol blue) prior to immunoblotting and kept on ice. Lysates were separated on a polyacrylamide gel (10 % or 12 % depending on the size of the protein of interest) via SDS-PAGE (120 V) and afterwards proteins were transferred onto a 0.2 µm nitrocellulose membrane or PVDF membrane (Bio-Rad) (100 V, 65 min). Membranes were processed according to manufacturer's protocol and incubated in 5 % skim milk or BSA (solved in 0.1 % Tween in PBS) at 4 °C overnight or at RT for at least 2 h. Primary antibodies were diluted in 5 % BSA and 0.1 % Tween in PBS and incubated with the membrane for 3-4 h at room temperature or at 4 °C overnight. After wash steps with 0.1 % Tween in PBS, the secondary antibodies in 0.1 % Tween in PBS were added to the membrane (2-3 h). Lastly, the membrane was washed again and fluorescence signals were detected and analysed with the Pharos FX™ Plus Molecular Imager using the Quantity One® software (Bio-Rad). If HRP conjugated secondary antibodies were used, membranes were incubated with ECL Pus Western Blotting Substrate (Pierce, Thermo Fisher) for 5 min and signals were visualised with MF-ChemiBis documentation system (DNR).

2.10 Flow cytometry

Cells were harvested and washed twice in staining buffer (5 % FBS, 0.1% NaN_3 in PBS). About $1x10^6$ cells were resuspended in 50 µL staining buffer containing cell surface antibodies and incubated at 4 °C in the dark for 20-30 min. To remove residual antibodies, cells were washed twice in 1 mL staining buffer. If unconjugated antibodies were used, the previous steps were repeated with the secondary antibodies. For fixation, cells were thoroughly resuspended in 250 µL fixation buffer (2 % PFA in PBS) and kept on ice for 20 min. After two wash steps in Perm/Wash buffer (0.1 % Triton™ X-100, 1 % FBS in PBS) cells were incubated in 50 µL Perm/Wash buffer with intracellular staining antibodies for 20-30 min on ice. Cells were washed twice in 1 mL Perm/Wash buffer, resuspended in 500 µL PBS and kept dark and on ice until flow cytometry analysis. Flow cytometry data was collected with BD FACSCanto™ II analyser using the BD FACSDiva software (BD Biosciences). Data was analysed using FlowJo® analysis software.

2.11 Cell sorting

Cells with a fluorescent marker (either GFP or mCherry) were sorted using the FACS Aria III (BD) or the MoFlo Astrios (Beckman Coulter) cell sorter. Cells were harvested shortly before sorting, washed twice in PBS and resuspended in PBS with 0.5% FBS. If adherent cell lines were used, cells were filtered through a 40 µm cell strainer to avoid the formation of cell aggregates. Cells were kept on ice until sorting. GFP or mCherry positive cells were selected and sorted into a separate tube. Sorted cells were centrifuged at 1500 rpm for 3 mins and the sorting buffer was discarded. Cells were washed in PBS, centrifuged again and seeded in a 96 or 24-well (depending on yield) in cell culture medium.

2.12 Quantitative PCR

Cells were harvested and washed in PBS, before cell pellets were lysed in TRIzol® Reagent (Life Technologies) for 10 min and stored at -80 °C. The RNA was extracted adding chloroform in a ratio of 1:5, mixing thoroughly and incubating the mixture for 5 min at RT to allow separation of the aqueous and organic phase. After centrifugation at 12,000 rcf for 15 min at 4 °C the organic phase was discarded and 20 ng glycogen were added to the aqueous phase. RNA was precipitated in isopropanol (1:2 ratio to amount of TRIzol®) for 10 min and centrifuged at 12,000 rcf for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed with 70 % ethanol, centrifuged again for 5 min and after removal of the ethanol, left at RT to dry. RNA pellets were dissolved in autoclaved DEPC-treated water at 60 °C for 10 min. For consecutive reverse transcription the RNA concentration was measured using NanoDrop™ (Thermo Scientific). To exclude DNA contamination 1 µg RNA was treated with RQ1 DNase (Promega) and incubated for 30 min at 37 °C. DNAse was heat inactivated at 65 °C for 15 min with EDTA. For reverse transcription Sensifast RT (Bioline) was used and the following incubation pattern was applied: 25 °C 10 min, 42 °C 15 min, 85 °C 5 min. 2x ITaq Universal Sybr Green Supermix (Bio-Rad) and the primers in Table 3 were used to set up the qPCR samples in duplicates. RNA levels were analysed with Stratagene Mx3005P™ qPCR machine. Primers for MHC class I and were purchased from Bio-Rad (PrimePCR™, SYBR® Green Assay, H2-D1 Mouse and GAPDH Mouse). Fold change in target genes was calculated using the $\Delta\Delta C_T$ method relative to the internal control GAPDH.

Table 3 Sequences of primers used for quantitative PCR experiments.

Name	Sequence
GAPDH (F) Hu	5‘-ACAGTCCATGCCATCACTGCC-3‘

GAPDH (R) Hu	5‘-GCCTGCTTCACCACCTTCTTG-3‘
GAPDH (F) Ms	5’-CGTCCCGTAGACAAAATGGT-3’
GAPDH (R) Ms	5’-TCAATGAAGGGGTCGTTGAT-3’
MNV-NS3 (F)	5’-TTGTTGGCATCAAGGACACCTG-3’
MNV-NS3 (R)	5’-TGGATGGAATGAAGGGCTCC-3‘
IFNβ (F) Human	5’-CATTACCTGAAGGCCAAGGA-3’
IFNβ (R) Human	5’-CAATTGTCCAGTCCCAGAGG-3’
IFNβ (F) Mouse	5’-TCCCTATGGAGATGACGGAG-3’
IFNβ (R) Mouse	5’-ACCCAGTGCTGGAGAAATTG-3’
IL-6 (F)	5‘-GATGGATGCTACCAAACTGGA-3‘
IL-6 (R)	5‘-TCTGAAGGACTCTGGCTTTG-3‘
TNFα (F)	5’-CAGCCTCTTCTCATTCCTGC-3’
TNFα (R)	5’-ATGAGAGGGAGGCCATTTG-3’
WNV/KUNV (F)	5’-TCAAGAATAACTTGGCGATCCA-3’
WNV/KUNV (R)	5’-TCACCTAGGACCGCCCTTT-3’
X-31 (F)	5’-ATGCAACGGTTCAAGTCATCCT-3’
X-31 (R)	5’-GTCAAGTGCAAGATCCCAATGAT-3’

2.13 Plaque Assay

To identify viral titres, plaque assays were performed on RAW264.7 cells. Cells were seeded in 6-well plates 24 h before the assay was performed to reach 50 % confluency the next day. This allows growth for a further 48 h without leading to overgrowth. A serial dilution (1:10) of viral

samples was performed in DMEM and the 10^{-3} to 10^{-8} dilutions were tested in the plaque assay. The media was removed from the cells and replaced with the serial dilutions in duplicates. Plates were incubated for 1 h at 37 °C with regular rocking to ensure viral binding and avoid drying of the monolayer. Next, an LMP agar overlay (9.4 mL 1 x DMEM, 0.33 mL FBS, 0.2 mL 0.9M $NaHCO_3$, 0.33 mL 1M HEPES, 0.12 mL Glutamax, 3.1 mL 1.5% LMP agarose for one 6-well plate) was added to the wells and set at 4 °C for 30 mins. Afterwards, plates were incubated at 37 °C for 48 h before fixing the cells with 10 % formalin for 1 h. To visualise occurring plaques, cells were stained with 0.2% crystal violet (in 10% methanol/PBS).

2.14 ELISA

Cell culture supernatants were analysed for their cytokine concentration using mouse specific ELISA kits for the following cytokines: IFNβ (LEGEND MAX™, BioLegend) and TNFα (ELISAkit.com). All ELISA analyses were performed according to the manufacturer's protocol. In short, tissue culture supernatants were used undiluted and samples as well as standards were applied in duplicates to the 96-well pre-coated assay plate and incubated for 2 h. Wells were washed 4x in assay wash buffer, before adding the assay specific biotin-labelled detection antibody. Plates were incubated for 2 h at room temperature, before wells were washed again 4x with the assay wash buffer. A streptavidin conjugated HRP was added afterwards and incubated for a further 45 min. Plates were washed thoroughly 5-6x with wash buffer to avoid background signals and the TMB substrate was added. Plates were checked every 3-5 min to observe the colour change. The reaction was stopped using the assay stop solution. Absorbance at 450 nm and 570 nm (background) was measured using the CLARIOstar® or FLUOstar® microplate reader (both BMG LABTECH). Cytokine concentrations were calculated using the ELISAanalysis.com website.

Chapter 3

MNV manipulates the surface expression of MHC class I proteins

3.1 Introduction

The immune response to MNV infection is highly dependent on the innate immune response and antiviral proteins such as cytokines. Another important group of proteins in the detection and defence against viral infections are the MHC class I proteins. These proteins form complexes with peptides within the cell which are in turn presented on the cell surface. These peptides are usually generated through proteasome-mediated degradation which affects host proteins as well as viral proteins. Host and viral peptides are transported into the ER via TAP (Vyas, Van der Veen *et al*. 2008). In the ER a complex of MHC class I proteins and peptides is formed with the aid of the PLC comprising TAP, tapasin, MHC class I, ERp57 and calreticulin, leading to the incorporation of the antigen peptide into the binding groove of MHC class I (Park, Lee *et al*. 2006, Wearsch and Cresswell 2008, Wearsch, Peaper *et al*. 2011). Successfully formed complexes are then transported to the cell membrane via the Golgi network. MHC class I proteins present their antigens to $CD8^+$ T cells causing the activation of an immune response if a viral antigen is presented. Additionally, a decrease in MHC class I protein levels on the cell surface alerts NK cells and leads to their activation and the elimination of the affected cell. MHC class I proteins have been the target of many viruses, because of their importance and impact in triggering an immune response to the infection.

Based on the importance of MHC class I proteins in the immune defence against viruses, we were interested in investigating the interaction of MNV with the MHC class I expression pathway. We reveal that MNV exerts an inhibitory effect on the intracellular as well as the surface expression of MHC class I proteins in infected cells. This phenotype is likely to be caused by the viral protein NS3, as we observed a significant surface reduction of MHC class I proteins in NS3

transfected cells. Rescue experiments with the proteasome inhibitor MG132 imply a potential degradation of MHC class I proteins during MNV infection, which could be either directly or indirectly initiated by NS3.

3.2 Results

3.2.1 MNV infection reduces the surface expression of MHC class I proteins

Many viruses, such as HIV and HCMV (Schwartz, Maréchal *et al*. 1996, Ahn, Gruhler *et al*. 1997) are known to interfere with the transport or synthesis pathway of MHC class I molecules to reduce the detection of viral antigens by CD8+ T cells as well as avoiding detection by NK cells to inhibit an immune response. MHC class I proteins play a major role in the presentation and detection of viral antigens and are an essential element for the establishment of an antiviral immune response. Therefore, we were interested to determine if MNV replication alters the synthesis and cell surface expression of MHC class I proteins. To test this hypothesis, we investigated the most important functional expression of MHC class I proteins, the expression on the cell surface of infected cells. For this, RAW264.7 cells were infected with MNV (MOI 5) and analysed via flow cytometry (Figure 10 A and B) and immunofluorescence (IF) imaging (Figure 10 C). Mock or MNV infected cells were harvested at 12h after infection and stained with an anti-MHC class I antibody at the cell surface and an anti-NS5 antibody (viral marker) within the cell. Infected cells were selected via positive staining with the anti-NS5 antibody (15-30% of the population) and analysed for their MHC class I surface expression. Flow cytometry analysis revealed a significant decrease in the MHC class I surface expression in cells that were infected with MNV (NS5-positive) compared to mock infected cells (Figure 10 A). Quantitation of the reduction of MHC class I expression was evaluated by measuring the median fluorescence intensity and revealed that only ~70% of the total MHC class I protein was expressed on the surface of infected cells when compared to mock infected cells (Figure 10 B) The decrease in MHC class I surface expression was further verified by IF analysis (Figure 10 C). Cells were stained on the surface with an anti-MHC

class I antibody, before fixation and permeabilisation, to stain for the viral protein NS5 within the cell. Cells positive for anti-NS5 staining again showed a reduction in MHC class I signal compared to mock infected cells (Figure 10 C), consistent with our flow cytometry analysis. This decrease in MHC class I surface proteins was found to be specific for infected cells, because 'bystander' cells, which were exposed to the virus but uninfected with MNV (NS5-negative), had a significantly higher amount of MHC class I proteins on their cell surface compared to MNV infected cells (Figure 10 B, C). To further our analysis we investigated the total amount of MHC class I protein at different time points post-infection by western blot analysis of cell lysates (Figure 10 D). In mock infected cells there was consistent production of MHC class I, however upon infection with MNV we observed a steady and drastic decline in the amount of MHC class I proteins from the 12 to 15 h.p.i. time points. These observations indicate that MNV not only reduces the cell surface expression of MHC class I protein, but appears to reduce MHC class I protein levels in general.

Combined these results indicate that MNV impedes the cell surface expression of MHC class I proteins in infected macrophages and that this may be mediated via down-regulation of the total protein amount of MHC class I in the infected cells.

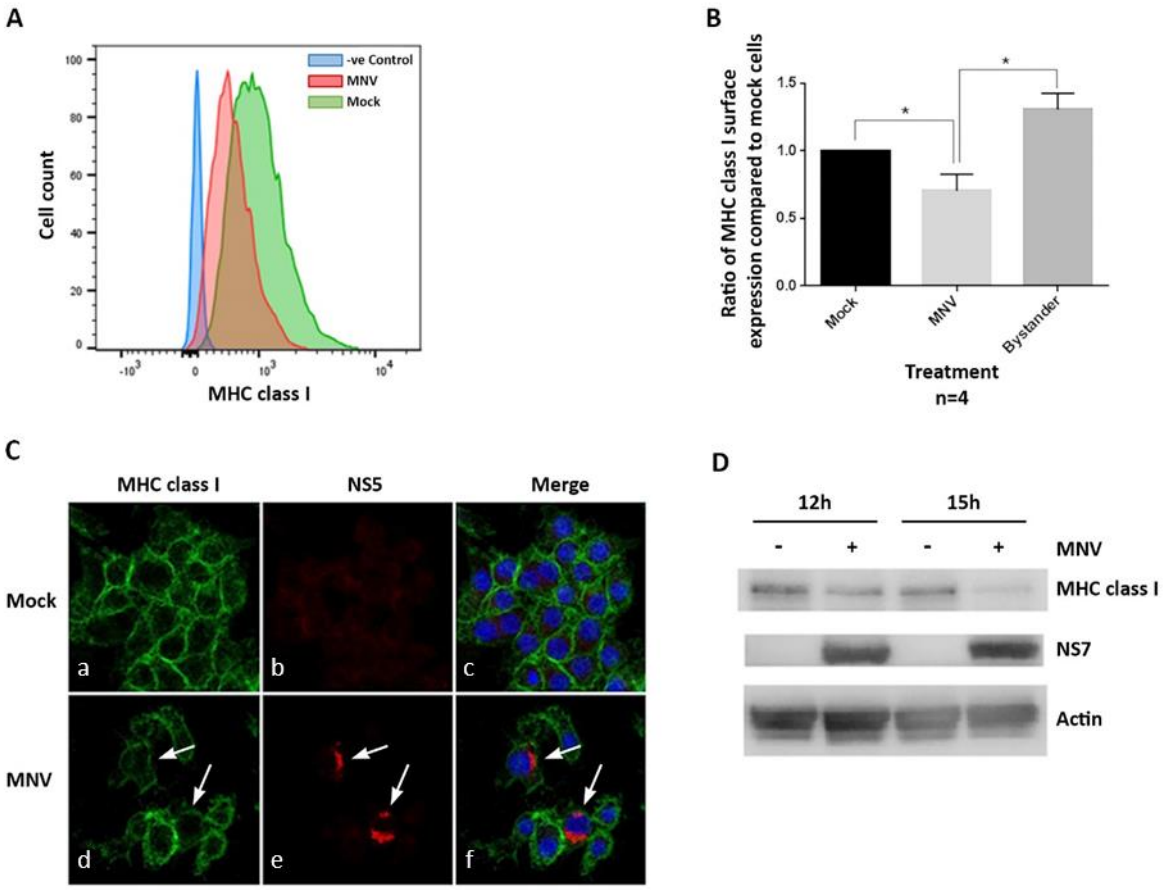


Figure 10 ***MNV infection leads to reduced protein and surface expression of MHC class I proteins in mouse macrophages.*** *RAW264.7 macrophages were either infected with MNV (MOI 5) for 12 h or left untreated and stained with MHC class I specific FITC conjugated antibodies on the cell surface and viral protein NS5 antibodies within the cell. MNV infected cells were gated for the NS5-positive population.* ***A*** *Representative histogram of MHC class I surface expression in MNV infected (red) and uninfected cells (green). Blue area represents isotype control cells.* ***B*** *MFI ratio of the MHC class I signal of MNV infected and bystander cells (exposed to virus, but NS5 negative) compared to mock treatment (n=3, average +/- SEM, *p<0.05).* ***C*** *Immunofluorescence analysis of MNV infected (Panel d, e, and f) and mock infected cells (Panel a, b and c) stained with MHC class I antibodies on the cell surface (Panel a and d) and NS5 antibodies within the cell (b and e). DAPI served as the nuclear stain for the merged image (Panel c and f). Infected cells are indicated by white arrows.* ***D*** *Immunoblot analysis of MNV infected or uninfected macrophages after 12 h and 15 h. Whole lysate samples were probed with anti-actin, anti-NS7 and anti-MHC class I antibodies.*

3.2.2 Effect of MNV on the surface expression of MHC class I is robust and rapid

To ensure that the observed effect of MNV on the surface expression of MHC class I proteins is not specific for the mouse macrophages from a Balb/c background (H-2d) used in the previous experiment, we additionally investigated the expression of MHC class I proteins on the surface of

a dendritic cell line from a C57BL/6 background (H-2b) (Figure 11 A and B). DC 2.4 dendritic cells were infected with MNV (MOI 5) or left uninfected for 12 h. Cells were harvested and stained for MHC class I proteins on the cell surface and MNV NS5 within the cell. NS5-positive and infected cells showed a reduced level of MHC class I surface expression compared to uninfected cells, similar to our previous observations in RAW264.7 cells (Figure 11 A). The signal intensity for MHC class I surface staining was measured for MNV infected and uninfected cells and revealed about a 50% reduction of MHC class I proteins on the surface of infected cells compared to uninfected cells (Figure 11 B). We additionally used RAW264.7 cells to determine the time point from which the surface reduction in MHC class I proteins could be observed (Figure 11 C and D). Generally, MNV replicates in RAW264.7 cells within 12-15h, therefore cells were either infected with MNV or left untreated and harvested at 7, 8, 9, 10, 11 and 12 h.p.i., before staining with an anti-MHC class I antibody on the cell surface and an anti-NS5 antibody within the cell. A reduction in MHC class I surface protein levels was visible as early as 7 h.p.i. and was robustly established from 8 h.p.i. onwards.

These observations highlight the consistency of the MNV-induced decreased MHC class I surface expression across different cell types (dendritic cells and macrophages) and MHC class I backgrounds (H-2d and H-2b) and that it is induced early during infection, between 7-8 h.p.i.

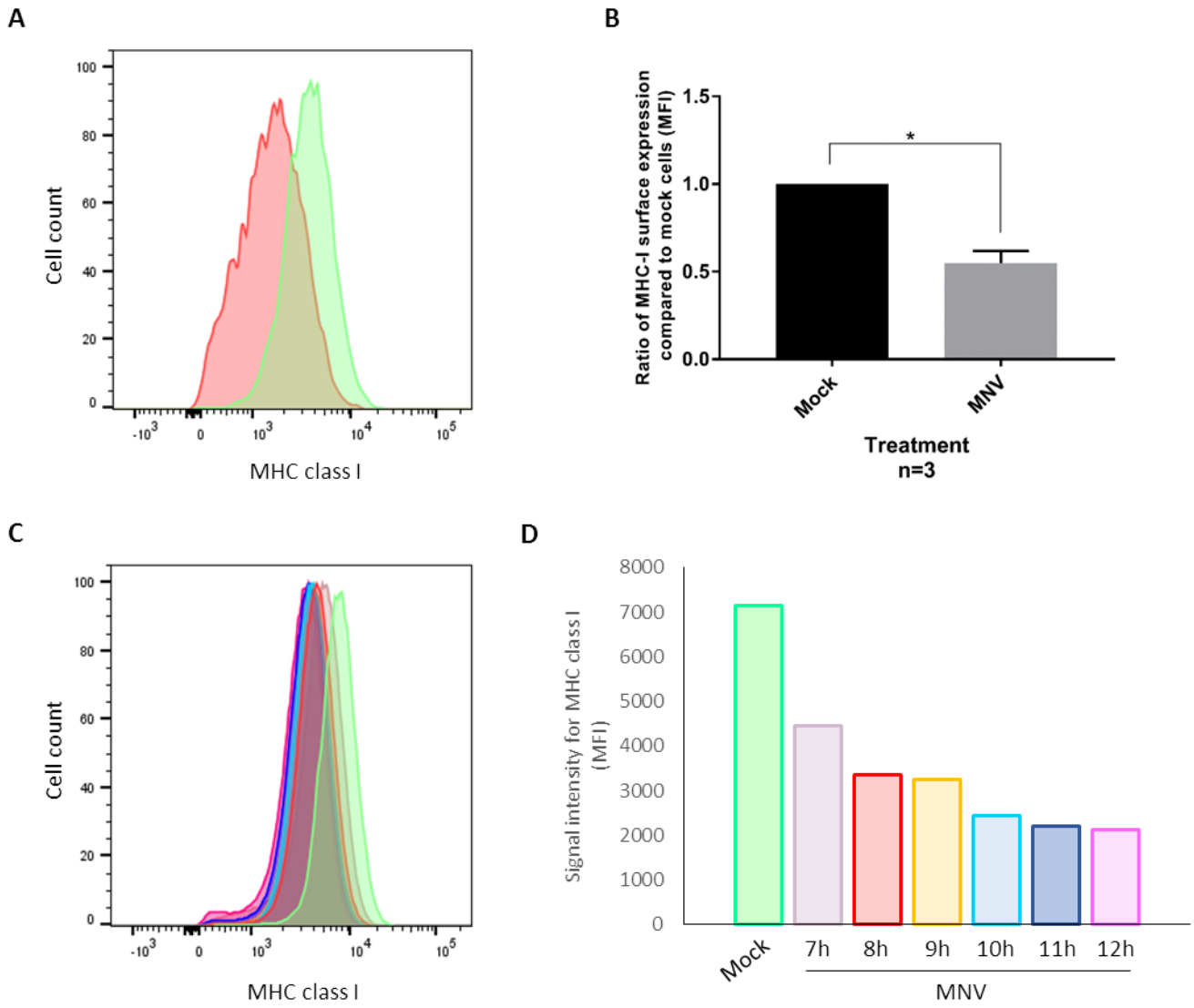


Figure 11 ***Effect of MNV on MHC class I surface expression is robust and rapid.*** *DC 2.4 dendritic cells (C57BL/6) were infected with MNV (MOI 5) or left uninfected. Cells were stained with anti-MHC class I antibodies 12 h.p.i. on the cell surface and with anti-NS5 within the cell. MNV infected cells were identified via NS5-positive staining.* ***A*** *Representative histogram of MHC class I surface signal on mock infected (green) and MNV infected cells (red).* ***B*** *Quantitative analysis of surface MHC class I signal (MFI) ratio compared to mock cells (n=3, average +/- SEM, *p<0.05).* ***C*** *RAW264.7 cells were infected with MNV (MOI 5) or left uninfected. Cells were stained 7, 8, 9, 10, 11 and 12 h.p.i. for MHC class I on the cell surface and NS5 within the cell. NS5-positive cells were gated as infected cells and are represented in the histogram. Representative graph for mock infected (green), and infected cells 7 h.p.i. (light pink), 8 h.p.i. (red), 9 h.p.i. (orange), 10 h.p.i. (light blue), 11 h.p.i. (dark blue) and 12h.p.i. (pink) (n=1).* ***D*** *Signal intensity for MHC class I on the cell surface of MNV infected or mock infected cells (n=1).*

3.2.3 Transcription of MHC class I mRNA is not affected by MNV infection

As we observed that MNV indeed affects MHC class I protein amounts and surface expression (Figure 10), we aimed to identify the step in the MHC class I synthesis pathway which MNV was inhibiting. To investigate if the reduction in MHC class I surface expression was associated with a decrease in MHC class I transcription, a quantitative RT-PCR was performed on RNA samples from mock and MNV infected cells (Figure 12 A, B). MHC class I mRNA levels were analysed at two time points (12 and 15 h.p.i.) using MHC class I and MNV (NS3) specific primers. We observed a significant increase in viral mRNA (NS3) levels in the infected cells confirming the successful infection of the macrophages (Figure 12 A). However, at neither of the time points investigated did we observe a change (either increase or decrease) in MHC class I mRNA levels between infected and uninfected macrophages (Figure 12 B). Assuming that mRNA degradation is negligible, we can exclude that our observed decrease in MHC class I surface protein levels is based on a reduction in MHC class I transcription and MHC class I mRNA levels.

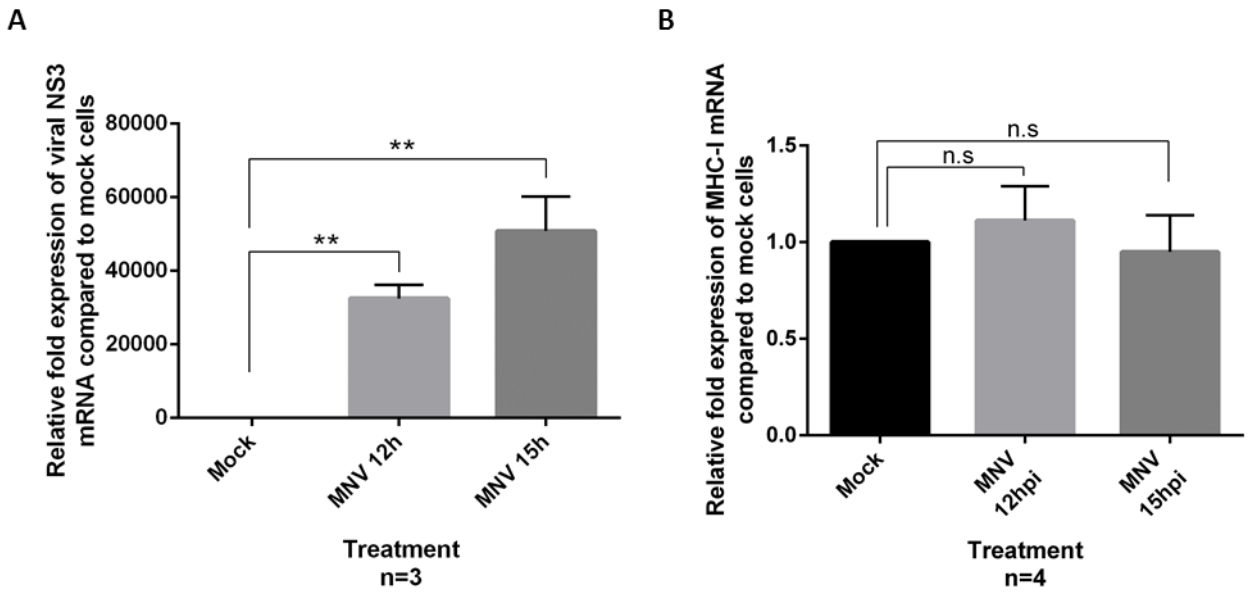


Figure 12 ***Transcription levels of MHC class I mRNA are not reduced during MNV infection.*** *RAW264.7 cells were infected with MNV (MOI 5) or left uninfected (mock). RNA samples were taken 12 h and 15 h after infection and analysed via RT-qPCR.* ***A*** *Relative fold expression of viral mRNA (NS3) in MNV infected cells compared to mock cells (n=3, average +/- SEM, **p<0.01).* ***B*** *Relative fold expression of MHC class I mRNA in MNV infected cells compared to mock cells (n=3, average +/- SEM, ns:p>0.05).*

3.2.4 MNV infection reduces intracellular MHC class I protein levels

Apart from a reduction in MHC class I transcription, a decrease in MHC class I expression on the cell surface could be caused by an inhibition in translation, interference with the MHC class I and peptide complex formation, transport inhibition or degradation of the protein. To investigate a possible change in the intracellular MHC class I protein levels in MNV infected cells, surface proteins including MHC class I were removed from mock and infected cells via trypsin treatment (Figure 13 A and B). Trypsin treated cells were lysed, analysed via immunoblotting and compared to non-trypsin treated cell lysates. Lysates of cells treated with trypsin represent the intracellular protein levels, whereas lysates of untreated cells contain both intracellular and surface proteins. Cell lysates of MNV infected cells which were left untreated, showed lower levels of MHC class I proteins compared to the untreated mock control cells, supporting our previous data in Figure

10. In the uninfected but trypsin-treated cells we observed a significant reduction in the amount of MHC class I detected by western blot, compared to the untreated control (Figure 13 A). This suggests that the majority of the total MHC class I protein detected in the untreated sample is in fact localised to the surface of the cells, and very little of the protein is retained intracellularly. Intriguingly, we observed a similar decrease in MHC class I protein levels between mock trypsin-treated cells and the MNV-infected untreated cells. This observation suggests that MNV is reducing the amount of MHC class I protein equivalent to that removed by trypsin treatment. In MNV infected and trypsin-treated cells the total amount of MHC class I protein was almost completely reduced (Figure 13 A and B), again suggesting that MNV infection results in a reduction of overall MHC class I protein levels rather than a defect in trafficking resulting in intracellular accumulation.

These results lead us to speculate that the reduction in MHC class I surface expression is either caused by an inhibition in translation or the degradation of the protein.

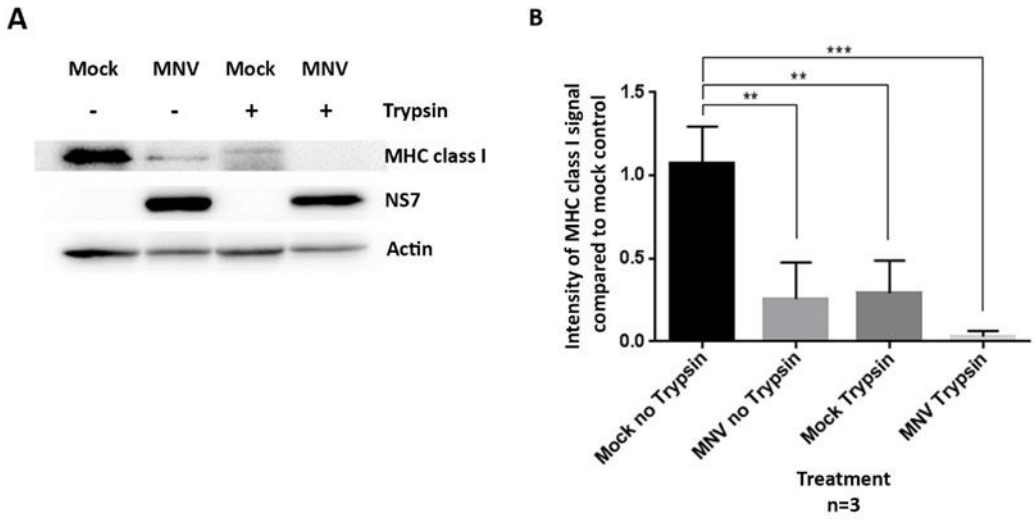


Figure 13 ***Removal of surface proteins via trypsin treatment reveals reduced intracellular MHC class I levels in MNV infected macrophages.*** *RAW264.7 cells were infected with MNV (MOI 5) for 12 h and harvested either mechanically or with trypsin to remove surface proteins. Cells were lysed and analysed via immunoblot analysis.* ***A*** *MHC class I, viral marker NS7 and loading*

control actin. ***B*** *MHC class I signal intensities were compared to the loading control and compared between treatments (n=3, average +/-SEM, **p<0.01, ***p<0.001).*

3.2.5 Treatment with the proteasome inhibitor MG132 leads to the partial restoration of MHC class I surface expression in MNV infected cells

Based on previously documented viral mechanisms interfering with the MHC class I expression pathway, we aimed to test if MNV infection leads to the degradation of MHC class I molecules by either targeting MHC class I proteins for proteasome degradation or actively degrading them. MG132 is a proteasome inhibitor which specifically acts on the proteolytic activity of the 26S proteasome (Lee and Goldberg 1998). Therefore, we utilised MG132 treatment to determine if we could rescue the reduced MHC class I surface expression observed during MNV infection. RAW264.7 cells were infected with MNV (MOI 5) and an hour later either treated with MG132 (0.5 µM) or left untreated. Cells were harvested at 12 h.p.i. and analysed via flow cytometry for their MHC class I surface expression (Figure 14 A and B). Infected cells were stained with an anti-NS5 antibody within the fixed and permeabilised cells. To exclude a detrimental effect of MG132 on the replication of MNV, we evaluated the NS5 signal in MNV infected cells with and without MG132 (Figure 14 C and D). MNV infected cells treated with MG132 showed similar levels of NS5 compared to MNV infected and untreated cells, indicating that MG132 did not adversely affect viral replication. NS5 positive and MG132 untreated cells showed a similar reduction in the surface expression of MHC class I proteins compared to uninfected cells as we had previously observed (Figure 10). When MNV infected cells were additionally treated with the proteasome inhibitor MG132 we observed a significant increase in the MHC class I surface expression compared to infected but untreated cells. The MG132 treatment did not result in a complete

restoration of the MHC class I surface expression in the infected cells, but did appear to restore ~50% of the amount of MHC class I on the surface.

Overall these observations indicate that MNV infection induces some degree of proteasome-mediated degradation of MHC class I proteins contributing to our data indicating a reduced surface expression of this immune molecule.

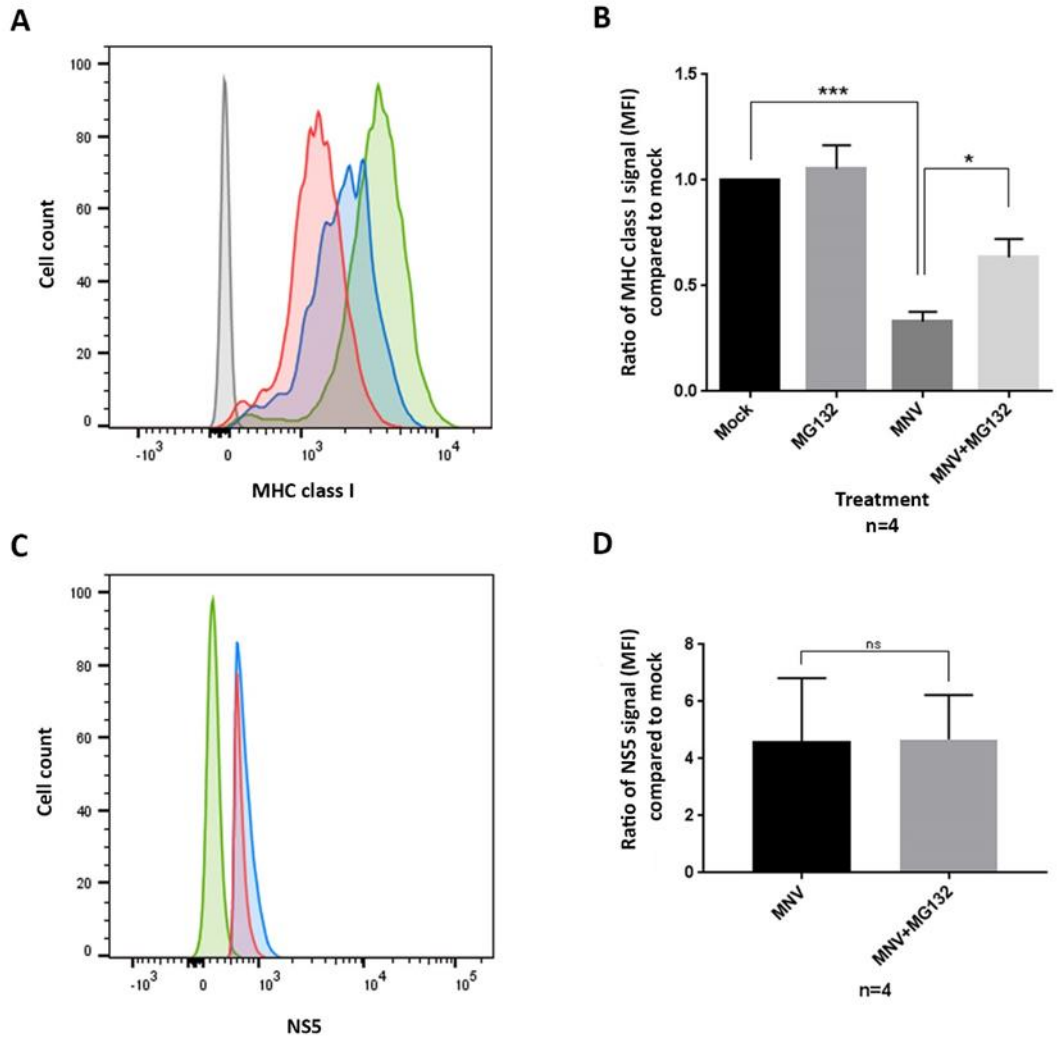


Figure 14 ***Treatment with proteasome inhibitor MG132 partly restores the reduction in MHC class I surface expression.*** *RAW264.7 macrophages were either infected with MNV (MOI 5) or left untreated for 12 h. The proteasome inhibitor MG132 (0.5 µM) was added 1 h post infection. Cells were stained with MHC class I specific FITC conjugated antibody on the cell surface and viral protein NS5 antibodies within the cell. MNV infected cells were gated for the NS5-positive population.* ***A*** *Representative histogram of MHC class I surface expression in MNV infected (red), MNV+MG132 treated (blue), mock infected (green) and isotype control cells (grey).* ***B*** *MFI ratio of the MHC class I signal for different treatments compared to mock treatment (n=4, average +/-*

*SEM, *p<0.05, ***p<0.001). **C** Representative histogram of NS5 signal in MNV infected (red), MNV infected and MG132 treated (blue) and mock infected cells (green). **D** MFI Ratio of the NS5 signal for MNV infected cells treated with MG132 or left untreated compared to mock infected baseline (n=4, average +/- SEM, ns:p>0.05).*

3.2.6 The MNV NS3 protein promotes a reduction in MHC class I surface expression when expressed individually.

Previous published studies on mechanisms of viral interference with MHC class I proteins, and our findings so far, lead us to hypothesise that one of the viral non-structural proteins is responsible for the observed reduction in the surface expression of MHC class I levels in MNV infected cells. To identify the protein potentially interfering with the MHC class I expression, we transfected recombinant cDNA plasmids encoding for the MNV NS proteins (NS1-2 – NS7; Hyde and Mackenzie 2010) individually into 293T cells and assessed their ability to restrict MHC class I surface expression. The transfected cells were harvested 18h after transfection and immunostained at the surface with an anti-HLA-ABC antibody. Cells were then fixed, permeabilised and immunostained for the 6xHIS epitope tag of the MNV NS proteins with an anti-6xHis antibody. Samples were analysed via flow cytometry and transfected cells were identified via the positive 6xHIS stain (Figure 15). We observed that the majority of the MNV NS proteins did not perturb the surface expression of MHC class I and for simplicity only the expression of NS1-2, NS3 and NS5 have been depicted in Figure 15. The expression of MNV NS4, NS6 and NS7 were assessed for their effect on MHC class I surface expression, but we detected no differences in MHC class I levels when compared to mock-transfected cells (data not shown). Interestingly, cells transfected with the plasmid encoding for the MNV NS3 protein showed a significant reduction in the surface expression of MHC class I proteins compared to mock-transfected cells, as well as to cells transfected with plasmids encoding the other MNV proteins NS1-2 and NS5

(Figure 15 B). Intriguingly, the observed reduction in MHC class I surface expression upon NS3 protein expression was similar to the cell surface reduction of MHC class I we have observed during an MNV infection.

This observation implies that the MNV NS3 protein is potentially responsible for our observed reduction in MHC class I surface expression during an MNV infection.

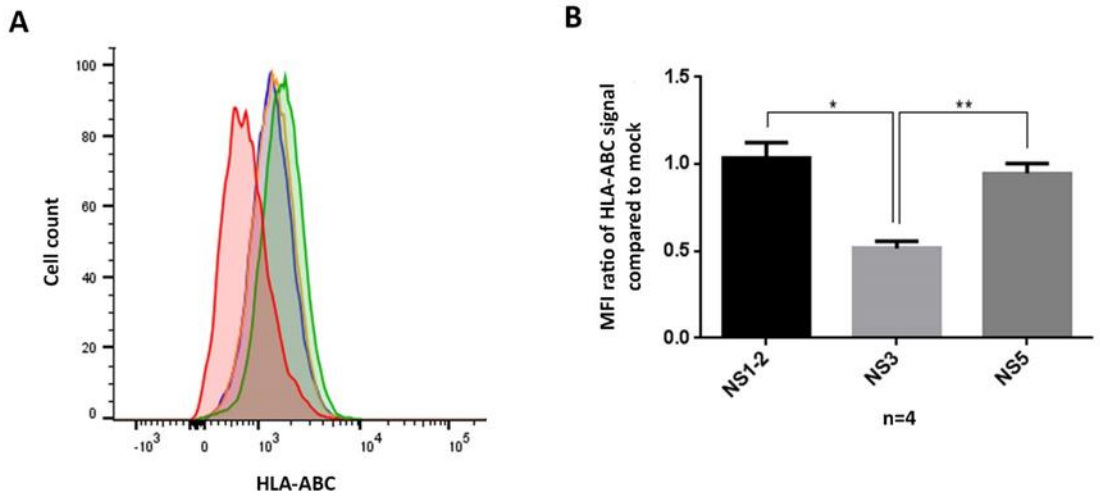


Figure 15 ***Expression of MNV NS3 in 293T cells reduces the surface expression of MHC class I proteins.*** *293T cells were transfected with MNV NS1-2, NS3, NS5 or left untreated. Cells were stained with human MHC class I specific APC conjugated antibody on the cell surface and the NS protein tag (6xHIS) within the cell. NS protein transfected cells were gated for the 6xHIS positive population.* ***A*** *Representative histogram of MHC class I surface expression in NS protein transfected 293T cells. NS1-2 (blue), NS3 (red), NS5 (orange) and mock transfected cells (green).* ***B*** *MFI ratio of the MHC class I signal for the different NS proteins compared to mock treatment (n=4, average +/- SEM, *p<0.05, **p<0.01).*

3.3 Discussion

NoVs cause a quick and acute infection in hosts, seemingly avoiding our rapid immune detection and delaying clearance. MHC class I proteins play a critical role in the immune response against viruses by exposing viral antigens to the innate immune cells and initiating the adaptive T cell response. In this study, we showed that infection with MNV in mouse macrophages leads to a reduction in MHC class I expression levels at the surface of infected cells (Figure 10). The fact that MNV interferes with the MHC class I expression might contribute to its quick replication in infected hosts. This observation was specific for MNV infected cells, since surrounding 'bystander' cells, which had been exposed to the virus but remained uninfected, displayed MHC class I surface expression levels similar to mock infected cells (Figure 10 B). Further analysis revealed that the detected reduction in the surface expression of MHC class I proteins was consistent across different cell types (dendritic cells and macrophages) and MHC class I backgrounds (H-2b and H-2d) (Figure 11 A and B), reinforcing our initial observation. We determined that the reduction in MHC class I surface expression is induced from 7 h.p.i. onwards and robustly established at 8 h.p.i. (Figure 11 C). We have demonstrated that the decreased MHC class I surface expression levels were not due to a reduction in transcription of MHC class I (Figure 12 B), but seem to be caused by a reduction in intracellular levels of MHC class I proteins.

We hypothesised that MHC class I proteins were degraded in MNV infected cells and therefore attempted to rescue MHC class I protein expression with the proteasome inhibitor MG132. Treatment of MNV infected cells with MG132 lead to a partial, but not a complete rescue of MHC class I surface expression. At this stage, our observations are contradictory to some extent as there is a close and critical association of the MHC class I pathway with the proteasome.

Normally, the proteasome-mediated degradation of proteins provides the source of peptides to be loaded onto the MHC class I molecules in the ER. Thus, inhibiting the proteasome would decrease the amount of peptides generated and imported into the ER, where less MHC class I and peptide complexes can be made and located to the surface. However, we observed that blocking protein degradation, via the proteasome, had a significant effect on restoring the surface expression of MHC class I proteins in infected cells. We can only postulate that in the MNV-infected cells MHC class I is targeted for degradation via the proteasome, possibly via translocation into the cytosol and subsequent ubiquitination, and we aim to further our studies to elucidate this degradation mechanism. Ideally, targeting the potential degradation of the MHC class I proteins specifically without blocking the proteasome would provide a clearer picture on the degradation hypothesis, but has not been feasible so far.

We additionally identified that the MNV NS3 protein is responsible for the reduced surface expression. NS3 has been characterised as a nucleoside triphosphatase (NTPase) and we have recently shown that it is associated with the viral replication complex and the microtubules (Cotton, Hyde *et al*. 2016). NTPases have diverse activities and functions including an association with microtubule dynamics, intracellular transport and protein degradation (Paschal, Shpetner *et al*. 1987, Yoshimori, Yamamoto *et al*. 1991, Rabinovich, Kerem *et al*. 2002, Smith, Benaroudj *et al*. 2006, Marshansky and Futai 2008). Our current studies have not shown if NS3 directly acts on MHC class I proteins or if it is affecting its expression via manipulation of the MHC class I protein pathway, but we have shown that NS3 is sufficient for the surface reduction of MHC class I proteins. In contrast, studies on MNV VP2 have identified a crucial role of the protein on MHC class I expression through inhibition of MHC class I transcription (Zhu, Regev *et al*. 2013). Based

on our transcriptional analysis we cannot exclude that MNV infection might prevent the up-regulation of MHC class I transcription, but we have so far not been able to detect a down-regulation of MHC class I transcription during MNV infection (Figure 12 B). If only the up-regulation of MHC class I transcription would be affected during MNV infection, MNV infected and mock infected cells would display similar levels of MHC class I surface expression. Conversely, we have observed a reduction of MHC class I proteins on the intracellular and surface expression level in MNV infected cells (Figure 10 and Figure 13). Therefore, we speculate that VP2 might prevent the up-regulation of MHC class transcription, but MNV NS3 causes the observed reduction in MHC class I surface expression levels.

Our observations presented here are similar to other viruses such as HIV, HCMV and EBV that all interfere with the surface expression of MHC class I proteins, albeit via different strategies (Ahn, Meyer *et al*. 1996, Schwartz, Maréchal *et al*. 1996, Ahn, Gruhler *et al*. 1997). It is known that viruses utilise several strategies to manipulate the surface expression of MHC class I, which are employed at different stages throughout the MHC class I protein expression pathway. For example, EBV inhibits proteasome-mediated degradation which prevents the synthesis of viral peptides and thus the formation of viral antigen and MHC class I complexes (Levitskaya, Sharipo *et al*. 1997). Whereas, HSV and HCMV encode proteins which block the import of peptides, including viral peptides into the ER, (Hill, Jugovic *et al*. 1995, Ahn, Gruhler *et al*. 1997, Lehner, Karttunen *et al*. 1997). Additionally, the Nef protein of HIV causes the downregulation of MHC class I expression and inhibits the trafficking of MHC class I complexes from the Golgi to the cell surface (Schwartz, Maréchal *et al*. 1996, Roeth, Williams *et al*. 2004). We can now contribute

another mechanism whereby MNV induces proteasome-mediated degradation of the MHC class I protein itself, thus resulting in decreased protein levels and surface expression.

We tried to assess the effect of the reduced MHC class I surface expression on the CD8+ T cell and NK cell response, but due to technical issues and time constraints we have so far not been able to accomplish the analysis. The down-regulation of MHC class I surface expression will likely have critical effects on the ability of both the innate and adaptive immune response to detect and clear MNV infected cells. In future experiments we aim to elucidate if the reduction of MHC class I proteins is sufficient to avoid detection of NK cells and subsequently antigen-specific CD8+ T cells. NK cells can react to the downregulation of MHC class I proteins on the cell surface and could be activated by the observed phenotype in MNV infected cells. Furthermore, it would be interesting to interrogate whether the lower MHC class I expression on antigen-presenting cells, in particular dendritic cells, has an effect on the generation of a robust the CD8+ T cell response against MNV infections. MNV and HuNoV infections have been shown to induce little immune memory to homologous challenges and the response to vaccine treatment has been very variable so far (Parrino , Schreiber *et al*. 1977, Graham, Jiang *et al*. 1994, Hinkula, Ball *et al*. 1995, El-Kamary, Pasetti *et al*. 2010). This could be based on an insufficient CD8+ T cell response caused by the reduction in MHC class I surface expression, inhibiting the formation of a robust CD8+ T cell response and CD8+ T cell memory.

Overall, the reduction in MHC class I surface expression levels can be highly beneficial for the virus as less MHC class I peptide complexes are displayed on the cell surface which likely results in a reduced detection by surveilling immune cells. Manipulating the MHC class I

expression on the cell surface aids the virus to hide within the infected cell and to avoid or dampen the immune response, allowing for efficient and swift replication.

Chapter 4

Cytokine secretion and synthesis during MNV infection

4.1 Introduction

Norovirus infections are typically fast and acute, with the onset of illness averaging at 12h after exposure and the symptoms lasting for 24-48h (Graham, Jiang *et al*. 1994). These observations indicate that the innate immune system plays a major role in combatting the infection in humans. Type I IFN and STAT proteins are key molecules of the innate immune response and have been shown to be crucial during MNV infection. The lack of type I IFN and STAT1 in mice caused the inability to clear the infection and resulted in a lethal MNV infection (Wobus, Karst *et al*. 2004). The secretion of type I IFN and other cytokines is a common innate immune response to viral infections and helps to fight viral infections within the cell and reduce spread. Cytokines are a diverse group of small signalling proteins which includes chemokines, interferons and interleukins. Cytokines have numerous effects in intracellular as well as extracellular signalling. These proteins can either act by themselves or in combination with other cytokines. Apart from a co-operative effect they can also antagonise other cytokines. Different causes of cellular stress (e.g. infections, starvation or toxins) lead to a specific set of cytokines which is released by the affected cells. During viral infections the cytokines IFNα/β, TNFα, TGFβ, IL-6, IL-10, IL-12 and IL-15 are known to be activated to support antiviral signalling and clearance (Biron 1998). IFNα/β are especially potent antiviral cytokines which induce an antiviral state in neighbouring uninfected cells and promote the cytotoxic activity of NK cells (Biron 1997). Apart from alerting surrounding cells and immune cells, IFNβ can also have specific antiviral intracellular effects. IFNβ has been shown to be a regulator of many antiviral proteins such as virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (Viperin), Mx proteins, PKR and RNAse L (Der, Zhou *et al*. 1998, Stark, Kerr *et al*. 1998, Thomson and Lotze 2003, Fitzgerald 2011, Haller and Kochs 2011). PKR activation leads to the phosphorylation of eIF2α, a crucial elongation initiation factor in the

formation of the host translation complex. Phosphorylated eIF2α is no longer able to bind to the translation complex, which is unstable and inactive without eIF2α, causing the downregulation of host cell translation. A reduction in translation aims to disturb viral replication, because viruses are dependent on a functional host cell translation machinery, lacking a translation complex of their own (Kalvakolanu and Borden 1996, Samuel 1998). In contrast, RNAse L has a more direct effect on viral replication by degrading viral RNA (Silverman 1994, Dong and Silverman 1999, Terenzi, Ying *et al*. 1999). IFNβ can also act together with TNFα and has inhibitory effects on IL-12 (Hunter, Subauste *et al*. 1994, Cousens, Orange *et al*. 1997). TNFα is a pro-inflammatory cytokine which can either support cell survival by activating antiviral TNFα stimulated genes or induce apoptosis through the synthesis of ROS and the activation of caspases (Chen and Goeddel 2002, Wang, Du *et al*. 2008, Kim, Lee *et al*. 2010).

During viral infections, cytokines are mainly induced upon the detection of dsRNA, which is generated during viral replication (Vilcek 1996). Several PRRs which can detect dsRNA have been discovered so far. RIG-I and MDA5 are the major cytosolic receptors, whereas TLRs such as TLR3 are associated with cellular membranes. Binding of these receptors to dsRNA activates multiple signalling pathways, which lead to the downstream relocation of crucial transcription factors like NF-κB and IRF-3 and the induction of inflammatory cytokines, e.g. IFNβ and TNFα (Alexopoulou, Holt *et al*. 2001, Changotra, Jia *et al*. 2009, Thackray, Duan *et al*. 2012). Type I IFNs are induced during MNV infection mainly through the MDA5/MAVS pathway, leading to the relocation of IRF-3/IRF-7 into the nucleus and allowing IFNβ transcription (Changotra, Jia *et al*. 2009, Thackray, Duan *et al*. 2012). IFNβ is then translated and secreted to alert other cells and to mount the immune response within the infected cell. Additionally, the recognition of viral dsRNA

by the intracellular receptor TLR3 leads to the relocation of the transcription factor NFκB, which induces the transcription of inflammatory cytokines such as TNFα (Alexopoulou, Holt *et al*. 2001). Because of their importance in the antiviral immune response, the expression and secretion of cytokines has been targeted by many viruses such as IAV and polioviruses on different levels in the cytokine synthesis and secretion pathway (Dodd, Giddings *et al*. 2001, López, García-Sastre *et al*. 2003, Fernandez-Sesma, Marukian *et al*. 2006). Due to the importance of cytokines in the innate immune response to viruses, we were interested in uncovering the role of cytokines during MNV infection. We show that MNV infected cells display high amounts of cytokine mRNA expression, but secrete no or only low amounts of cytokines into the culture medium. This lack in cytokine secretion is mainly due to a reduced amount of intracellular cytokine levels, which is based on the shutdown of host translation.

4.2 Results

4.2.1 MNV infected cells release only low amounts of major cytokines

The innate immune response has been shown to be crucial during MNV infection (Wobus, Karst *et al*. 2004), specifically STAT1 and type I IFNs seem to play an essential role in combatting the infection. We were interested in investigating the innate immune response to MNV infection using the cytokines IFNβ and TNFα as representatives of two major immune response pathways. First, we tested the functional response to MNV infection by analysing the secretion of these two cytokines during MNV infection. For this, we infected mouse macrophages (RAW264.7) with MNV (MOI 5), treated them with the stimulant Poly(I:C), with Poly(I:C) and the secretion inhibitor Brefeldin A (BFA) or left them untreated (Figure 16). Cell culture supernatant samples were taken at 9h, 12h and 15h after infection and were analysed for cytokine secretion via ELISA. Untreated cells as well as cells stimulated with Poly(I:C) but treated with BFA secreted no or only low amounts of IFNβ and TNFα at any time point tested. In contrast to that Poly(I:C) stimulated cells released a high amount of IFNβ and TNFα into the tissue culture supernatant as early as 9h after treatment. Interestingly, cells infected with MNV showed a significantly lower amount of IFNβ and TNFα being secreted into the supernatant compared to Poly(I:C) treated cells. Cytokine levels observed for MNV infected cells were similar to Poly(I:C) and BFA treated cells. BFA acts on the ER to Golgi transport and inhibits protein secretion. The comparable low amounts of secreted cytokines into the supernatant of infected and Poly(I:C) and BFA treated cells, suggest that cytokine secretion might be inhibited in MNV infected cells, comparable to the effect of BFA.

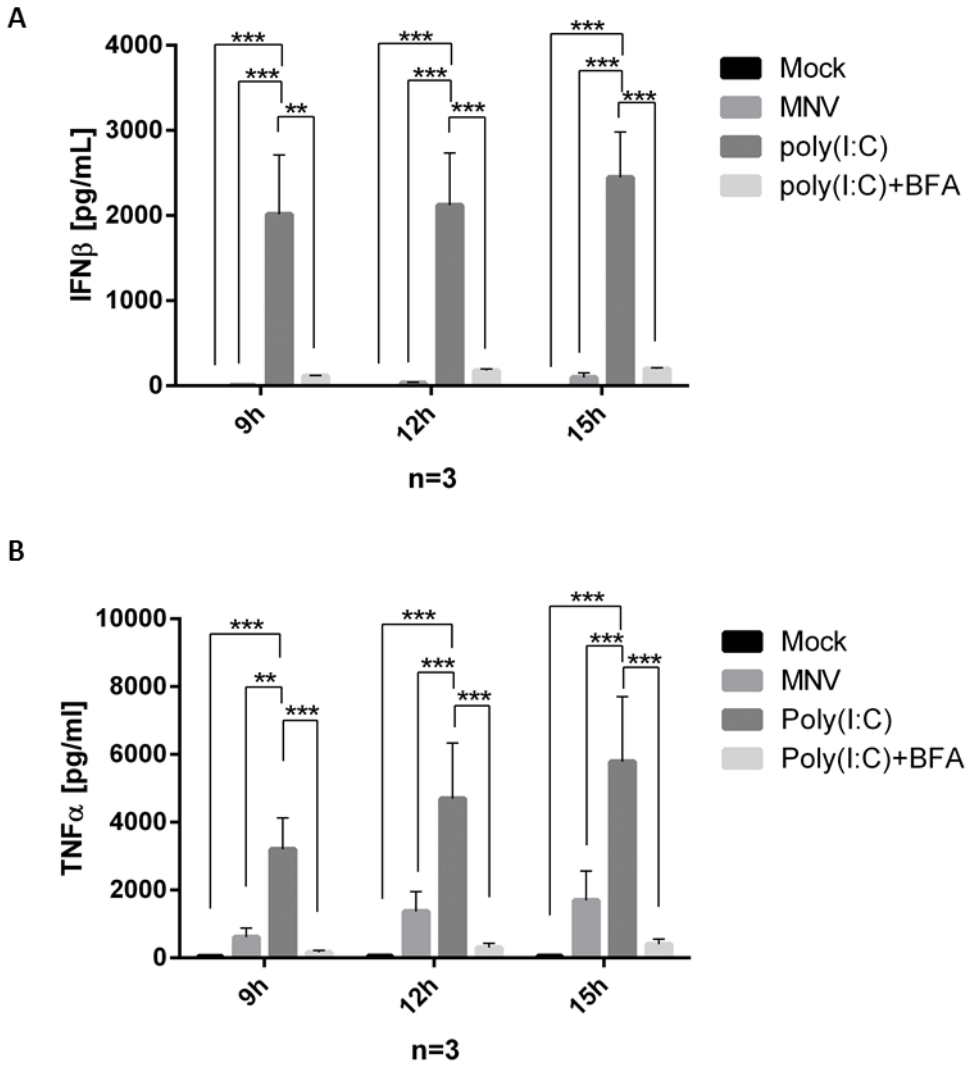


Figure 16 ***MNV inhibits the secretion of specific cytokines in macrophages.*** *RAW264.7 cells were infected with MNV (MOI 5), treated with Poly(I:C) (20 µg/mL), treated with Poly(I:C) (20 µg/mL) and BFA or left untreated and uninfected for 9 h, 12 h and 15 h. Cell culture supernatants were analysed for the secretion of the specific cytokines via ELISA.* ***A*** *Amount of IFNβ in cell culture supernatant [pg/mL] (n=3, average +/- SEM, **p<0.01, ***p<0.001).* ***B*** *Amount of TNFα in cell culture supernatant [pg/mL] (n=3, average +/- SEM, **p<0.01, ***p<0.001).*

4.2.2 General protein secretion is not affected by MNV infection

To test if the low amount of secreted cytokines from MNV infected cells is due to a block in protein secretion, we used the bidirectional reporter vector pBI-CMV5, which encodes a secreted *Metridia* luciferase (*Met*Luc). We introduced a fluorescence marker (mCherry) into the vector to

be able to identify pBI-CMV5-positive cells. RAW264.7 cells were transfected with the pBI-CMV5-mCherry vector, sorted for mCherry-positive cells via FACS and were either infected with MNV, treated with BFA or left untreated (Figure 17 A). Cell culture supernatants were analysed for their luciferase activity after 12 h of infection. Transfected cells treated with BFA showed lower activity for *Met*Luc in the supernatant, because the ER to Golgi transport is blocked by BFA. In contrast to that, untreated cells transfected with pBI-CMV5-mCherry secreted high amounts of *Met*Luc into the supernatant. When cells were transfected with pBI-CMV5-mCherry and additionally infected with MNV, there was no significant difference between infected and uninfected cells. Similar levels of *Met*Luc were secreted into the supernatant of infected cells, suggesting that MNV is not influencing the general protein secretion of the host cell.

In addition to this, we tested the effect of the NS proteins on cellular protein secretion (Figure 17 B). For this, we introduced the MNV NS proteins and VP1 into the pBI-CMV5 vector. 293T cells were transfected with these vectors and analysed for *Met*Luc activity in the cell supernatant and *Met*Luc levels within the cell. The ratio between intracellular and extracellular activity of *Met*Luc was calculated for all tested constructs. Cells transfected with pBI-CMV5 were positive for the secretion of *Met*Luc into the supernatant, whereas pBI-CMV5 transfected and BFA treated cells showed a block in secretion and only low amounts of *Met*Luc in the supernatant while luciferase levels within the cells accumulated. None of the non-structural proteins nor VP1 seemed to affect the secretion of *Met*Luc as all tested MNV proteins had a similar supernatant to intracellular luciferase activity ratio compared to pBI-CMV5 transfected cells. These observations imply that the low levels of IFNβ and TNFα secretion observed during MNV infection are not due

to an inhibition in general protein secretion, but might be caused by an inhibition at earlier stages of the protein synthesis pathway, e.g. transcription or translation.

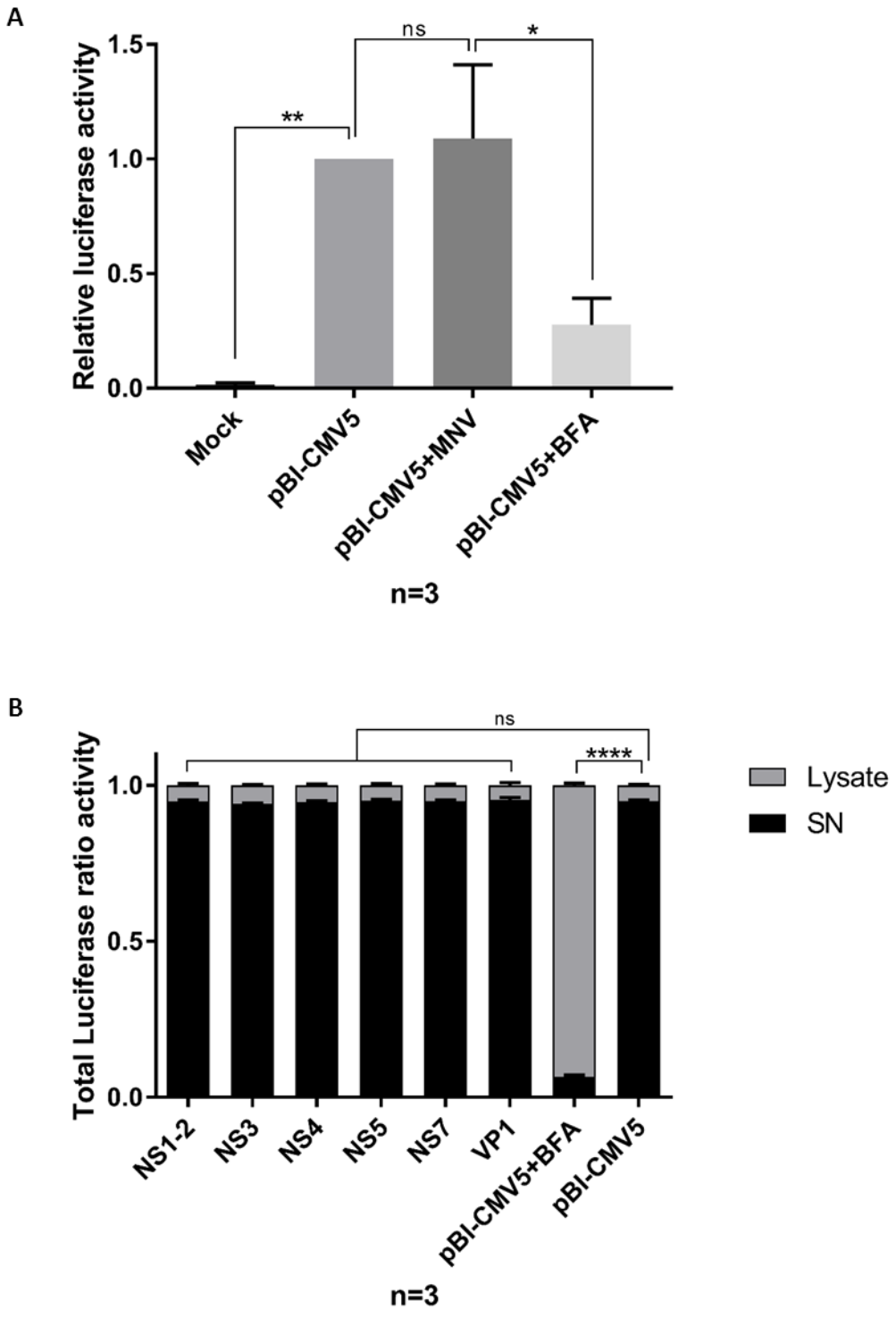


Figure 17 ***MNV does not affect general protein secretion***. ***A*** *RAW264.7 macrophages were transfected with the Metridia luciferase containing pBI-CMV5-mCherry vector. mCherry positive cells were sorted and infected with MNV, treated with BFA or left untreated. The relative luciferase activity was measured at 12 h.p.i. (n=3, average +/- SEM, ns:p>0.05, *p<0.05, **p<0.01).* ***B*** *293T cells were transfected with pBI-CMV5 vectors containing the individual MNV NS proteins. As controls pBI-CMV5 only and pBI-CMV5 and BFA treated cells were used. Supernatants and lysates were collected 24 h post transfection and the ratio between intracellular (lysate) and secreted (supernatant) luciferase activity was calculated (n=3, average +/- SEM, ****p<0.0001).*

4.2.3 MNV infection robustly induces the transcription of IFNβ and TNFα

To test if the transcription of IFNβ and TNFα is elevated during MNV infection, we infected mouse macrophages (RAW264.7) with MNV (MOI 5), treated them with Poly(I:C) or left them untreated and uninfected for 9 h, 12 h and 15 h. Transcription levels of IFNβ and TNFα were tested via RT-qPCR and compared to untreated cells (Figure 18). Poly(I:C) stimulation led to the induction of both IFNβ and TNFα transcription, observed through increasing mRNA levels compared to untreated cells. MNV infected cells showed similar increases in mRNA levels for both cytokines, indicating that the observed lack in cytokine secretion is not based on an inhibition of transcription of these cytokines.

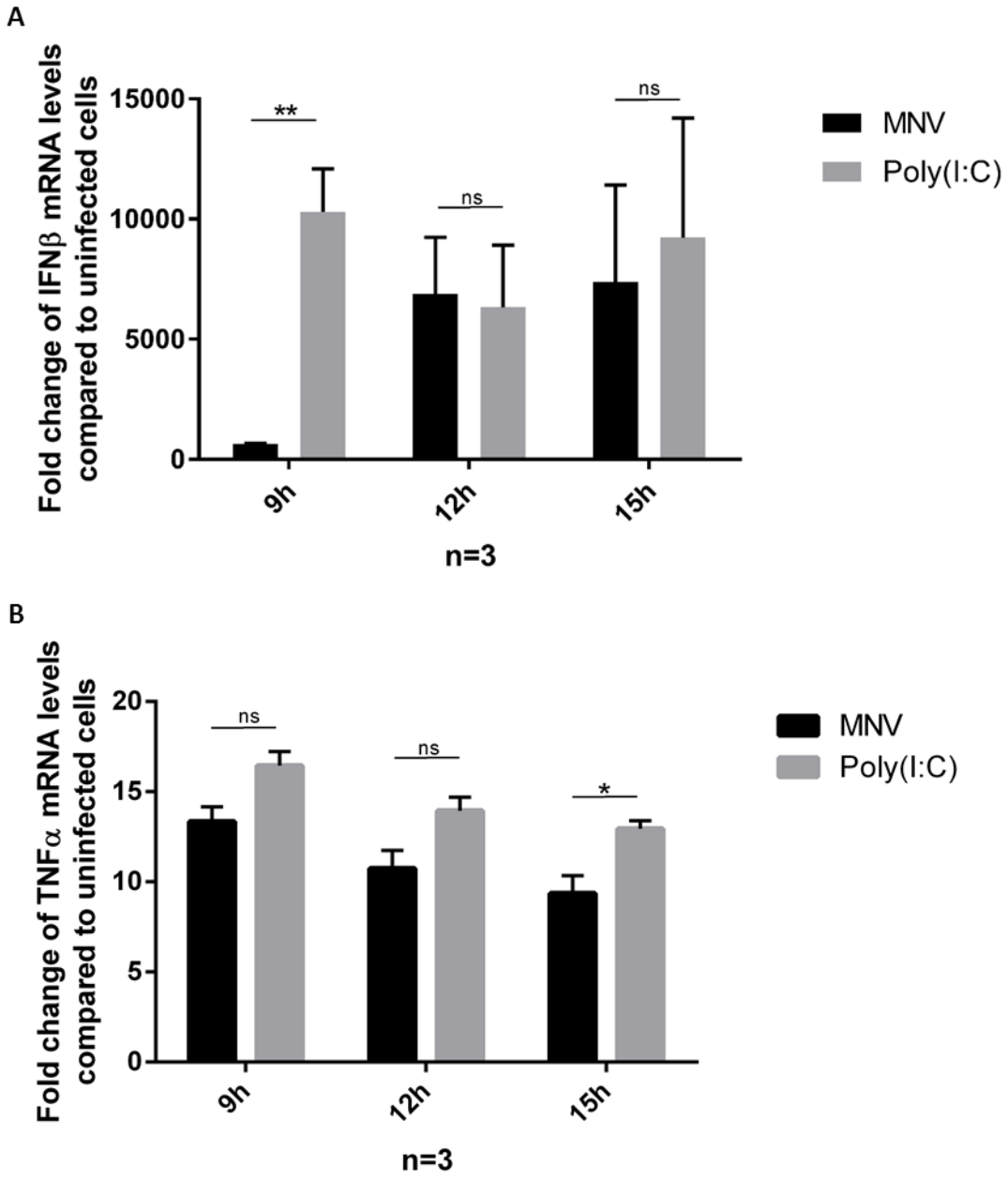


Figure 18 ***MNV infection induces the transcription of cytokines.*** *RAW264.7 cells were infected with MNV (MOI 5), treated with Poly(I:C) (20 µg/mL) or left untreated for 9 h, 12 h and 15 h. RNA samples were taken and analysed via RT-qPCR for the following cytokines (n=3, average +/- SEM, ns:p>0.05, *p<0.05, **p<0.01)* ***A*** *IFNß,* ***B*** *TNFα.*

4.2.4 Intracellular levels of TNFα are low in MNV infected cells despite induction on mRNA level

We have verified the induction of IFNβ and TNFα transcription as a response to MNV infection, but unexpectedly we were unable to detect corresponding high amounts of these cytokines in

the supernatant of MNV infected cells even though we confirmed that protein secretion is not affected by MNV infection. We therefore hypothesised that MNV either has an inhibitory effect on the translation of these cytokines or host proteins in general, or it may cause the degradation of cytokines. Intracellular levels of TNFα were investigated via immunoblot and immunofluorescence analysis to test if the observed low amounts of secreted cytokines are due to low levels of intracellular cytokines (Figure 19). We tried to determine IFNβ levels via IF analysis or immunoblotting with two different antibodies as well, but were unable to detect a signal with both techniques. RAW264.7 cells were infected with MNV, stimulated with Poly(I:C) or left untreated for 12 h (Figure 19 A). Cell lysates were analysed via immunoblot detection using an anti-TNFα antibody. Uninfected cells showed undetectable levels of intracellular TNFα, whereas Poly(I:C) treated cells displayed high levels of TNFα within the cells. In contrast to that, cell lysates of MNV infected cells contained only low amounts of TNFα, despite the induction of cytokine transcription. Additionally, cells were either infected with MNV (MOI 5), treated with Poly(I:C), infected with MNV and treated with Poly(I:C) or left untreated and uninfected (Figure 19 B). RAW264.7 cells were fixed 12 h.p.i. and stained with an anti-TNFα antibody. Mock treated cells showed background levels of TNFα within the cell, whereas the majority of Poly(I:C) treated cells displayed a bright signal for TNFα concentrating around the Golgi apparatus which was visualised through staining with an anti-GM130 antibody. The TNFα signal in MNV infected cells was low compared to Poly(I:C) treated cells and remained low even in the presence of Poly(I:C).

These observations support our findings in the immunoblot analysis revealing a low amount of intracellular TNFα despite increased mRNA levels of the cytokine. The immunofluorescence analysis also indicates that the reduced intracellular cytokine levels cannot

be rescued by additional stimulation with Poly(I:C). This suggests, that MNV might interfere with cytokine stability (i.e. degradation) or translation of host proteins in general or cytokines in particular.

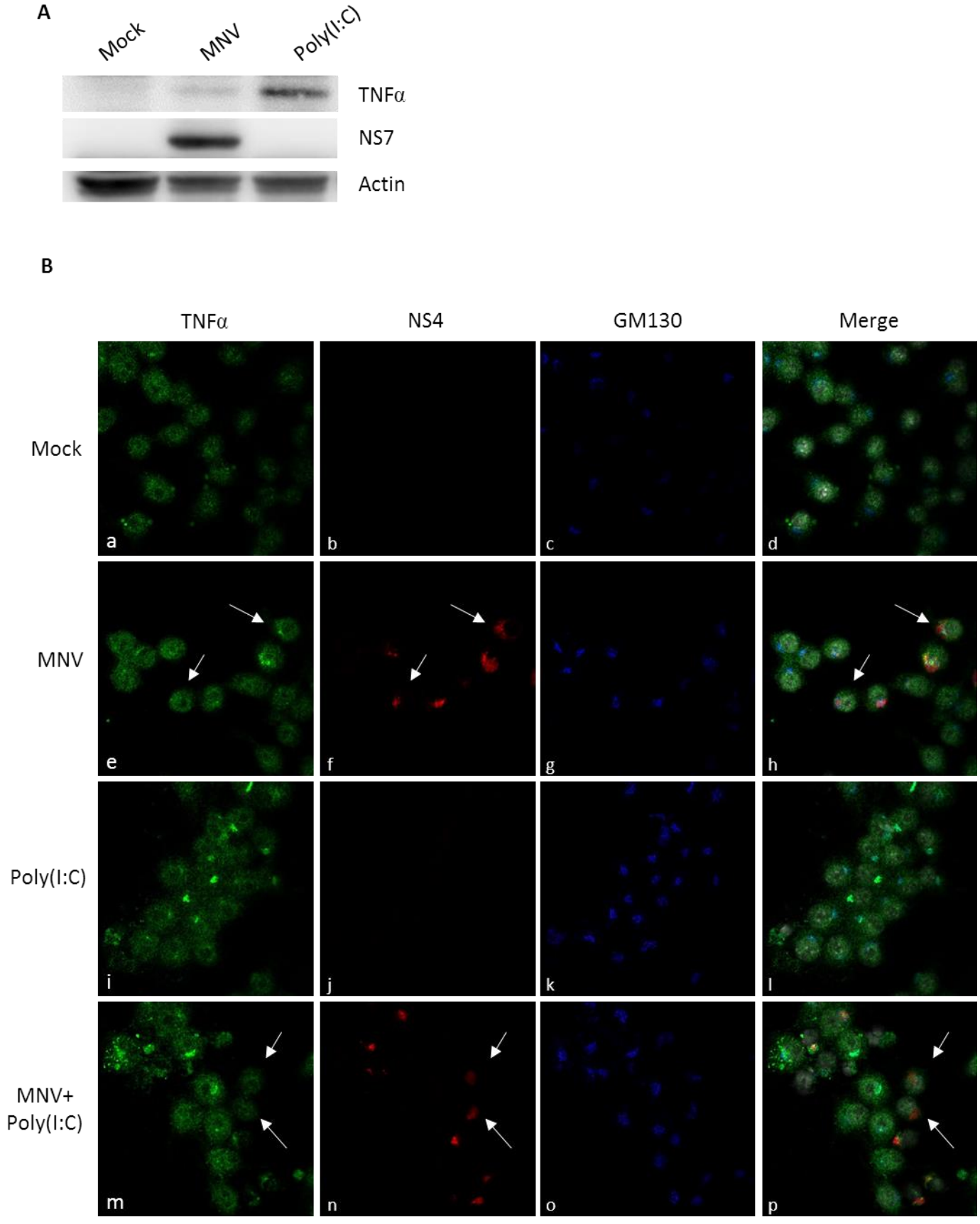

A
Mock
MNV
Poly(I:C)
TNFα
NS7
Actin
B
TNFα
NS4
GM130
Merge
Mock
MNV
Poly(I:C)
MNV+
Poly(I:C)
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Figure 19 ***Intracellular levels of TNFα are low in MNV infected cells.*** *RAW264.7 cells were either infected with MNV (MOI 5), stimulated with Poly(I:C), stimulated and infected or left untreated. Cells were fixed or harvested at 12 h.p.i.* ***A*** *Immunoblot analysis of cell lysates stained with anti-TNFα, anti-NS7 and anti-actin antibodies.* ***B*** *Cells were stained with anti-TNFα (Panel a, e, i and m), anti-NS4 (Panel b, f, j and n), anti-GM130 (Panel c, g, k and o) and DAPI for the merged image (Panel d, h, l and p). Stained samples were analysed via confocal immunofluorescence analysis. Arrows indicate MNV infected cells presenting a low TNFα signal.*

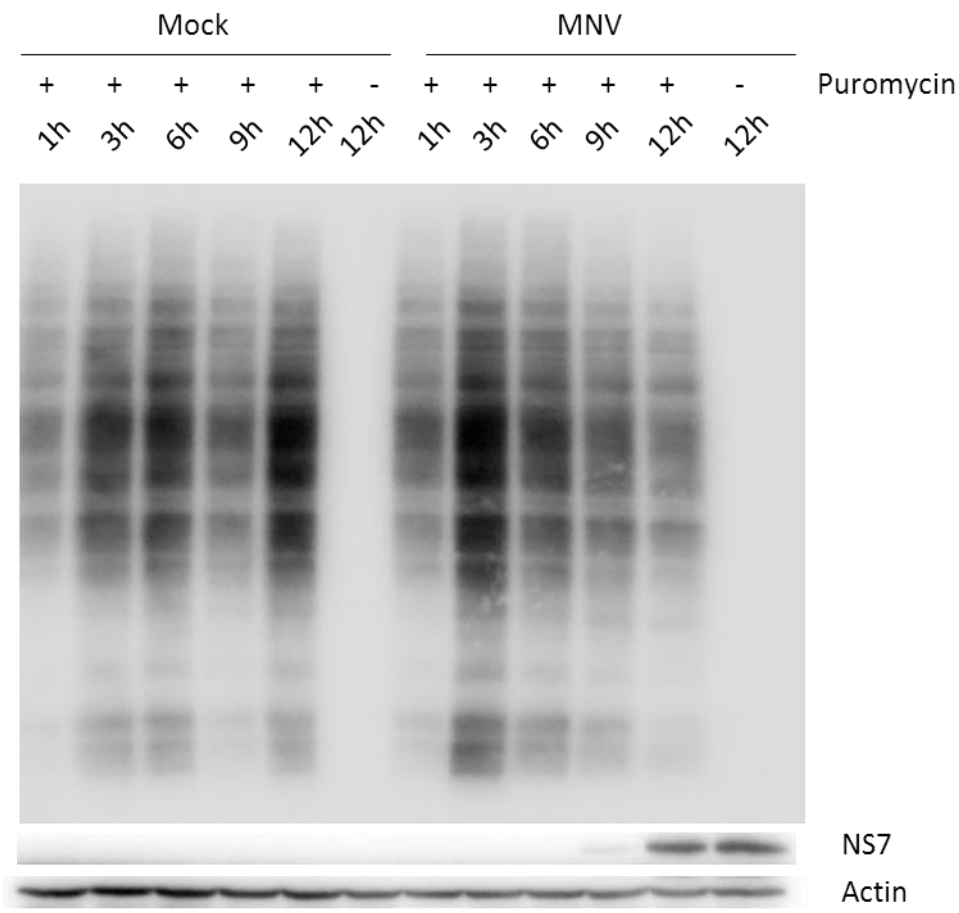
4.2.5 Host cell translation decreases in MNV infected cells over time

To uncover a potential effect of MNV on the translation of the host cell, RAW264.7 cells were infected with MNV or mock infected and treated with puromycin (10µg/mL) 20 mins before cells were harvested and lysed. Puromycin is an antibiotic which interferes with translation and leads to the premature termination of translation. Puromycylated proteins can be visualised with an anti-puromycin antibody and are an indication of the level of general host translation. Cell lysates of puromycin treated cells at 1, 3, 6, 9 and 12 h.p.i. were analysed via immunoblot staining (Figure 20 A). Mock infected cell lysates are positive for the staining with an anti-puromycin antibody at all time points, confirming the constant occurrence of translation in these cells. There is a robust increase in puromycylated proteins detectable in the cell lysates of infected cells at 3 h.p.i., which could be an indication of increased translation due to increased viral protein translation or an activated innate immune response. Interestingly, from 6 h.p.i. onwards and coinciding with detectable amounts of the viral NS7 protein, the signal for puromycin-positive proteins continuously decreases in the lysates of infected cells. This indicates that less translation is occurring in infected cells, especially from 9 h.p.i., which might be caused by a host cell translational shut off induced by the infection. Furthermore, RAW264.7 cells were infected with MNV (MOI 5) or left uninfected and analysed via immunofluorescence analysis (Figure 20 B). Cells were treated with puromycin 30 min prior to fixation after 6 h, 9 h and 12 h of infection and stained with an anti-puromycin antibody. Mock infected cells displayed a robust signal for

puromycin, indicating the occurrence of translation in these cells. There was no major difference in the puromycin signal for infected and uninfected cells after 6 h of infection, but from 9 h.p.i. onwards a drastically reduced puromycin signal was visible in MNV infected cells. If any residual puromycin signal was detectable, it was found to be around the RC of the virus. The number of infected cells presenting a low puromycin signal increased from 9 h.p.i. to 12 h.p.i. and formed the majority of the infected population at 12 h.p.i.

The decrease of host cell translation after 9 h of infection in the IFA is similar to our findings in the immunoblot analysis and supports the hypothesis that MNV interferes with the translation machinery of the host cell.

A


Mock
MNV
+ + + + + - + + + + + -
Puromycin
1h 3h 6h 9h 12h 12h 1h 3h 6h 9h 12h 12h
NS7
Actin

B

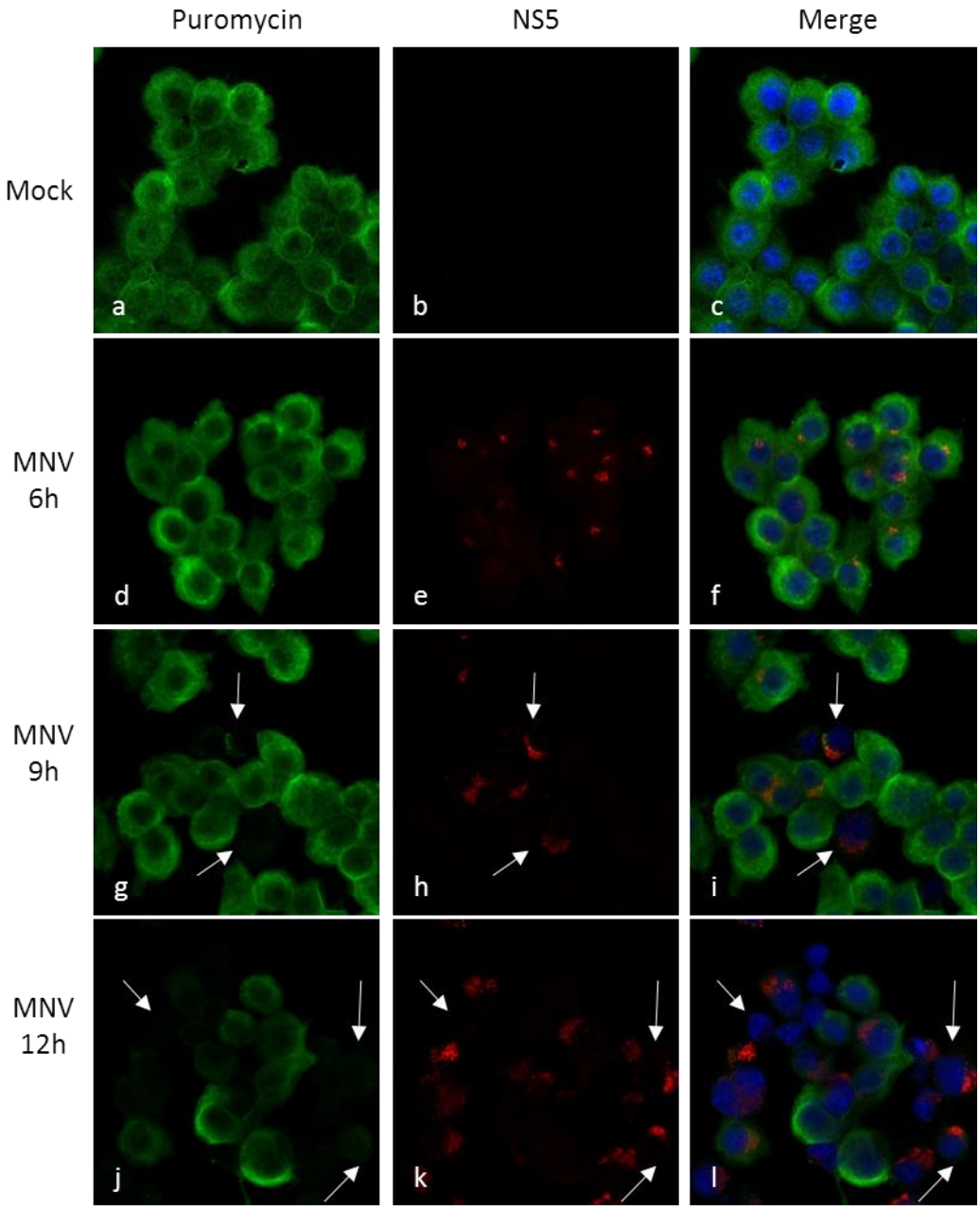


Figure 20 ***Host cell translation decreases in MNV infected cells.*** *RAW264.7 cells were either infected with MNV (MOI 5) or left uninfected and analysed for their translation using puromycin (10 µg/mL). Cells were treated with puromycin 20 min (A) or 30 min (B) prior to harvesting.* ***A*** *Immunoblot analysis of puromycin-treated cell lysates harvested at 1h, 3h, 6h, 9h and 12 h.p.i. The western blot was stained with anti-puromycin, anti-NS7 and anti-actin antibodies.* ***B*** *Immunofluorescence analysis of puromycin-treated cells at 6 h, 9 h and 12 h post infection. Cells were stained with anti-puromycin (Panel a, d, g and j), anti-NS5 (b, e, h and k) and DAPI for the merged image (Panel c, f, I and l). Arrows indicate MNV infected cells displaying a low signal for anti-puromycin.*

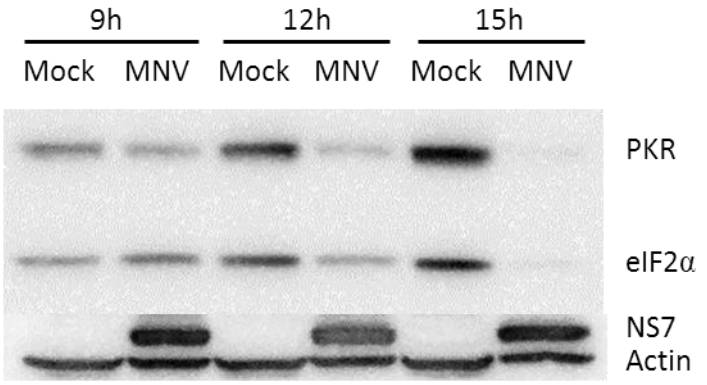
4.2.6 The role of PKR in the reduced host cell translation in MNV infected cells

One of the major regulators in the translational control of the host cell is PKR, which can be activated upon viral infections to inhibit host-dependent viral replication. PKR becomes activated through phosphorylation and can in turn lead to the phosphorylation of eIF2α. p-eIF2α is no longer able to bind to the translation complex and can therefore be an indicator of stalled translation. Having observed a decrease in translation in MNV infected cells (Figure 20), we explored the role of PKR in the translational shut down (Figure 21). RAW264.7 cells were infected with MNV (MOI 5) or left uninfected for 9 h, 12 h and 15 h and analysed via immunoblotting (Figure 21 A). Lysates were tested with antibodies against PKR and eIF2α and revealed a steady decrease in signal intensity for both proteins in MNV infected cell lysates compared to mock infected cells. This decrease was detectable from 9 h.p.i. for the PKR signal and 12 h.p.i. for the eIF2α signal, indicating that both proteins are susceptible to the host cell translation inhibition, but might have induced the shutdown of translation early during infection. Therefore, we analysed the PKR and p-PKR levels in MNV infected and mock infected cells early during infection at 1, 3, 6 and 9 h.p.i. via immunoblot analysis (Figure 21 B-D). PKR levels are decreased early during infection with differences detectable between MNV infected and mock infected cells from 3 h.p.i. onwards (Figure 21 B). Surprisingly, despite decreasing levels of PKR, p-PKR levels remain considerably stable during the course of infection, indicating that the majority of PKR within the infected cells is in a phosphorylated state (Figure 21 C and D). To uncover the activation status of PKR and eIF2α at the peak of replication (12 h.p.i.), cell lysates of RAW264.7 cells infected with MNV (MOI 5), stimulated with Poly(I:C) or left untreated and uninfected were analysed via immunoblotting using antibodies against PKR, p-PKR, eIF2α, p-eIF2α, NS7 and actin (Figure 21 E). Mock infected as well as Poly(I:C) treated cells showed robust signals for PKR and eIF2α, while

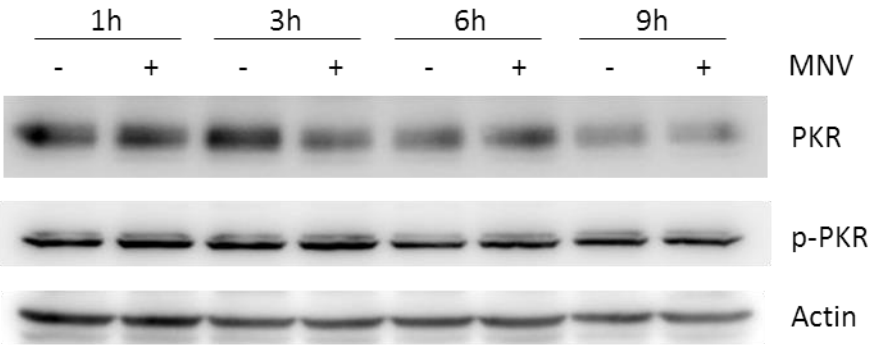
both proteins have a reduced signal in MNV infected cells, similar to our previous observation (Figure 21 A). Intriguingly, compared to the low signal of the overall amount of PKR and eIF2α in infected cells, the signal for the phosphorylated and activated forms of both proteins seems to be slightly elevated (Figure 21 E). The signal for p-PKR in mock infected and Poly(I:C) treated cells is lower than the overall signal for PKR, indicating that the majority of PKR within the cell is in an inactive, non-phosphorylated state. In contrast to that, the ratio of the total PKR to p-PKR is high in MNV infected cells, which suggests that most of the PKR proteins present in MNV infected cells are in an active, phosphorylated state (Figure 21 F). Similar to p-PKR, p-eIF2α levels are increased compared to the total level of eIF2α in MNV infected cells (Figure 21 G). Increased levels of p-PKR and p-eIF2α, compared to levels of unphosphorylated forms of both proteins, can affect host cell translation. However, the effectiveness of the increased levels of p-PKR and p-eIF2α in MNV infected cells remains to be elucidated.

In conclusion, our findings indicate that PKR/eIF2α could be causing the host translation shut down, but might not be the only inducer causing the inhibition of host cell translation.

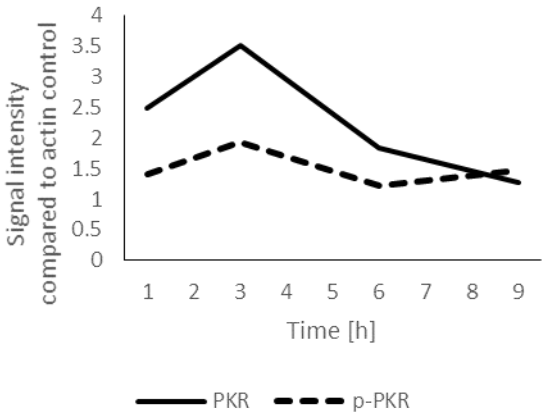
A


9h
12h
15h
Mock
MNV
Mock
MNV
Mock
MNV
PKR
eIF2α
NS7
Actin

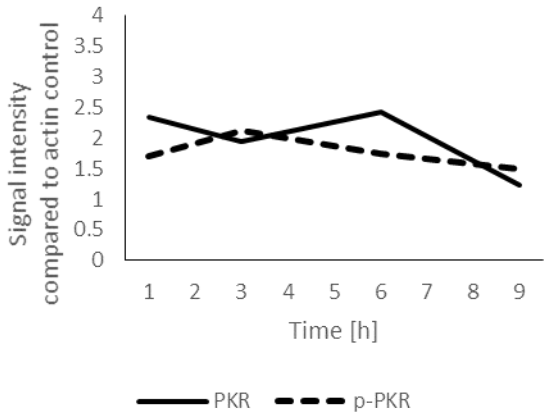
B


1h
3h
6h
9h
- + - + - + - +
MNV
PKR
p-PKR
Actin

C


Signal intensity compared to actin control
4
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9
Time [h]
PKR
p-PKR

D


Signal intensity compared to actin control
4
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9
Time [h]
PKR
p-PKR

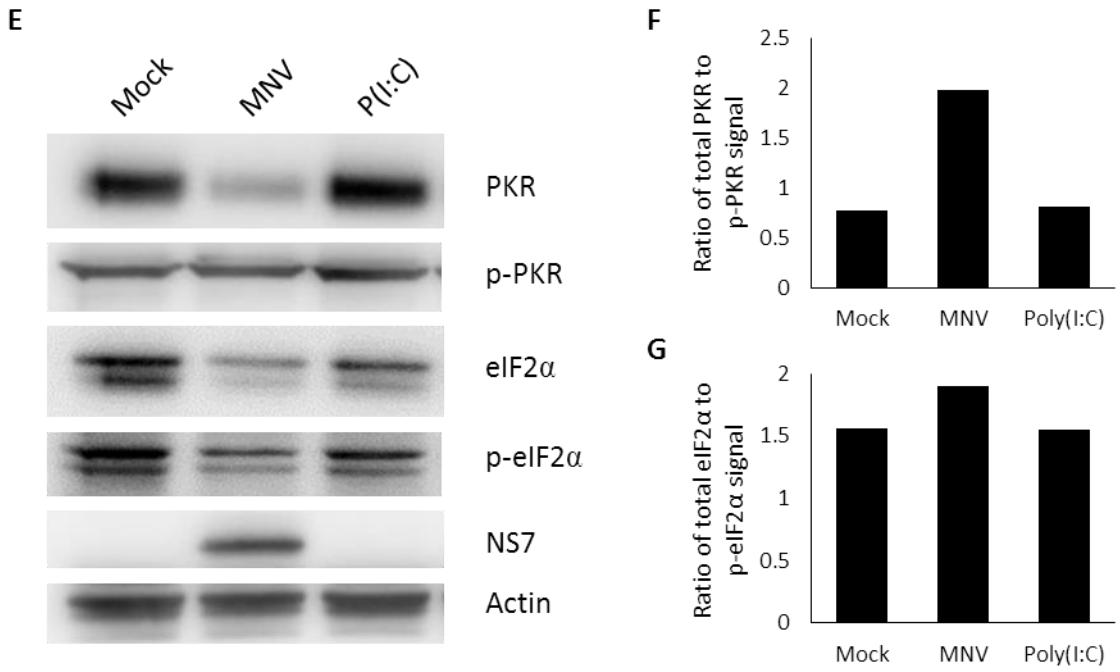


Figure 21 ***Role of PKR in the inhibition of host cell translation during MNV infection. A*** *Immunoblot analysis of RAW264.7 cells infected with MNV or left uninfected for 9 h, 12 h and 15 h. Cell lysates were stained with anti-PKR, anti-eIF2α, anti-NS7 and anti-actin antibodies. RAW264.7 cell were infected with MNV (MOI 5) or left uninfected. Cells were harvested at 1, 3, 6 and 9 h.p.i. and analysed via immunoblot analysis.* ***B*** *Immunoblot of whole cell lysates stained with anti-PKR, anti-p-PKR and anti-actin antibodies.* ***C*** *Signal intensity for anti-PKR and anti-p-PKR stain for mock infected cell lysates (compared to actin signal, n=1)* ***D*** *Signal intensity for anti-PKR and anti-p-PKR stain for MNV infected cell lysates (n=1)* ***E*** *Immunoblot analysis of RAW264.7 cells infected with MNV, treated with Poly(I:C) or left untreated and uninfected. Cell lysates were harvested 12 h.p.i. and stained with anti-PKR, anti-p-PKR, anti-eIF2α, anti-p-eIF2α, anti-NS7 and anti-actin antibodies.* ***F*** *Ratio of total PKR to p-PKR (n=1) and* ***G*** *total eIF2α to p-eIF2α signal from immunoblot analysis (n=1).*

4.3 Discussion

The release of cytokines such as IFNβ and TNFα during viral infections plays a crucial role in the innate immune response against viruses. The transcription and translation of cytokines is elevated in infected cells, mostly due to the recognition of PAMPs, e.g. dsRNA. Cytokines are then secreted into the extracellular space where they can either bind to receptors on neighbouring cells or bind to receptors on the infected cell itself to enhance the antiviral response. In this study, we have shown that MNV infected cells increase the transcription of cytokine (IFNβ and TNFα) mRNAs (Figure 18), indicating that PRRs like MDA5 have successfully detected the viral infection and activated an antiviral response against it.

Intriguingly, MNV infected cells do not secrete cytokines which would aid to overcome and contain the acute infection (Figure 16). Based on our studies using the *Met*Luc reporter system for protein secretion, we can exclude that the low amounts of secreted cytokines from infected cells are based on the inhibition of general protein secretion (Figure 17). Instead, we uncovered that only very low amounts of translated cytokines can be detected within the infected cells, further confirming that there is no secretion inhibition, which would cause the accumulation of cytokines within the cells (Figure 19). The difference in intracellular protein levels for TNFα compared to the mRNA levels indicates an interference of the virus with the translation of host cell proteins. We investigated host cell translation using puromycin as a marker for translational activity (Figure 20). Even though translation seemed to be upregulated early during the infection (3 h.p.i.), there was a continuous decrease in the amount of puromycylated proteins at later stages of the infection, indicating a reduction in global host cell translation. Based on the

immunoblot and immunofluorescence analysis, MNV starts to affect host cell translation 6-9 h.p.i., reducing host cell translation to a minimum in the majority of infected cells by 12 h.p.i.

The shutdown of host cell translation is one of the major defence mechanisms against viral infections. Viral replication is dependent on host cell translation, because viruses lack their own translational machinery. Therefore a reduction in host protein translation should also lead to a decreased translation of viral proteins and interfere with efficient viral replication. Surprisingly, translation of viral proteins like NS7 does not seem to be affected by the reduced host protein translation, because intracellular amounts of NS7 increase from 6 h.p.i. onwards, while host protein translation subsides (Figure 20 A). This observations strongly suggest that MNV employs a different mechanism to initiate translation, independent of cellular protein translation (Hanson, Zhang *et al*. 2012). This would be a great advantage for the virus, not only by forcing the cell to preferentially translate viral proteins, but also by diminishing the innate immune response by preventing the translation of immune effectors such as cytokines.

The HuNoV VPg and MNV NS5 protein have been shown to interact with the translation initiation complex through eIF4GI and eIF4E, suggesting a role of NS5 in the initiation of viral protein translation (Daughenbaugh, Fraser *et al*. 2003, Daughenbaugh, Wobus *et al*. 2006). Initiation of viral protein translation, which is independent of the cellular cap-dependent protein translation, could be mediated by NS5 and allow viral protein translation to occur in the absence of cellular protein translation (Chung, Bailey *et al*. 2014).

To uncover how MNV is causing the reduced level of host protein translation, we investigated the PKR/eIF2α pathway, which is a major regulator of translation (Figure 21). Activated and phosphorylated PKR inhibits protein translation by phosphorylating elongation

initiation factor eIF2α. PKR can get activated and phosphorylated via binding to dsRNA, which is generated during MNV infection. The further upregulation of PKR levels in infected cells is part of the antiviral defence and is induced through IFNβ (Meurs, Chong *et al*. 1990). Interestingly, IFNβ secretion is significantly impaired in MNV infected cells, which indicates that PKR synthesis might not be promoted during MNV infection. However, PKR is expressed on a low level in a variety of cell types and can be activated by dsRNA in the absence of IFNβ (Clemens 1997, Nanduri, Carpick *et al*. 1998). We observed high levels of activated p-PKR in MNV infected cells compared the total amount of PKR within the cell (Figure 21 E and F). In contrast to that, mock infected and Poly(I:C) treated cells displayed lower levels of p-PKR compared to the total amount of intracellular PKR.

These findings suggest that PKR induces the shutdown of translation at early stages in infection, but intracellular PKR levels decrease over time due to the translational shutdown and the initial lack of IFN-induced transcription of PKR. Immunoblot analysis of PKR levels early during infection supported this hypothesis, revealing decreasing levels of PKR in MNV infected cells from 3 h.p.i., while p-PKR levels were more stable over time and represent the majority of PKR within the infected cells (Figure 21 B-D). The increased ratio of p-PKR to unphosphorylated PKR in MNV infected cells could be crucial and sufficient to inhibit the host cell translation. It is important to note that eIF2α levels, similar to PKR, are decreased in MNV infected cells and can be very sensitive to changes in the p-PKR to PKR ratio despite the overall low levels of PKR in MNV infected cells.

A recent study on the role of PKR during Chikungunya virus (CHIKV) infection discovered that cytokines like IFNβ and IL-6 can still be synthesised in the context of PKR activation and the reduction in host cell translation to counteract the infection (Clavarino, Cláudio *et al*. 2012). The

key protein to enable the specific translation of antiviral proteins was shown to be GADD34. GADD34 or MyD116 is controlled via activating transcription factor 4 (ATF4) and counteracts the effect of PKR by dephosphorylating eIF2α. Even though the expression of GADD34 did not rescue the global translational shutdown, it was still able and necessary to allow the translation of IFNβ and IL-6. This was not only shown to be true for CHIKV infection, but also for cytosolic dsRNA in general (Clavarino, Cláudio *et al*. 2012). Considering that dsRNA is generated during MNV replication, it would be interesting to uncover the role of GADD34 during MNV infection. Based on the observations by Clavarino *et al*, the translational shutdown through PKR might not be sufficient to restrict IFNβ and TNFα translation and might involve an additional interference of MNV with GADD34. In future experiments, the expression level of GADD34 in MNV infected cells should be investigated and it would be interesting to test if our observed inhibition in cytokine translation and secretion can be rescued by GADD34 overexpression.

Another protein to explore in future studies is PABP, because it is important in the establishment of the translation complex. Coxsackie virus, poliovirus as well as FCV cleave PABP to stall or reduce host translation (Kerekatte, Keiper *et al*. 1999, Kuyumcu-Martinez, Belliot *et al*. 2004, Rivera and Lloyd 2008). Interestingly, purified VPg of NoV MD145 has been shown to also be able to cleave PABP, when added to cell lysates (Kuyumcu-Martinez, Belliot *et al*. 2004). A recent study investigating the translational repression of Viperin and ISG15 in MNV infected cells, proposed the translational shutdown is caused by cleavage of PABP through MNV NS6 (Emmott, Sorgeloos *et al*. 2017). When NS6 was transfected into 293T cells it was able to induce cleavage of PABP and the overexpression of a non-cleavable PABP mutant partially restored the Viperin levels in MNV infected cells. It remains to be elucidated if the host translation shutdown is

triggered through one pathway alone or induced by the interplay of several regulators of the translation machinery.

Many viruses try to evade antiviral effectors or activation of antiviral signalling altogether. IAVs express the NS1 protein which prevents the dsRNA-mediated induction of IFNβ transcription by interfering with the transcription factors NF-κB and IRF-3 as well as the cytosolic dsRNA receptor RIG-I (Talon, Horvath *et al*. 2000, Wang, Li *et al*. 2000, Mibayashi, Martínez-Sobrido *et al*. 2007). In this case an antiviral immune response is inhibited at the recognition of viral infection, preventing any further downstream signalling. Other viruses interfere with cytokine secretion to prevent further activation of signalling pathways and systemic effectors. The 2B and 3A proteins of poliovirus have been identified to interfere with cytokine secretion. 3A, which is related to MNV NS4, inhibits the ER-to-Golgi transport in infected cells (Dodd, Giddings *et al*. 2001). A mutation in 3A, which affects its ability to inhibit the secretory pathway, showed increased secretion of cytokines, compared to cells transfected with the wild type 3A (Doedens and Kirkegaard 1995, Doedens, Giddings *et al*. 1997).

Based on our findings, MNV seems to employ a strategy similar to picornaviruses and alphaviruses to evade the innate immune response by inducing the inhibition of host cell translation (Etchison, Milburn *et al*. 1982, Lloyd, Jense *et al*. 1987, Lloyd, Grubman *et al*. 1988, Macadam, Ferguson *et al*. 1994, McInerney, Kedersha *et al*. 2005, Ryman and Klimstra 2008). During MNV infection shutdown of host translation is caused via activation of PKR to phosphorylate eIF2α and inhibit the translation and secretion of cytokines. Intriguingly, MNV triggers the shutdown of cellular protein translation without disturbing viral protein synthesis and

translation. Our future studies will be focusing on unveiling the mechanisms leading to the specific inhibition of cellular protein translation while ensuring viral protein translation.

Chapter 5

The role of GEF-H1 during MNV infection

5.1 Introduction

Previous studies have investigated the interaction of MNV with the cytoskeleton of the host cell. Interestingly, tubulin, a major component of microtubules has been shown to interact and co-localise with the MNV RC and the MNV protein NS3 (Hyde, Gillespie *et al*. 2012, Cotton, Hyde *et al*. 2016). Microtubules are highly dynamic structures, which can undergo fast and drastic changes through the depolymerisation and polymerisation at the end of the tubular fibres. They are generated through polymerisation of α- and β-tubulin dimers and form a network throughout the cell. This network is anchored at the MTOC, which harbours of a specific form of tubulin, γ-tubulin. Microtubules have several functions within cells including the motility and structure of the cell as well as providing a scaffold for the intracellular transport of proteins and vesicles. The latter function is mediated through the microtubule motor proteins dynein and kinesin. Experiments using Nocodazole, a drug that leads to the depolymerisation of microtubules, showed that a functional microtubule network was needed for the successful generation of a concentrated MNV RC during virus replication (Hyde, Gillespie *et al*. 2012). This indicates that MNV may use the host microtubule network for the transport of host and viral proteins and benefits from the structural scaffold to set up an efficient replication complex. Recently, our lab observed a direct interaction between β-tubulin and vesicular structures induced upon transient expression of the MNV non-structural protein NS3 (Cotton, Hyde *et al*. 2016). The motility and size of the NS3 induced vesicles was observed to be dependent on a functional microtubule network, implying that NS3 might be responsible for the microtubule-dependent formation of the MNV RC. Additionally, MNV not only seems to be dependent on the microtubule network, but it also causes changes to it. As early as 12h after infection with MNV the formation of strongly polymerising microtubule bundles in the cells periphery were observed (Hyde, Gillespie *et al*. 2012). Intriguingly, a similar phenotype of

microtubule bundling has been observed in cells which express GEF-H1 which, unlike other GEFs, is able to bind microtubules. GEFs are regulators of GTPases, activating the GTPase by mediating the exchange from GDP to GTP. GEF-H1 has been shown to have several regulatory functions in the cell through its ability to activate the GTPases Rho and Rac (Ren, Li *et al*. 1998). It is involved in the connection of microtubules with the actin cytoskeleton, the regulation of tight junctions and vesicle transport (Chang, Nalbant *et al*. 2008, Guilluy, Swaminathan *et al*. 2011, Pathak, Delorme-Walker *et al*. 2012). Intriguingly, GEF-H1 has also been proposed to play a role in the innate immune response against microbial pathogens. *Shigella*, a gram-negative bacteria which causes diarrhoea in humans, by invading the intestinal epithelium through the tight junctions of the intestinal barrier. GEF-H1 is involved in the invasion of *Shigella* past the intestinal epithelial barrier through its association with tight junctions. GEF-H1 gets recruited to tight junctions upon *Shigella* infection and co-localises with *Shigella* entry sites. In addition, GEF-H1 deficient cells lack the NOD1 and RhoA mediated activation of NF-κB during infection with *Shigella* (Fukazawa, Alonso *et al*. 2008). During IAV infection, GEF-H1 has been proposed to play a major role in the innate immune response and the activation of transcription factors which lead to the expression of IFNβ (Chiang, Zhao *et al*. 2014). The authors proposed a model whereby GEF-H1 is activated upon detection of dsRNA derivatives by cellular sensors such as RIG-I and MDA5. GEF-H1 is released from microtubules and can act on TBK1 which in turn activates the regulatory kinase IKKε. IKKε phosphorylates the transcription factor IRF3, which translocates into the nucleus to promote IFNβ transcription. Due to the involvement of GEF-H1 in the innate immune response against IAV and its interaction with the microtubule network, we were interested in the role of GEF-H1 during MNV infection. Considering its interaction with the microtubule network and the

recognition of intracellular pathogens, GEF-H1 could be an important factor in the host defence against MNV or a target for MNV proteins.

5.2 Results

5.2.1 MNV infection leads to the formation of microtubule fibres

Previous observations have shown that infection with MNV leads to changes in the cytoskeleton of infected cells. The most obvious effect observed was in the microtubule network with the induction of microtubule bundles (Hyde, Gillespie *et al*. 2012). Instead of an evenly distributed, spread out network throughout the cytoplasm, staining with β-tubulin revealed thick microtubule fibres in the periphery of the cell (Hyde, Gillespie *et al*. 2012). These findings were verified in RAW264.7 cells via immunofluorescence analysis (Figure 22). Microtubules were visualised through staining with an anti β-tubulin antibody and virus positive cells were identified with an anti-VPg antibody. The formation of microtubule fibres could be observed from 12h onwards after infection with MNV and was specific for infected cells compared to uninfected cells. Uninfected cells showed no bundling of microtubules and displayed a network-like microtubule staining.

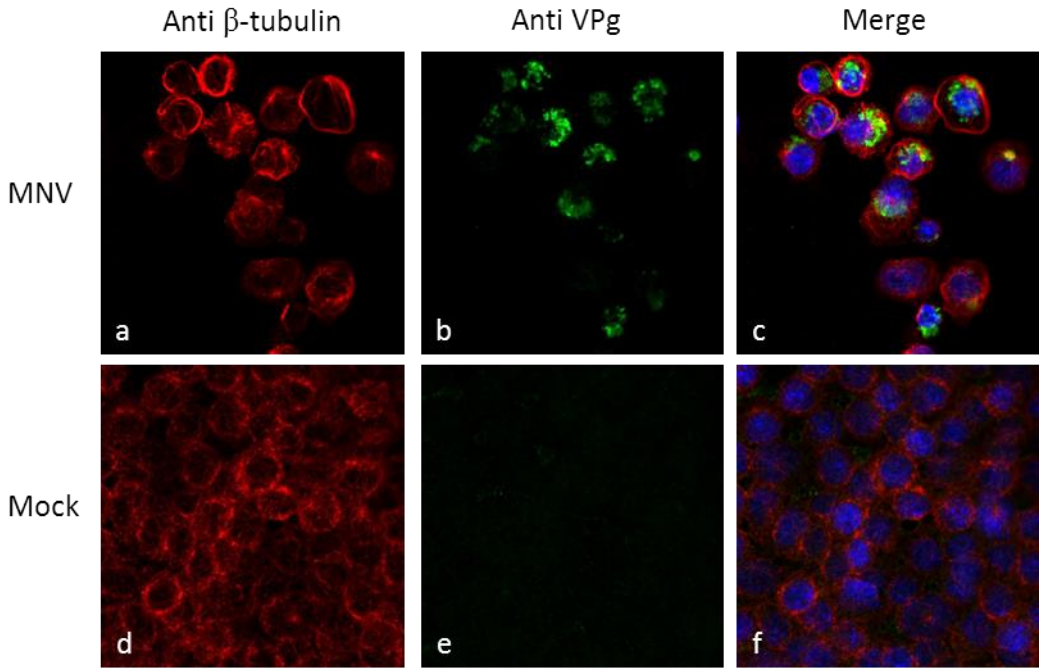


Figure 22 ***Infection with MNV induces the formation of microtubule bundling****. Murine macrophages (RAW264.7) were either infected with MNV (MOI 5) for 12 h or left uninfected. Cells were fixed 12 h.p.i. and labelled with an anti β-tubulin (panel a and d; red) and an anti VPg antibody (panel b and e; green) for confocal microscopy analysis. Panel c and f show the merged image including the nuclear stain (DAPI; blue).*

5.2.2 Overexpression of wildtype GEF-H1 leads to the formation of microtubule bundling in mammalian cells

To interrogate the role of GEF-H1 during MNV infection, we utilised recombinant cDNA plasmids that encoded WT GEF-H1 tagged to GFP and mutant GEF-H1. C53R and ΔDH are loss of function mutants, lacking the ability to associate with the microtubules or missing the catalytic function, respectively. S885A in contrast is a constitutively active mutant, which can still bind to the microtubules (Chiang, Zhao et al. 2014). These plasmids were transfected in Vero cells, and the localisation and interaction with microtubules of expressed GFP-tagged GEF-H1 WT as well as the mutant forms C53R, ΔDH and S885A was determined via immunofluorescence analysis (Figure 23). Microtubules were visualised via anti α-tubulin antibody staining. Cells transfected with wildtype GEF-H1 as well as the S885A mutant contained thick microtubule fibres similar to the

microtubule bundling observed in MNV-infected cells. Both GEF-H1 forms co-localised with α-tubulin, a major component of microtubule fibres, whereas the C53R and ΔDH mutants failed to induce the fibre formation. The C53R mutant was observed to form aggregates within the cytoplasm of transfected cells and did not co-localise with α-tubulin. The ΔDH mutant partially co-localised with α-tubulin, but did not appear to induce thick microtubule fibres like the wildtype. These results imply that the ability to bind to microtubules as well as the GTPase functionality of the GEF are important for the observed microtubule bundling.

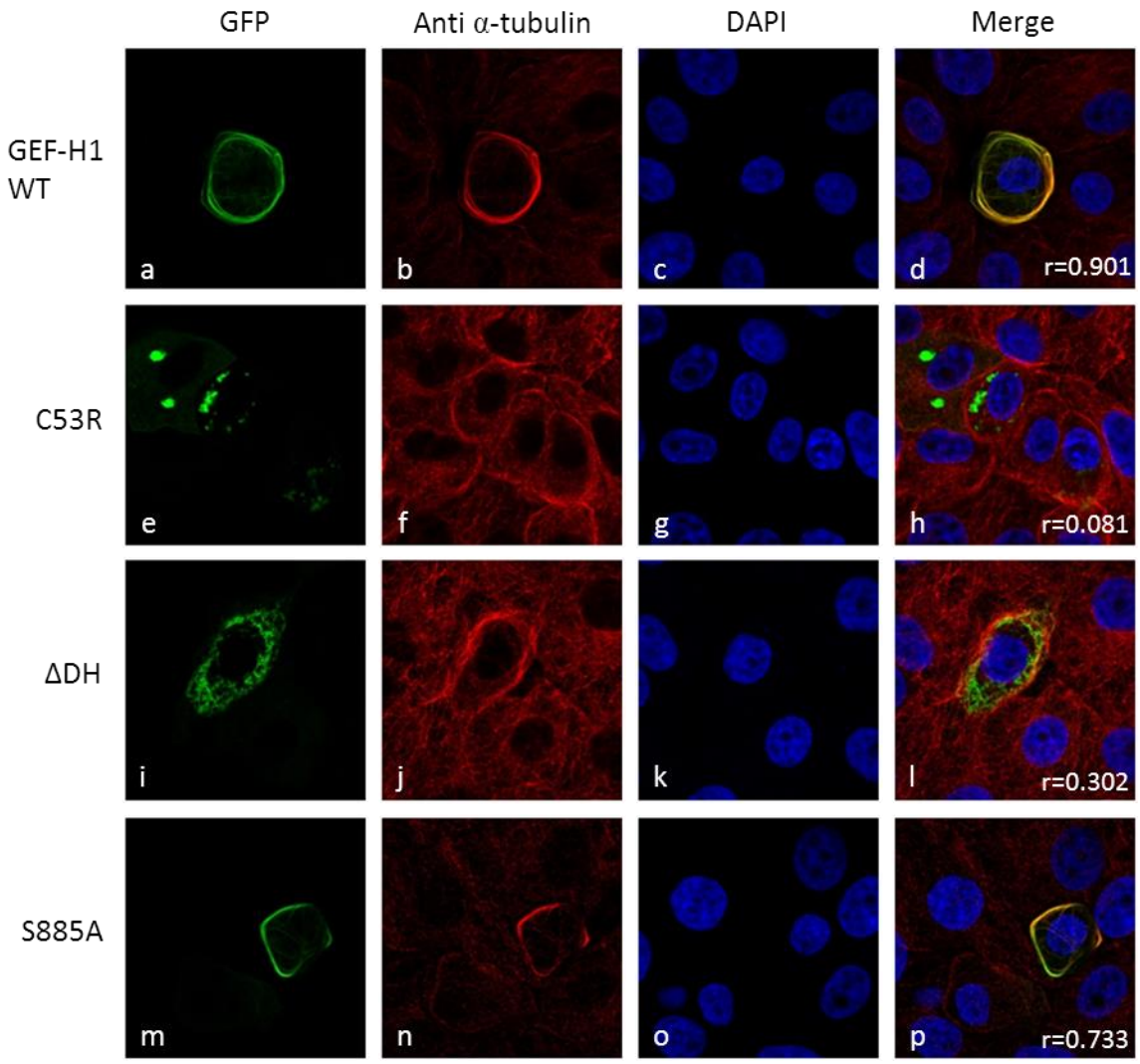


Figure 23 ***Expression of GEF-H1 WT and mutants leads to a distinct cellular localisation pattern.*** *Vero cells were transfected with GFP-tagged GEF-H1 forms (WT, C53R: unable to associate with microtubules, ΔDH: no GEF activity, S885A: constantly active GEF). Cells were fixed 24 h.p.t and labelled with an anti-α-tubulin antibody for confocal microscopy analysis. Panels a, e, i and m show the GFP signal of the GEF-H1 forms (green), panels b, f, j and n display the staining with an anti α-tubulin antibody (red). Panels c, g, k and o represent the nuclear staining with DAPI (blue) and panels d, h, l and p are showing the merged pictured of all channels. Macrophages expressing WT GEF-H1 and S885A show the induction of a microtubule fibre formation. The co-localisation was quantified with the Pearson's coefficient and is indicated in the merged image as a yellow hue.*

5.2.3 Expression of GEF-H1 leads to changes in the localisation of the RC during MNV infection

Based on our previous observations showing that GEF-H1 induces similar microtubule bundles compared to the microtubule fibre formation during MNV infection, we aimed to investigate the role of GEF-H1 during MNV infection. GEF-H1 has been shown to be a crucial regulator in the viral

sensing pathway during IAV infection and could additionally be important for the detection of MNV (Chiang, Zhao et al. 2014). To determine if the expression of GEF-H1 and its mutants affected MNV infection, murine macrophages were transfected with the different GFP-tagged GEF-H1 encoding plasmids and subsequently infected with MNV. Cells were fixed 12 h.p.i. and stained with antibodies against the viral protein NS4, as well as the nuclear stain DAPI and analysed via immunofluorescence microscopy (Figure 24). Cells transfected with the C53R mutant (lack of microtubule binding) displayed no change in the morphology or location of the MNV RC, which is located in the perinuclear region and forms a single aggregate. However, expression of wildtype GEF-H1, ΔDH and S885A mutants lead to significant changes in the morphology and location of the MNV RC. We observed that instead of one large perinuclear located RC, several smaller RCs could be observed distributed throughout the infected cell. These RCs were distributed in a non-polar fashion and generally observed around the cell periphery.

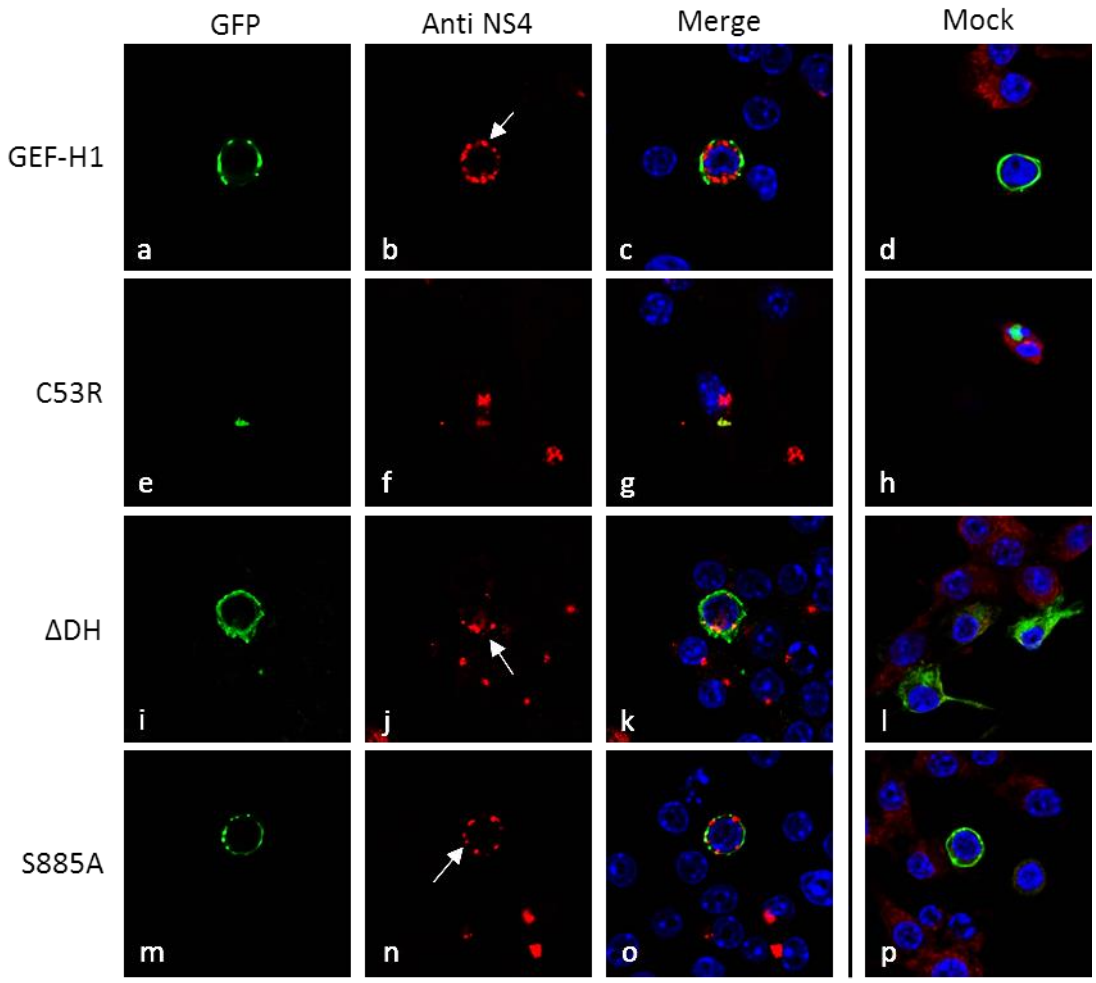


Figure 24 ***Expression of GEF-H1 WT induces the dispersion of the MNV RC****. Murine macrophages (RAW264.7) were transfected with GFP-tagged GEF-H1 forms (WT, C53R: unable to associate with microtubules, ΔDH: no GEF activity, S885A: constantly active GEF) and at 12 h.p.t. were subsequently infected with MNV (MOI 5). Cells were fixed at 12 h.p.i. and immune-labelled with an anti-NS4 antibody for confocal microscopy analysis. The GFP signal of the different GEF-H1 forms is displayed in panels a, e, i and m (green), while panel b, f, j and n show the staining with the anti NS4 antibody (red). Panel c, g, k and o show the merged image including the nuclear stain (DAPI; blue). Panels d, h, l and p represent the merged image of GEF-H1-GFP transfected, but uninfected cells. Macrophages expressing the microtubule-associated forms of GEF-H1 (WT, ΔDH, S885A) all showed a dispersion of the MNV RC (white arrows).*

5.2.4 The MNV protein NS3 co-localises with GEF-H1 and alters its distribution

To elucidate the effect of GEF-H1 on MNV or the virus on GEF-H1, we co-expressed the viral NS proteins and the major capsid protein VP1 together with wildtype GFP-tagged GEF-H1 in Vero cells. Cells were fixed 24h after transfection and stained with antibodies against the 6xHIS tag of the viral proteins for immunofluorescence analysis (Figure 25). Most viral proteins did not affect

the localisation of GEF-H1 or co-localise with it, nor did GEF-H1 have an influence on the viral protein localisation (only NS1-2 and NS4 are shown in Figure 25, panel a-d and panel i-l). However, co-expression of NS3 and GEF-H1 altered the localisation and appearance of GEF-H1 as well as NS3 (Figure 25, panel e-h). Upon co-expression with NS3, the GEF-H1 expression did not induce microtubule bundling, but was instead observed to be evenly distributed throughout the cell and the cell periphery except for the nucleus and vesicle like structures. We additionally observed that the NS3 staining mainly co-localised with GEF-H1 and was also observed to have a more diffuse cytoplasmic staining compared to the expression of NS3 alone (Hyde and Mackenzie 2010, Cotton, Hyde *et al*. 2016).

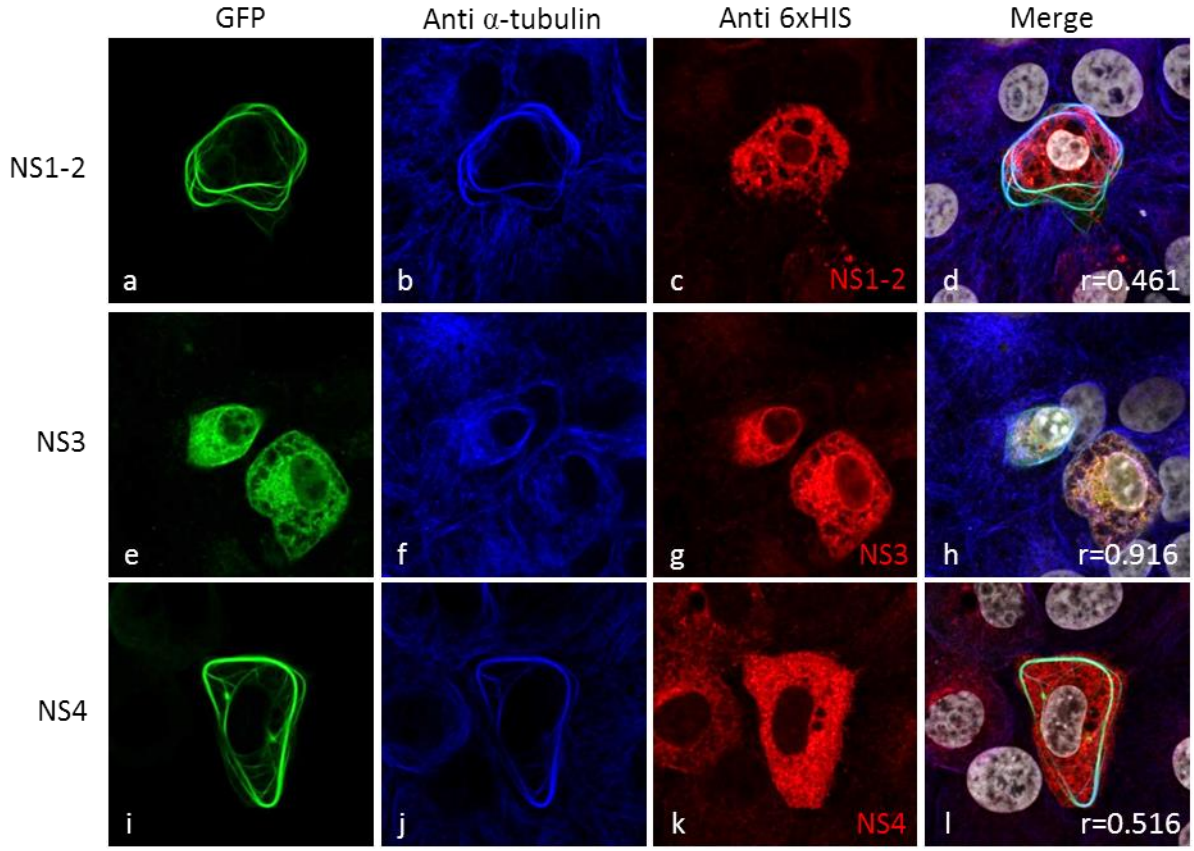


Figure 25 ***Co-expression with NS3 changes GEF-H1 WT morphology****. Vero cells were co-transfected with GEF-H1-GFP WT and the HIS-tagged viral proteins. Cells expressing GEF-H1-GFP is shown in panel a, e and I (green). Panel b, f and j displays the staining with an anti α-tubulin antibody (blue), while panel c, g and k show the antibody staining for anti 6xHIS (red). Panel d, h and l*

represent the merged image of all channels including the nuclear stain (grey). Co-transfected cells displayed the typical bundle-like structures of GEF-H1 in the cell periphery, except for cells co-transfected with the non-structural protein 3 (NS3). NS3 and GEF-H1 WT appeared to co-localise and a rather reticular distribution of GEF-H1 could be observed. Additionally, GEF-H1 WT appeared to change the distribution of NS3 as well compared to cells transfected with NS3 only. The co-localisation was quantified with the Pearson's coefficient and is indicated in the merged image.

After observing that wildtype GEF-H1 co-localised with the expressed viral protein NS3 we were interested in investigating the interaction of NS3 with the GEF-H1 mutants. For this, Vero cells were used to co-express NS3 with the three different GEF-H1 mutants C53R, ΔDH and S885A. Cells were stained with anti 6xHIS antibody and analysed via immunofluorescence microscopy (Figure 26). Surprisingly, the cellular distribution of all mutants seemed to be affected when cells co-expressed NS3. This included the C53R mutant which lacks the ability to bind to the microtubules and did not have an effect on the location and size of the MNV RC. Comparable to the co-expression of NS3 and wildtype GEF-H1, the morphology of the mutants was dispersed and diffuse. Co-expression of NS3 and the constitutively active mutant S885A appeared to have the lowest changes in morphology, including the formation of microtubule fibres. Those fibres were less thick and prominent than previously observed in the single expression of the protein, but were still inducible in contrast to the wildtype GEF-H1.

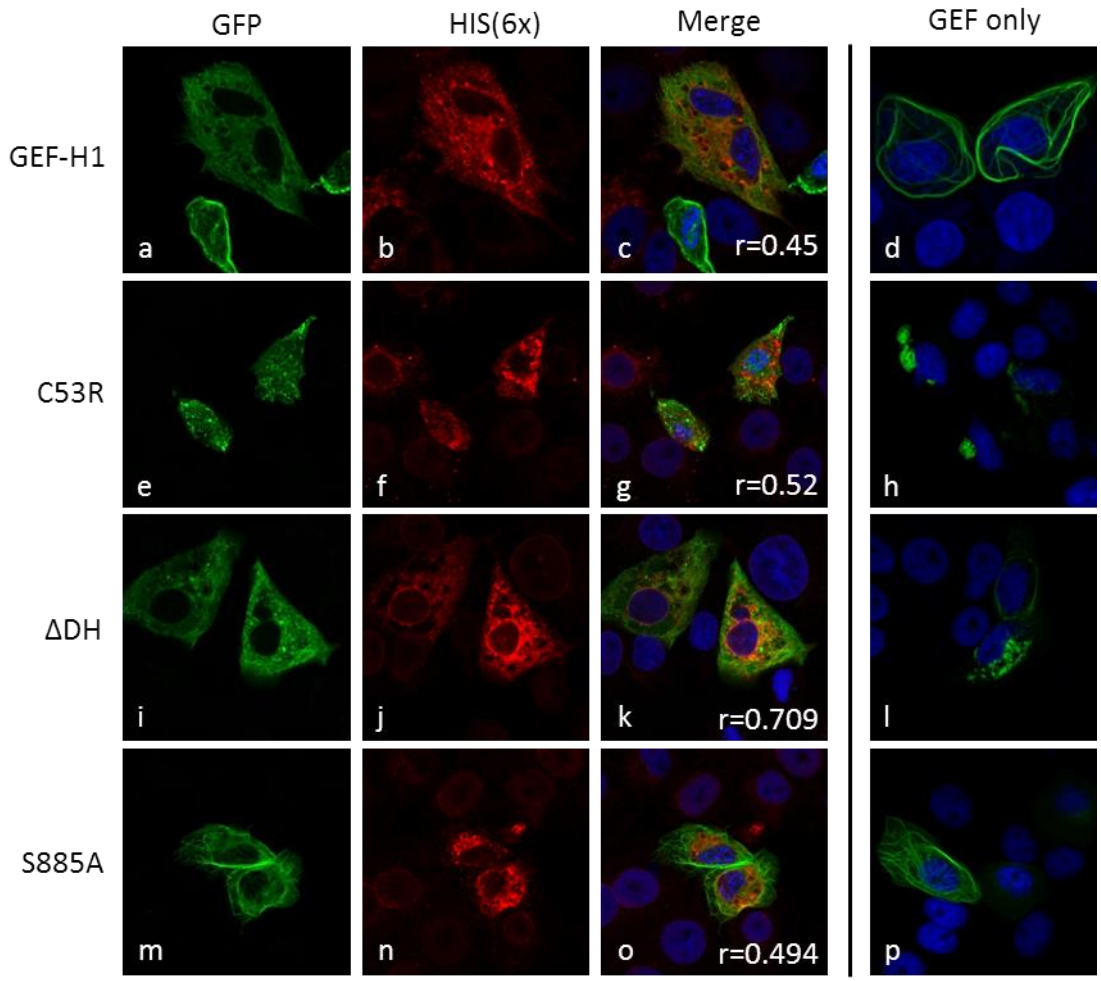


Figure 26 ***Expression of NS3 changes the location and morphology of the different GEF-H1 forms****. Vero cells were co-transfected with the HIS-tagged viral protein NS3 and the four different GEF-H1-GFP forms (WT, C53R, ΔDH, S885A). The GFP signal of the different GEF-H1 forms is displayed in panel a, e, i and m, while panels b, f , j and n show the staining with the anti 6xHIS antibody. Panel c, g, k and o show the merged image including the nuclear stain (DAPI). Panel d, h, l and p represent the merged image of GEF-H1-GFP transfected, but uninfected cells. Co-transfected cells displayed a dispersed morphology of GEF-H1 compared to cells transfected with the GEF-H1 forms only (panel d, h, l and p). The co-localisation was quantified with the Pearson's coefficient and is indicated in the merged image (panel c, g, k and o).*

5.2.5 The MNV protein NS3 directly interacts with GEF-H1

To further validate the observed co-localisation of NS3 and GEF-H1 in the immunofluorescence analysis, both proteins were co-expressed in 293T cells and analysed via co-precipitation to determine if these two proteins interacted directly. The expressed NS3 protein was precipitated by utilizing its 6xHIS tag and a HIS bead based assay. The precipitate was subsequently analysed

by immunoblotting and examined for the presence of NS3 (anti 6xHIS antibody) and GEF-H1 (anti-GEF-H1 antibody) (Figure 27). A 6xHIS positive signal corresponding to the size of NS3 (39 kDa) was detected in the precipitate of the lysate of NS3 only and NS3 and GEF-H1 expressing cells, confirming the successful precipitation of NS3. GEF-H1 could be detected in the lysate of GEF-H1 only and GEF-H1 and NS3 transfected cells, verifying the successful expression and co-expression of the protein. Analysing the NS3 precipitate, GEF-H1 could be detected in cells lysates co-expressing NS3. This indicates that GEF-H1 not only co-localises with NS3, but can also bind to it.

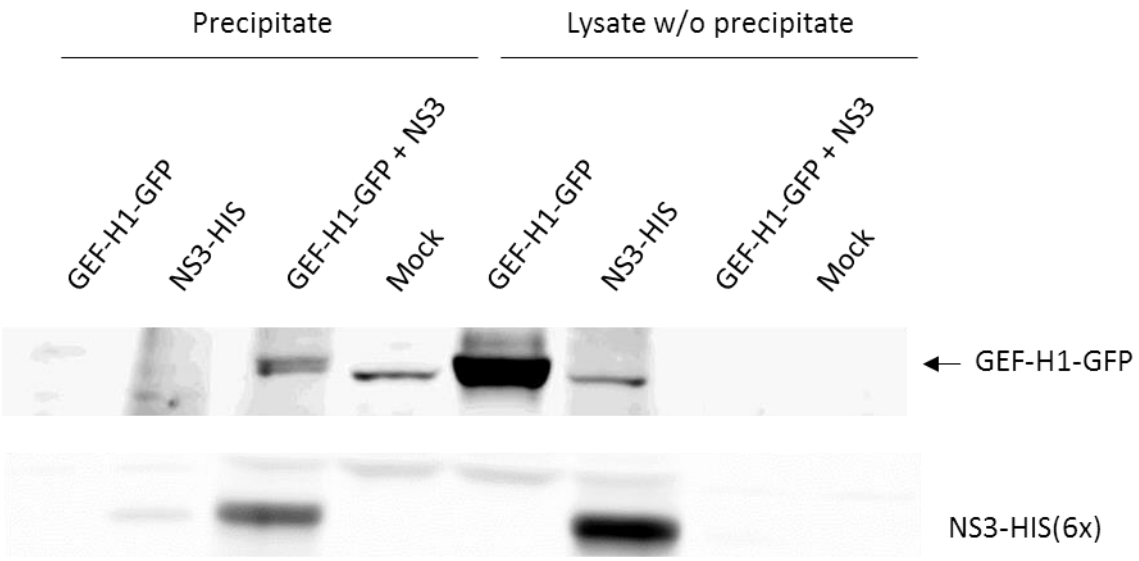


Figure 27 ***GEF-H1 WT associates with viral protein NS3.*** *For further verification of the interaction between NS3 and GEF-H1 WT, 293T cells were co-transfected with both proteins and cell lysates were analysed via immune precipitation. Lysates were incubated with cOmplete His-Tag Purification Resin to pull down the His-tagged NS3 and possible interaction partners. When cells were co-transfected with NS3 and GEF-H1 WT, GEF-H1 WT-GFP could be detected in the pull down with NS3. Precipitate: proteins bound to cOmplete His-Tag Purification Resin. Lysate: proteins which did not bind to the resin.*

During our co-expression studies, we not only observed that co-expression of NS3 and GEF-H1 lead to a distribution of GEF-H1, but we had also noticed an effect of NS3 on the overall expression level of GEF-H1. To test if NS3 was influencing the expression of GEF-H1, we co-expressed GEF-H1 with several MNV NS proteins, including NS3, and analysed these cells via flow cytometry. In

addition to the GFP tag of GEF-H1, we stained the cells with an anti 6xHIS antibody against the expressed MNV NS proteins. Cells were selected for a positive NS protein (anti 6xHIS) stain and then examined for their GEF-H1-GFP signal (Figure 28 A). There was no significant difference between cells positive for the NS proteins NS1-2, NS3 and NS5, with all of them having similar co-expression of GEF-H1-GFP in the co-transfected cells. We then investigated the level of GEF-H1-GFP expression and divided the GFP-positive population in LOW and HIGH expressing cells. HIGH GEF-H1-GFP expressing cells corresponded with microtubule fibre formation, emitting a strong and bright signal (Figure 28 B). Focusing on the amount of HIGH GEF-H1-GFP expressing cells in the NS protein positive population, we saw a significant decrease in the NS3 and GEF-H1-GFP co-expressing cells. Only 1.18 % of the NS3 and GEF-H1-GFP positive cells displayed a HIGH GEF-H1-GFP expression. We did not observe this decrease in the NS1-2 or NS5 transfected cells co-expressing GEF-H1-GFP. NS1-2 and NS5 positive cells co-expressing GEF-H1-GFP showed a similar percentage of about 20 % of HIGH GEF-H1-GFP expressing and microtubule fibre containing cells. These results suggest that NS3 does not completely inhibit the expression of GEF-H1 when co-expressed, but might interfere with the level of GEF-H1 being expressed, the activity of GEF-H1 and the formation of microtubule bundling. This correlates with our IFA in Figure 25 and Figure 26.

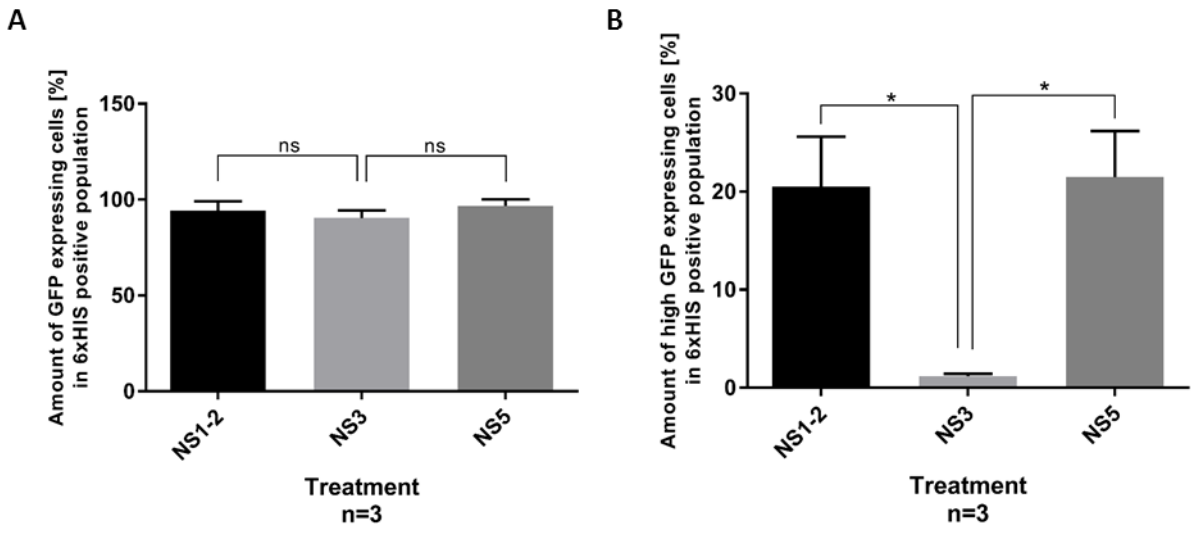


Figure 28 ***Co-expression with NS3 inhibits high expression of GEF-H1 WT.*** *293T cells were either transfected with GEF-H1-GFP WT or co-transfected with the NS viral proteins NS1-2, NS3, and NS5. Cells were fixed 18 h.p.t. and stained with anti-6xHIS antibody for flow cytometry analysis. Cells expressing a high amount of GFP (fluorescence intensity > 105) were selected as an indicator for GEF-H1 bundling. The percentage of* ***A*** *overall and* ***B*** *high GFP-expressers versus GFP-positive cells was calculated and is displayed in the graph above. Cells co-transfected with GEF-H1 WT and NS3 show a reduction in high GFP expressing cells compared to cells co-transfected with other NS proteins (n=3, average +/- SEM, ns:p>0.05, *p<0.05).*

5.2.6 Endogenous GEF-H1 and its interaction with MNV replication

Based on the interesting previous results focusing on the overexpression of GEF-H1, we aimed to investigate the interaction of endogenous GEF-H1 with MNV replication and its viral proteins. For this we obtained a GEF-H1 specific antibody and analysed the location and interaction of GEF-H1 during infection. Mouse macrophages were infected for 12h and 18h (MOI 5), fixed and subsequently immune-stained with anti GEF-H1 and anti NS4 antibodies (Figure 29). Uninfected cells displayed a cytoplasmic and microtubule-like staining pattern for GEF-H1 (Figure 29 a-d), indicating the association of GEF-H1 with microtubules but we did not observe thick fibre structures indicative of microtubule bundling. The MNV-infected cells displayed only minor changes in the morphology of GEF-H1 at 12h (Figure 29 e-h) or 18h (Figure 29 i-l) after infection without a clear induction of microtubule fibres. However, we did observe that GEF-H1 partially

co-localised with the MNV replication complex in the perinuclear region at both time points analysed.

This result suggests that GEF-H1 might be recruited or sequesters within the MNV replication complex. This redistribution could be attributed to either (i) a host response involved in the sensing of MNV and the activation of the antiviral immune response, (ii) be required for MNV replication, or (iii) be targeted by the virus to avoid immune detection and dampen the anti-viral response.

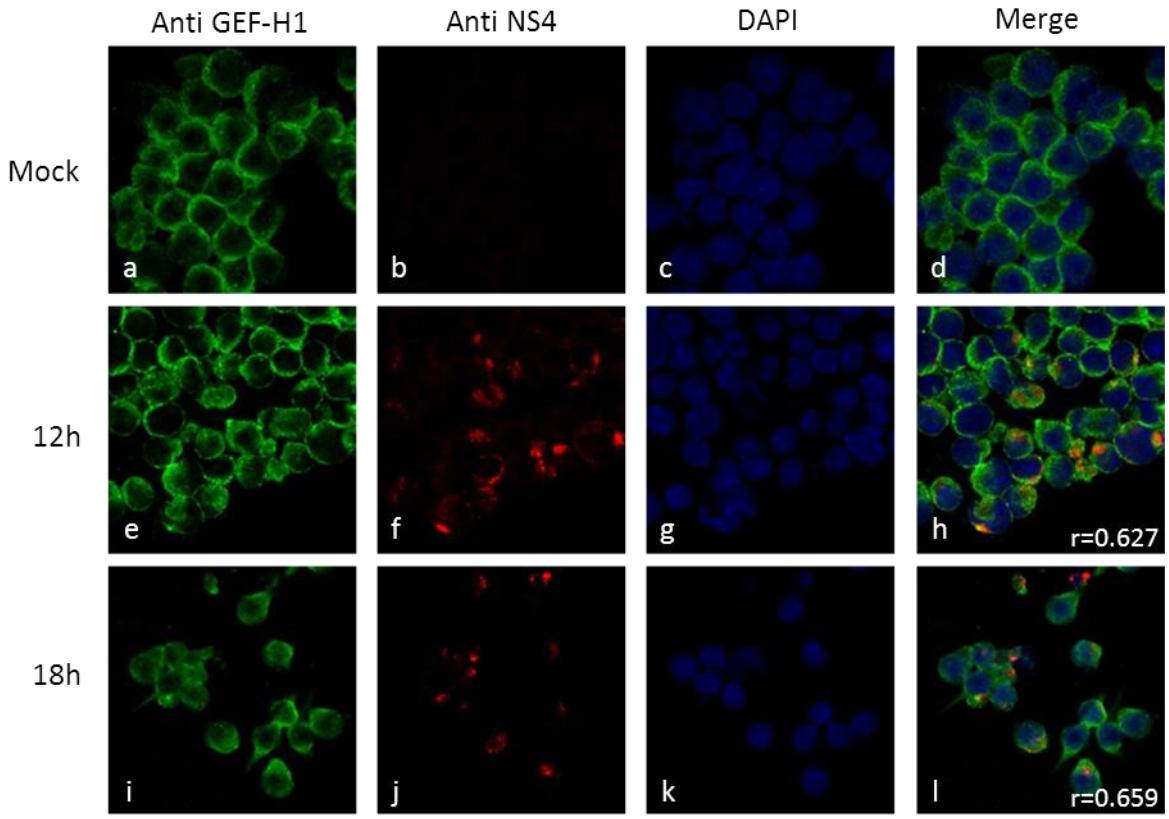
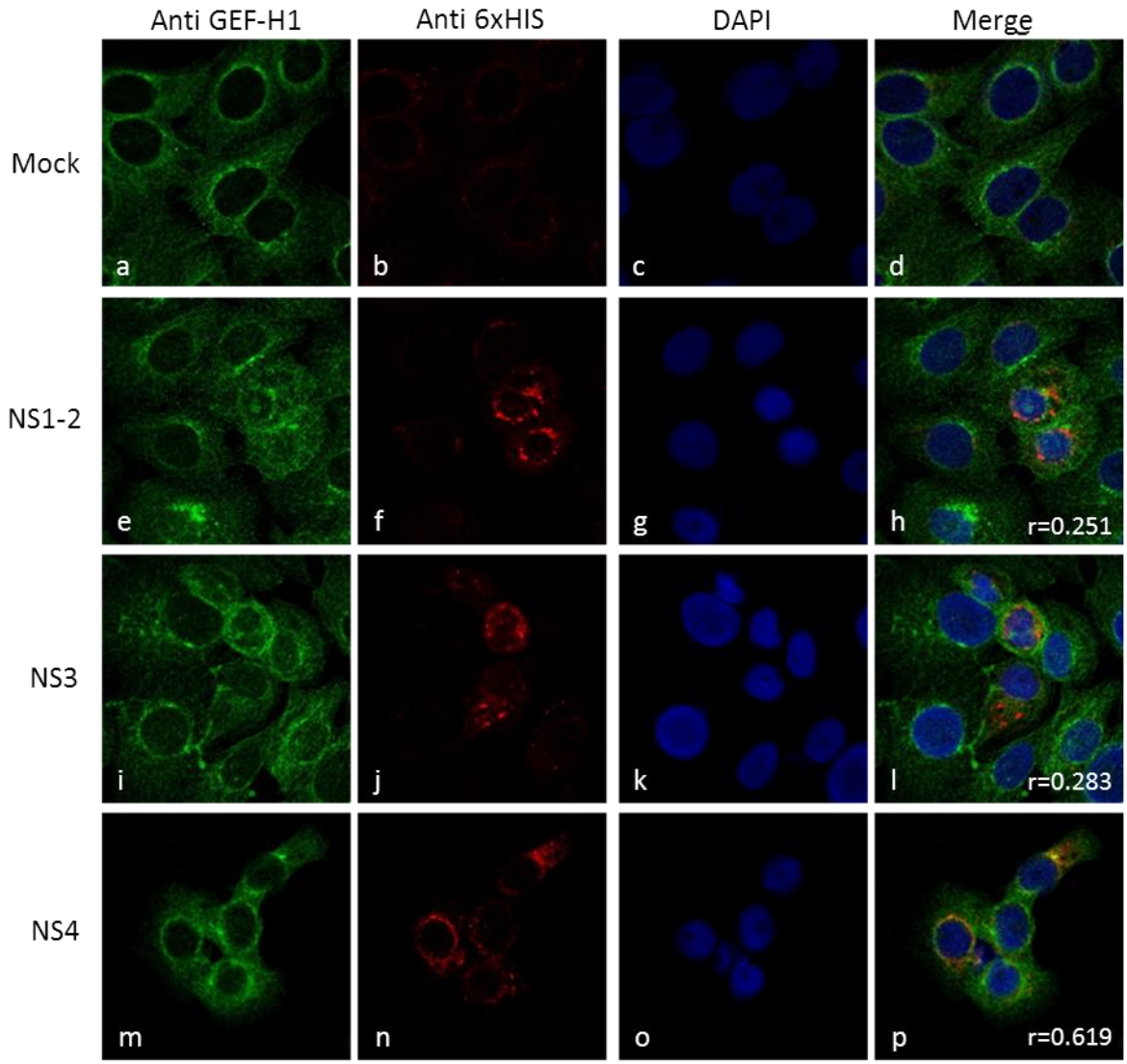


Figure 29 ***The replication complex (RC) of MNV co-localises with endogenous GEF-H1 in the perinuclear area.*** *RAW264.7 cells were infected with MNV (MOI 5) and fixed at 12 h and 18 h after infection. Panels a, e, i and m show the staining for the anti GEF-H1 antibody (green), panels b, f, j and n display the staining with an anti-NS4 antibody (red). Panel c, g, k and o represent the nuclear staining with DAPI (blue) and panels d, h, l and p show the merged pictured of all channels. Stained cells were analysed via confocal microscopy and the co-localisation was quantified with the Pearson's coefficient.*

After confirming the association of MNV and endogenous GEF-H1 during infection, we expressed the single MNV non-structural proteins and VP1 in Vero cells and investigated their interaction with endogenous GEF-H1 via immunofluorescence analysis (Figure 30). The MNV NS proteins were visualised through antibody staining with an anti-6xHIS antibody. Surprisingly, we did not detect a significant amount of co-localisation between endogenous GEF-H1 and NS3 (Figure 30i-l); an association we had previously observed during co-expression of both of this proteins (Figure 25e-h). Cells that expressed the viral proteins NS4 and NS1-2 showed some co-localisation or changes in the GEF-H1 morphology, respectively, which we had not observed in the co-expression of the NS proteins and GEF-H1-GFP.


Anti GEF-H1
Anti 6xHIS
DAPI
Merge
Mock
NS1-2
NS3
NS4
a
b
c
d
e
f
g
h
r=0.251
i
j
k
l
r=0.283
m
n
o
p
r=0.619

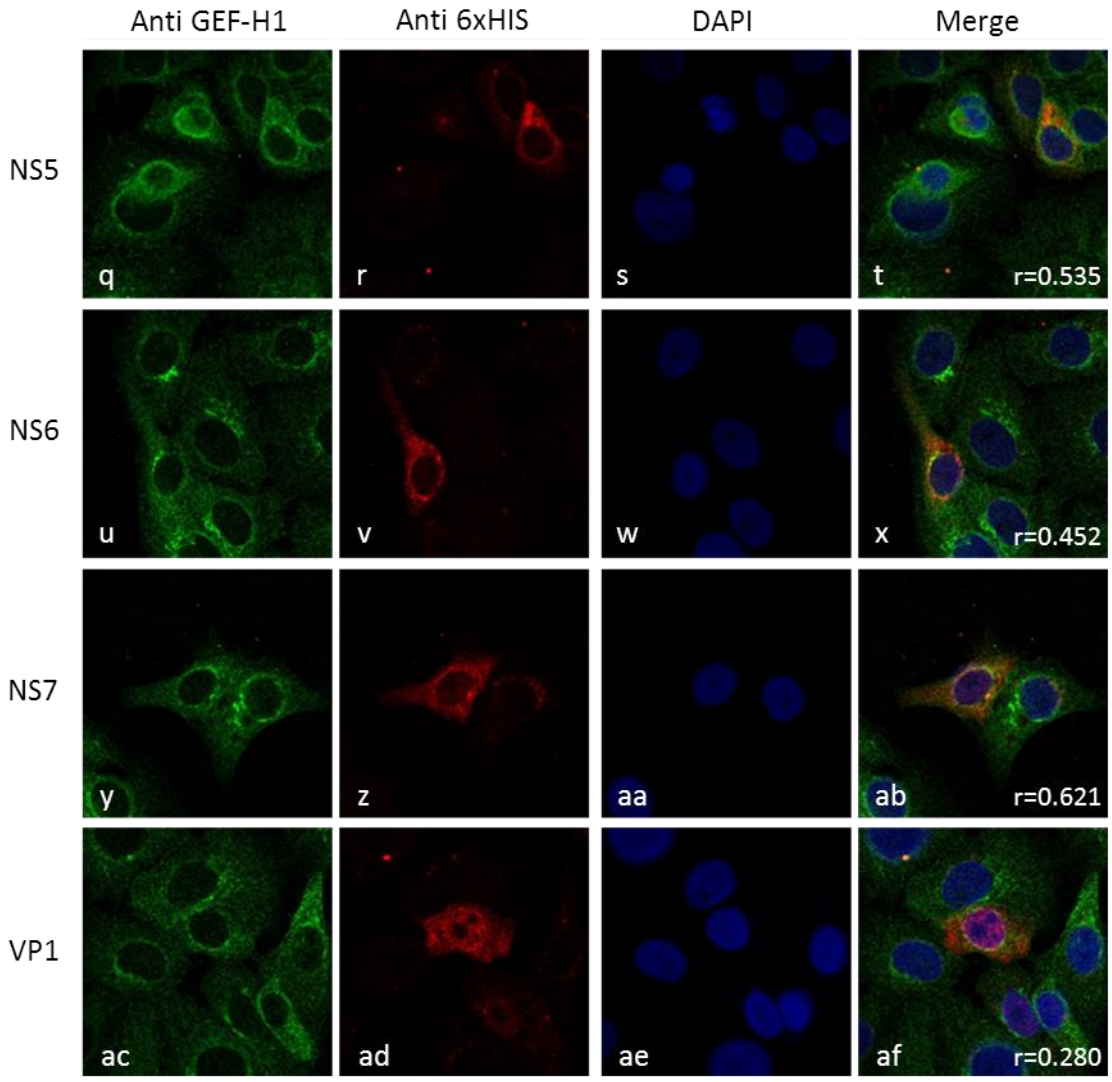


Figure 30 ***Some NS proteins change the cellular location of endogenous GEF-H1 and co-localise with it****. HeLa cells were transfected with the HIS-tagged viral proteins (NS proteins and VP1) and fixed 24h after transfection. Panel a, e, i, m, q, u, y and ac show the staining for the anti GEF-H1 antibody, panel b, f, j, n, r, v, z and ad display the staining with an anti 6xHIS antibody. Panel c, g, k, o, s, w, aa and ae represent the nuclear staining with DAPI and panel d, h, l, p, t, x, ab and af are showing the merged pictured of all channels. Stained cells were analysed via confocal microscopy and the co-localisation was quantified with the Pearson's coefficient.*

5.2.7 siRNA knockdown of endogenous GEF-H1 does not affect MNV replication complex formation or localisation

To disentangle the interaction of GEF-H1 and MNV and further investigate the function of GEF-H1 during MNV infection, we used siRNA to knock down endogenous GEF-H1 in murine

macrophages. Cells were treated with siRNA specific for GEF-H1 twice to ensure efficient knock down of the protein before they were infected with MNV (MOI 5) for 12h. The successful knock down of GEF-H1 was verified via immunoblotting and immunofluorescence (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Macrophages treated with GEF-H1 siRNA had a significantly lower signal for endogenous GEF-H1 compared to cells that were treated with control siRNA or left untreated. Even though the knockdown was robust in the immunoblot analysis, our IFA revealed a few cells still expressing GEF-H1 at a level comparable to control cells in the siRNA treated cells. If the number of these cells was less than 5% the knockdown was considered successful. Staining for the viral protein NS4 revealed that there was no significant difference in the amount of infected cells or the shape and location of the replication complex in GEF-H1 siRNA treated cells or control treated cells. These findings indicate that GEF-H1 is not essential or required for MNV replication and the lack of GEF-H1 did not seem to promote viral replication.

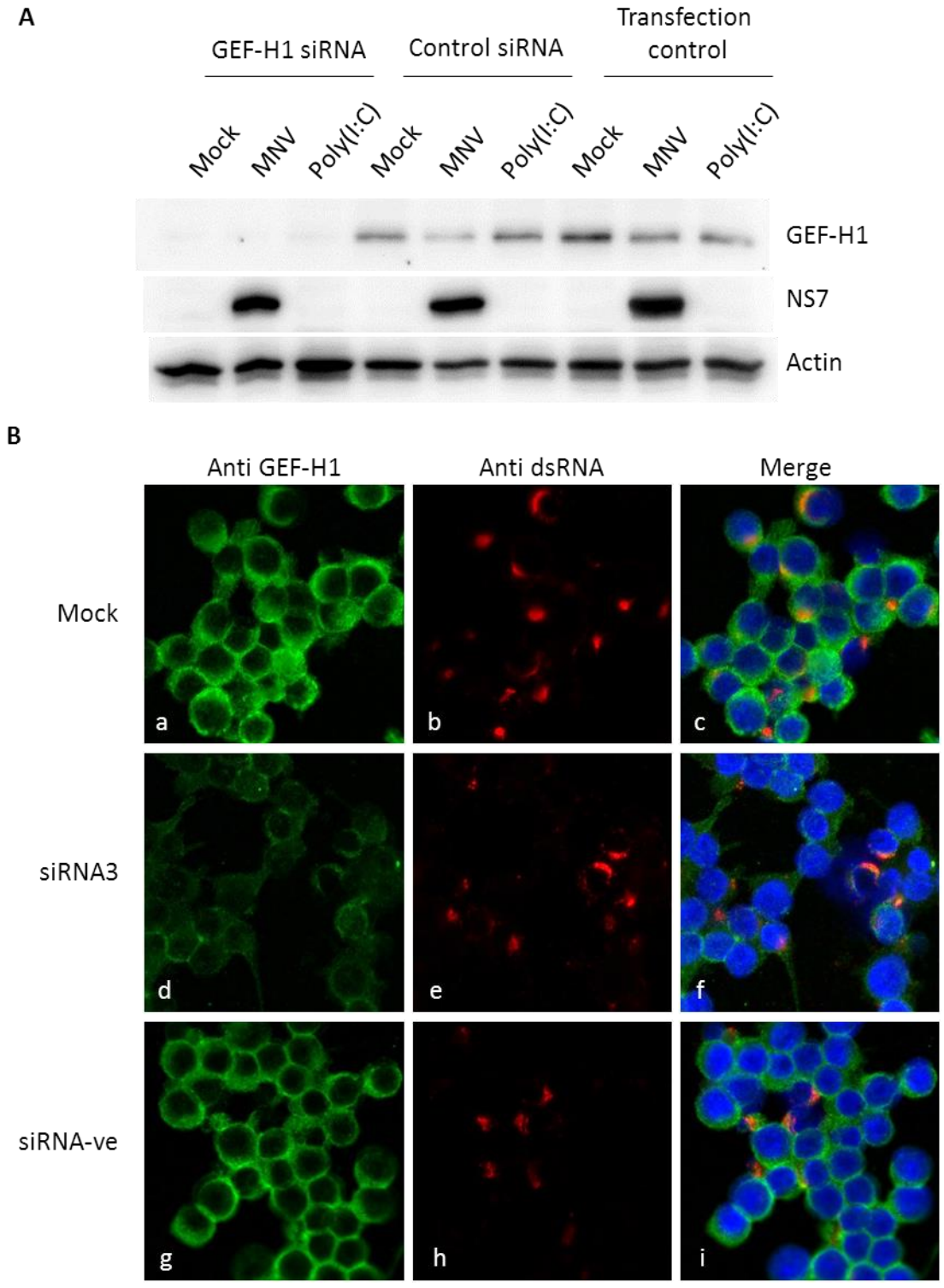


Figure 31 ***Treatment with siRNA3 (GEF-H1) leads to the knock down of endogenous GEF-H1 in RAW264.7 cells.*** *RAW264.7 cells were treated twice with GEF-H1 siRNA (siRNA3) or control siRNA (siRNA-ve), before cells were infected with MNV (MOI 5) for 12 h.* ***A*** *Immunoblot analysis of whole cell lysates stained with antibodies against GEF-H1, NS7 and actin.* ***B*** *Panel a, d, and g show the staining for the anti GEF-H1 antibody, panel b, e and h display the staining with an anti dsRNA antibody. Panel c, f and i represent the merged pictured of all channels including the nuclear stain (DAPI). Stained cells were analysed via confocal microscopy.*

5.2.8 siRNA knockdown of endogenous GEF-H1 does not affect MNV genome replication or infectious virus production

To extend the previous observation that GEF-H1 does not influence the formation or localisation of the viral replication complex, we determined if GEF-H1 played any other role during the MNV lifecycle. Thus, we enumerated the production of infectious virus as well as the amount of viral mRNA produced during replication in GEF-H1 knockdown cells compared to untreated cells. For thus murine macrophages were treated twice with GEF-H1 siRNA or control siRNA and infected with MNV for 12h. Supernatants and RNA samples were harvested and analysed via plaque assay and RT-qPCR, respectively (Figure 32). We observed no significant difference in viral titres between cells that were treated with GEF-H1 siRNA, control siRNA or left untreated (Figure 32 B). This indicates that GEF-H1 does not influence the assembly or release of progeny infectious MNV particles. A similar observation was made when we quantitated the amount of viral RNA generated during MNV infection (Figure 32 A). Again, there was no significant difference in the amount of viral RNA produced between cells treated with GEF-H1 siRNA, control siRNA or the untreated cells. Combined these results put into question the postulated critical role GEF-H1 plays in viral sensing.

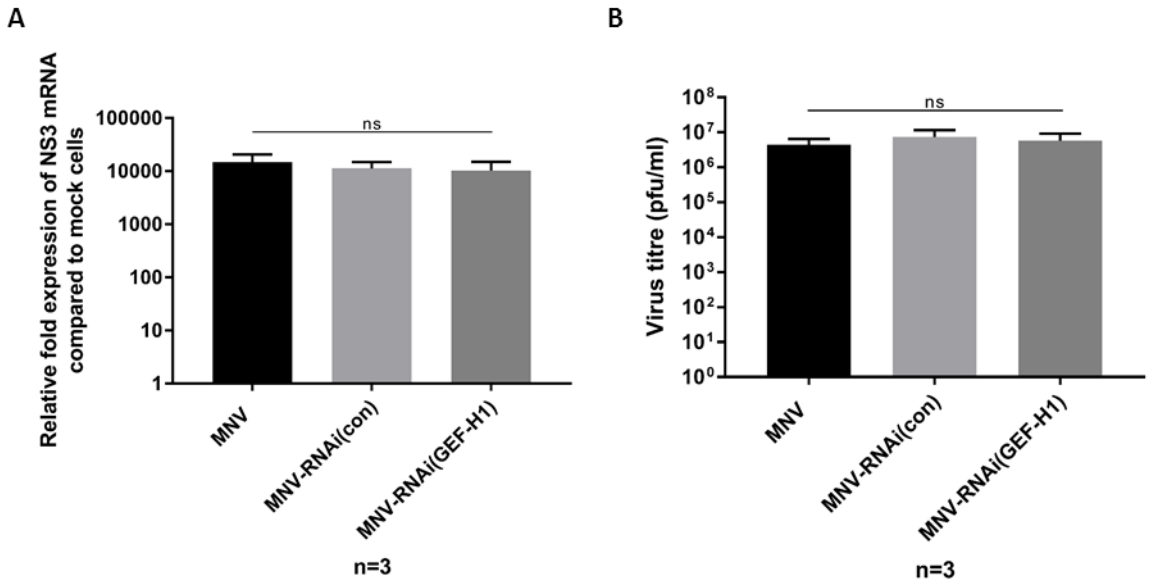


Figure 32 ***Knock down of endogenous GEF-H1 with siRNA3 in RAW264.7 cells does not affect MNV replication.*** *RAW264.7 cells were treated twice with GEF-H1 siRNA (siRNA3) or control siRNA (siRNA-ve), before cells were infected with MNV (MOI 5) for 12 h.* ***A*** *Cells were lysed and RNA was extracted. Relative fold expression of MNV mRNA compared to mock cells was analysed via RT-qPCR (n=3, average +/- SEM, ns:p>0.05).****B*** *The supernatant of the infected cells was collected and used to determine viral titres via plaque assay.*

5.2.9 Suppression of GEF-H1 expression does not affect the production of cytokines in response to Poly(I:C) stimulation or MNV infection

The knockdown of GEF-H1 in murine macrophages did not lead to a change in viral replication unlike previous studies performed with IAV (Chiang, Zhao *et al*. 2014). The role of GEF-H1 among others has been characterised as an adaptor protein to enhance host viral sensing via the RIG-I/MDA5 pathway. A downstream effect of this signalling pathway is the induction of type I interferon, mainly IFNβ. To test if the reduction of endogenous GEF-H1 in RAW264.7 cells affects the induction of IFNβ, we again treated cells with GEF-H1 siRNA, control siRNA or left them untreated, before infecting them with MNV for 12h or treating them with poly(I:C). Poly(I:C) is a dsRNA analogue and was used as a positive control to induce IFNβ transcription. After infection or stimulation RNA samples were harvested and analysed for the expression of IFNβ mRNA as

well as TNFα mRNA as a control via RT-qPCR (Figure 33). Poly(I:C) treated and MNV infected cells both showed the induction of IFNβ and TNFα compared to uninfected cells, but the amount of mRNA which was produced differed between both cytokines. In the case of IFNβ, poly(I:C) treatment generated lower amounts of IFNβ mRNA compared to MNV infected cells, whereas the levels of TNFα transcription did not differ significantly between MNV infected and poly(I:C) treated cells. The IFNβ mRNA levels did not differ significantly between GEF-H1 siRNA treated, control siRNA treated and untreated cells when cells were infected with MNV. This result suggests that GEF-H1 is not essential for the induction of type I IFN in MNV infected cells, because there was no reduction in IFNβ mRNA levels when GEF-H1 was knocked down. The induction of TNF is not directly linked to the dsRNA sensing pathway and was used as a control. Similar to IFNβ there was no change in TNFα mRNA levels between GEF-H1 knock down, or control treated cells, highlighting the fact that GEF-H1 is not needed for the expression of IFNβ mRNA.

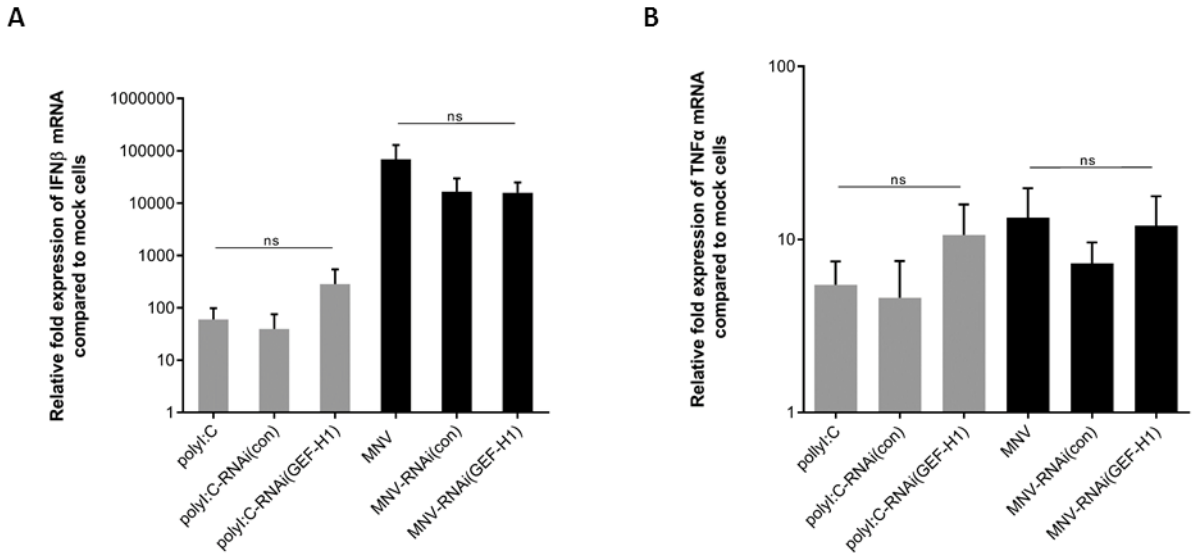


Figure 33 ***Knock down of endogenous GEF-H1 with siRNA3 in RAW264.7 cells does not lead to significant changes in the mRNA expression of IFNβ and TNFα.*** *RAW264.7 cells were treated twice with GEF-H1 siRNA (siRNA3) or control siRNA (siRNA-ve), before cells were either infected with MNV (MOI 5), treated with Poly(I:C) or left untreated for 12 h. Cells were lysed and the RNA was extracted. Relative fold expression of* ***A*** *IFNβ and* ***B*** *TNFα mRNA compared to mock cells was analysed via RT-qPCR (n=3, average +/- SEM, ns:p>0.05).*

The data we have obtained so far indicates that GEF-H1 is not essential for the induction of IFNβ during MNV infection and that a reduction in endogenous GEF-H1 does not promote nor restrict viral replication. These observations stand in stark contrast to studies on Influenza A virus highlighting the importance of GEF-H1 to induce type I IFN (Chiang, Zhao *et al*. 2014). That study also showed that the lack of GEF-H1 leads to higher viral titres and viral mRNA levels. To test our model and to verify the results presented in the above mentioned study, we repeated our GEF-H1 siRNA experiments with influenza strain X31, a mouse specific Influenza A virus strain, as well as MNV and West Nile Virus (WNV) as a control. Mouse macrophages were used to generate GEF-H1 knockdown cells via siRNA treatment. Knockdown as well as control cells were infected with MNV, X31 and WNV (all MOI 5) and harvested at the virus specific peak replication time points (12h, 18h and 21h post infection, respectively). Cells were harvested and analysed for the mRNA levels of IFNβ, TNFα and viral mRNA via RT-qPCR (Figure 34). We analysed the change in IFNβ, TNFα and viral mRNA induction by comparing infected siRNA treated cells (GEF-H1 siRNA and control siRNA) to infected but untreated cells. As expected, there was no significant change in the TNFα mRNA levels for any of the viruses when cells were treated with GEF-H1 or control siRNA, because GEF-H1 has so far not been described to play a role in the TNFα signalling pathway. Contradicting the studies on GEF-H1 and IAV, we were not able to detect a difference in IFNβ mRNA levels in GEF-H1 knockdown cells infected with X31 (Figure 34 A). Additionally, we did not observe an increase in X31 viral RNA in the GEF-H1 siRNA treated and X31-infected cells (Figure 34 C). Interestingly, GEF-H1 siRNA treated cells infected with WNV displayed significantly lower levels of IFNβ mRNA compared to untreated cells indicating a supporting effect of GEF-H1 in the IFNβ induction during WNV infection. However, this effect did not correlate with an increase in WNV mRNA. Surprisingly, GEF-H1 siRNA treated and MNV infected cells showed a significant

increase in the levels of viral mRNA compared to control siRNA treated cells. However, the overall increase was rather modest and most likely does not have a biological significance during replication.

Overall, we propose that suppression of GEF-H1 via siRNA did not contribute significantly to the induction of the innate immune response, particularly the induction of IFNβ, nor does it affect the replication kinetics of a range of RNA viruses including both positive-sense (*e.g.* MNV and WNV) and negative-sense viruses (*e.g.* influenza virus). We would thus argue against the postulated role of GEF-H1 in promoting the activation of the innate immune response during RNA virus infection.

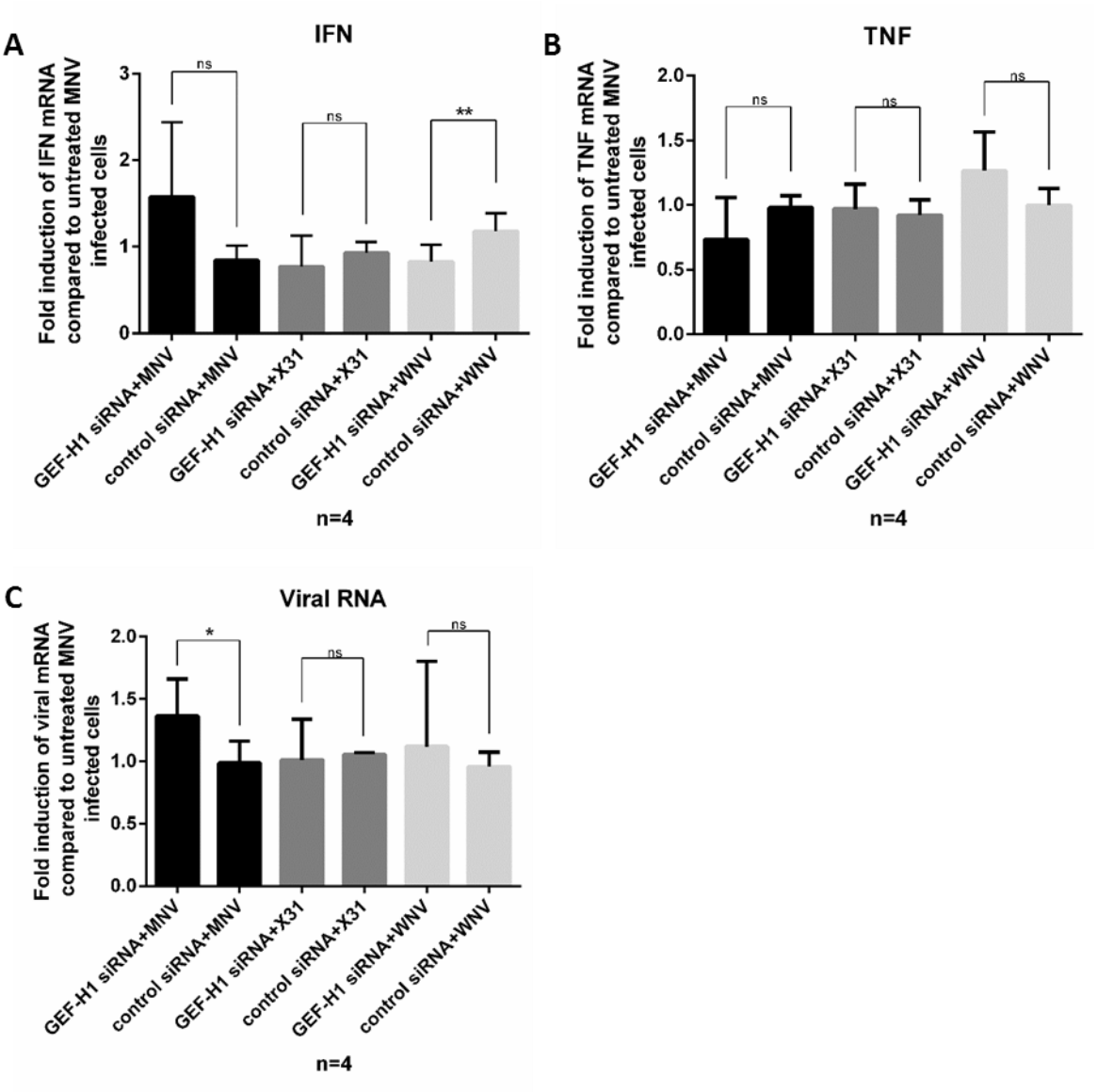


Figure 34 ***Knock down of endogenous GEF-H1 with siRNA in RAW264.7 cells leads to changes in the expression of IFNβ and viral mRNA in MNV and WNV infected cells.*** *RAW264.7 cells were treated twice with GEF-H1 siRNA, control siRNA or left untreated, before cells were either infected with MNV (MOI 5), X31 (MOI 5) or WNV (MOI 1). Cells were lysed and the RNA was extracted. Relative fold expression of* ***A*** *IFNβ and* ***B*** *TNFα* ***C*** *viral mRNA compared to infected but untreated cells was analysed via RT-qPCR (n=4, average +/- SEM, ns:p>0.05, *p<0.05).*

5.3 Discussion

In this Chapter, we aimed to determine the contribution of the host protein GEF-H1 during the replication cycle of MNV. We investigated GEF-H1 as it is a protein that is known to bind microtubules and to regulate and activate the innate immune response upon sensing of an RNA virus infection. Our initial studies focussed on over-expression of wild-type and mutant GEF-H1 proteins and their potential interaction with the MNV NS3 protein. We subsequently investigated the association of endogenous GEF-H1 with MNV and its contribution to innate immune regulation.

GEF-H1 and the MNV NS3 proteins have both been observed to associate with microtubules (Krendel, Zenke *et al*. 2002, Pathak and DerMardirossian 2013, Cotton, Hyde *et al*. 2016) thus we investigated whether these two proteins interacted with or affected the function of each other. During our transient transfection studies we observed that cells expressing GEF-H1 alone display a distinct staining for GEF-H1, which co-localises with the microtubule component tubulin and leads to the formation of microtubule bundles. However, co-expression of the MNV NS3 protein and GEF-H1 significantly affected the morphology and localisation of GEF-H1. GEF-H1 and NS3 co-expressing cells displayed a rather diffuse labelling for GEF-H1 without the formation of microtubule fibres. In addition, when co-expressed with NS3, GEF-H1 no longer appeared to co-localise to the microtubules, but displayed a more prominent partial co-localisation with NS3. The association of both proteins was confirmed via immune precipitation, suggesting a direct interaction between both of these proteins.

Although we have not explored the interaction between NS3 and GEF-H1 in great detail we hypothesise that NS3 could affect GEF-H1 via three different mechanisms (Figure 35). First of

all by direct association with GEF-H1, e.g. through binding and therefore inhibiting GEF-H1 or by changing the phosphorylation status of GEF-H1. GEF-H1 is postulated to be inactive and binds to microtubules in its phosphorylated state, whereas dephosphorylation leads to the release from the microtubules and the subsequent activation of GEF-H1 (Chiang, Zhao *et al*. 2014). The hypothesis of a direct interaction would be supported by the findings that co-expression of NS3 seemingly leads to the detachment of GEF-H1 from the microtubules and that GEF-H1 and NS3 co-localise and can be precipitated together. Another possibility is an indirect interaction of NS3 with GEF-H1 through their common binding partner, the microtubules, and more specifically tubulin. NS3 has been shown to partially co-localise with the microtubule marker β-tubulin and it's mobility in the cells is dependent on the microtubule network (Cotton, Hyde *et al*. 2016). As previously shown, GEF-H1 also co-localises with tubulin and could therefore be affected by changes in the microtubule network. Changes like that could be the result of another protein binding to tubulin, such as NS3. The expression of NS3 and subsequent binding of the protein to the microtubules could interfere with the binding of GEF-H1 to tubulin and indirectly release GEF-H1 from the microtubules, resulting in a similar phenotype as a direct interaction of both proteins would. However, this hypothesis is contested by the direct association of NS3 and GEF-H1 in the immunoprecipitation assay supporting a direct interaction. On the other hand, in the setting of an MNV infection, expression of GEF-H1 and its mutants lead to changes in the size and number of viral RCs, except for the C53R mutant, which is not able to bind to the microtubules. This indicates that removing the ability of GEF-H1 to bind to the microtubules and its potential mediator, abolishes the effect that GEF-H1 expression has on the MNV RC, changing the location and amount of MNV RCs. A third hypothesis to explain the findings of the effect of NS3 on GEF-H1 is the ability of NS3 to reduce the high expression of GEF-H1. NS3 may inhibit the ability of

GEF-H1 to induce microtubule fibres by limiting the amount of GEF-H1 produced in cells and thereby reduce the ability to bind to the cytoskeleton network. In this case GEF-H1 is not released from the microtubules, but fails to establish an interaction with tubulin and the formation of microtubule fibres from the start. The reduction of GEF-H1 expression could be either specific for GEF-H1 or an overall effect of NS3 on protein expression. The effect of MNV in general and NS3 in particular on protein expression is something we are currently investigating.

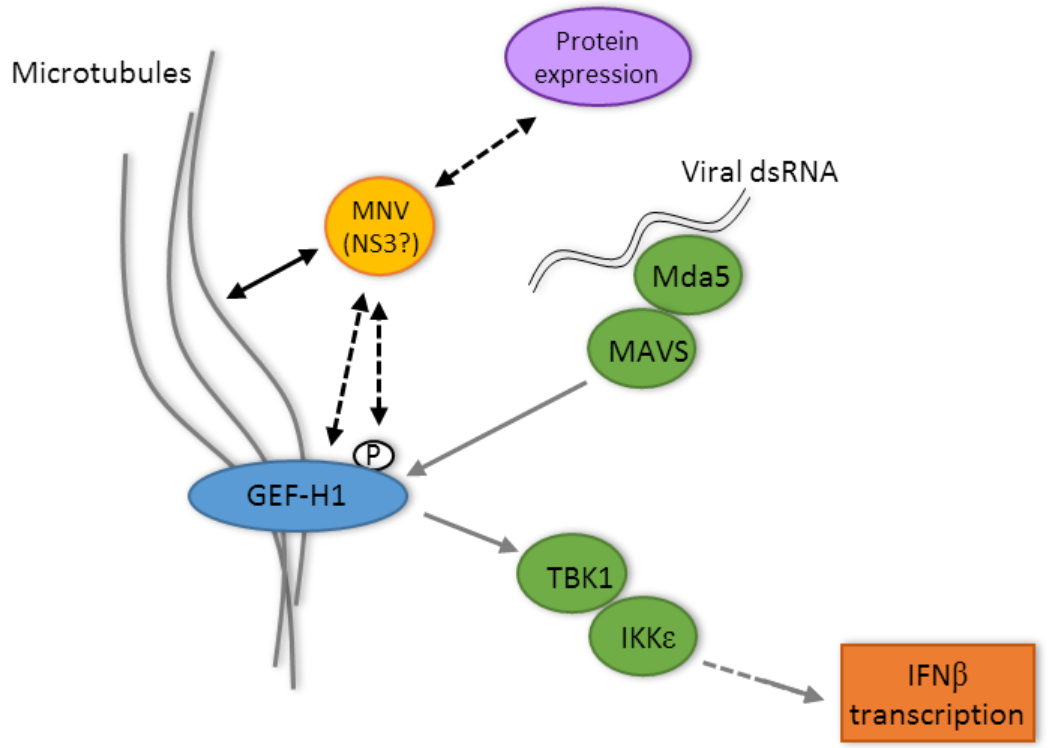


Figure 35 ***Schematic model of the potential interaction of MNV/NS3 and GEF-H1***

After we observed the drastic changes in the number and size of the MNV RC when GEF-H1 WT, S885A and ΔDH were overexpressed, we analysed the effect and interaction of endogenous GEF-H1 and MNV. Immunofluorescence analysis showed a partial association of endogenous GEF-H1 with the MNV RC in the perinuclear region close to the MTOC. Apart from

the co-localisation we did not observe any changes in the morphology of GEF-H1 during MNV infection. In the model proposed by Chiang *et al*, GEF-H1 aids in viral sensing once it has been released from the microtubules. This release is associated with the dephosphorylation of the protein and a reduction in microtubule binding. Based on this model, we expected to observe a change from the microtubule-like staining of GEF-H1 in the immunofluorescence microscopy analysis due to the activation of GEF-H1 during infection and its release from the microtubules. As we did not observe this change in localisation it indicates that either GEF-H1 is not playing a major role and not activated upon MNV infection, or GEF-H1 is blocked by the virus either through direct binding or by inhibiting the dephosphorylation of the protein. To further disentangle the role of GEF-H1 during MNV infection, GEF-H1 expression was suppressed via siRNA treatment in mouse macrophages. Cells treated with GEF-H1 specific siRNA displayed a marked reduction (~90%) in the expression of GEF-H1. However, knockdown of GEF-H1 did not reduce the level of IFNβ mRNA produced during an MNV infection, indicating that GEF-H1 was not essential for dsRNA sensing or activating the innate immune response during infection. Viral RNA as well as viral titres were used to investigate the ability of the virus to replicate within the cell in the presence or absence of GEF-H1. The lack of GEF-H1 did not increase viral replication, hinting on the fact again that GEF-H1 might not play a role in combatting MNV infection. Conversely, GEF-H1 siRNA treated cells did not show a reduction in viral replication either, illustrating that MNV replication is not dependent on the presence GEF-H1 to facilitate replication. All these observations indicate that the postulated role of GEF-H1 in the viral sensing pathway might either be specific for IAV or not as crucial as it has been hypothesised. To reconcile our results with the published observations, we tested two other viruses, X31 (IAV) and WNV, in our GEF-H1 siRNA knockdown model and similar to MNV we failed to see an effect of GEF-H1 on viral replication as

well as the induction of IFNβ mRNA. At this stage we cannot exclude the possibility that MNV interferes with GEF-H1 during infection and that the knockdown of GEF-H1 basically mimics an infection. To test if this hypothesis and to verify if the protein is inhibited by the virus, we can overexpress the constitutively active mutant S885A and analyse if MNV replication is affected. Despite various efforts of transfecting RAW264.7 cells with WT GEF-H1-GFP and GEF-H1-GFP mutants, we were unable to generate a cell population expressing GEF-H1-GFP in high enough numbers to make a robust assumption about the effect of the tested proteins in the context of MNV infection. Transfection efficiencies ranged from 1-12% with the majority of the cell population being untransfected. Therefore, we made use of the GFP marker in the transfected cells and sorted for GFP-positive cells via FACS. Unfortunately, low transfection efficiencies in combination with a cytotoxic effect of GEF-H1 overexpression and the sensitivity of RAW264.7 cells to cell sorting lead yields of GFP-positive cell which were not high enough to be analysed. To approach the transfection efficiency issue from a different angle, we generated Lentivirus plasmids with a pLenti6 backbone and the sequence for GEF-H1 WT and its three mutants (C53R, ΔDH, S885A), including a GFP tag. We were able to successfully produce Lentivirus particles including the sequence for GEF-H1-GFP in 293T cells. 293T and Vero cells were found positive for GFP-expressing cells 48h after Lentivirus particle exposure, but only minimal numbers of RAW264.7 were found to be GFP-positive (<1%). In conclusion, due to technical issues and time constraints we have so far not achieved to obtain a cell population, which is dominantly positive for GEF-H1, but we will continue to work on an efficient delivery system.

Overall, we have observed that the cellular microtubule associated protein GEF-H1 partially associates with the MNV RC. However, this association between GEF-H1 and MNV RNA

replication does not appear to be essential as siRNA treatment specific for GEF-H1 did not result in any decrease or increase in MNV replication. It is important to note that the siRNA treatment is not a complete deletion of the protein, which might have affected our results. Additionally, there might be other pathways compensating for the loss of GEF-H1 which lead to no observable changes in MNV replication. However, we did observe that the MNV NS3 protein can associate with GEF-H1 during transient over-expression of both these proteins. Intriguingly, NS3 could affect and negate the microtubule-bundling properties of GEF-H1 indicating that the MNV NS3 protein most likely affects microtubule dynamics, and supports previous studies from our lab on the connections between MNV, NS3 and microtubules (Hyde, Gillespie *et al*. 2012, Cotton, Hyde *et al*. 2016). Additionally, we have discounted the postulated role of GEF-H1 as an initiator of the innate immune response during RNA virus replication.

Chapter 6

General Discussion

HuNoVs cause sporadic local outbreaks of gastroenteritis and regular pandemics which affect millions of people worldwide, leading to a major burden on the health system and an estimated economic cost of about 60 billion dollars every year (Bartsch, Lopman *et al*. 2016). Recent outbreaks, especially in enclosed environments such as hospitals, childcare and aged care facilities, highlight the significance and impact of the virus. So far, no efficient antiviral drug or vaccine has been developed, which impedes the ability to control outbreaks and to protect the most vulnerable individuals (*i.e.* children, the elderly and the immunocompromised), within the population. This indicates the importance of ongoing NoV research to be able to acquire a better understanding of the interaction between the virus and the immune response of the host cell.

In this study, we investigated the role of the host immune response during MNV infections as a model to further our knowledge about NoVs. MHC class I proteins are a crucial component of the immune response against viruses and are responsible for the presentation of host and viral peptides on the cell surface. We have shown that MNV infection leads to the reduced surface expression of MHC class I proteins, which is caused by a reduction in intracellular levels of the protein (Chapter 3, Figure 10 and Figure 13). By manipulating the intracellular levels of MHC class I, the virus is targeting MHC class I proteins early in the antigen presentation pathway and reducing the risk of surface exposure of viral antigen. Time course experiments (Chapter 3, Figure 11) revealed that the reduction in MHC class I surface expression occurs as early as 7 h post infection, coinciding with the start of detectable translation of viral proteins. Further studies are needed to uncover if this observation is due to viral proteins being available to target MHC class I from that point onwards, or if the occurrence of viral proteins within the cell calls for a preventative measure to avoid viral peptides from being exposed on the cell surface.

We have identified that proteasomal degradation of MHC class I is a crucial contributor in the reduction of intracellular MHC class I levels, however the treatment of MNV infected cells with MG132 (Chapter 3, Figure 14) only lead to a partial rescue of the MHC class I surface expression. There are several explanations for this observation and each of them should to be addressed in future experiments. Apart from proteasome-mediated degradation, additional lysosome-mediated degradation could be the cause of reduced MHC class I levels. Treating cells with a combination of proteasome and lysosome inhibitors would be a great way to start disentangling the degradation pathways. Another explanation could be the dual role of the proteasome in the MHC class I pathway. On the one hand it can cause the degradation of MHC class I and reduce its intracellular levels, on the other hand it generates the vast pool of peptides which is needed to form the MHC class I and peptide complexes, before they are presented on the cell surface. The fact that MG132 treatment was able to partially restore MHC class I levels on the surface highlights not only the impact of the proteasome on MHC class I degradation, but also indicates the importance of alternative pathways which generate peptides. Alternative pathways which have been identified so far are the endo/phagocytic pathway and autophagy (Shen, Sigal *et al*. 2004, Tiwari, Garbi *et al*. 2007, English, Chemali *et al*. 2009). MNV has been shown to cause the induction of autophagosomes, even though these fail to mature in the presence of MNV (O'Donnell, Hyde *et al*. 2016). Last but not least, it needs to be considered if the partial rescue is caused by an indirect effect on protein levels. By blocking the function of the proteasome and degradation of most proteins, protein levels are either increasing due to continued synthesis or stabilised in the case of translation inhibition. In chapter 4 we have shown that MNV affects the translation of host proteins which most likely includes MHC class I proteins. Even though the addition of MG132 would not restore the synthesis of the proteins, it would help

to stabilise the existing levels of MHC class I proteins. The lack of continuous translation and synthesis of MHC class I proteins during the translational shutdown could also explain the partial but incomplete rescue of MHC class I surface expression following MG132 treatment. To uncover if MHC class I protein levels are decreased due to degradation or inhibition of translation, PKR KO cells could be utilised in future studies. Without PKR as the initiator of host translation inhibition, MHC class I translation should be unimpeded and changes in the intracellular and surface levels of the protein would be solely due to degradation.

The decrease in MHC class I levels seems to be caused by the MNV NS3 protein, which is essential and sufficient for the reduction of MHC class I on the cell surface. So far, little is known about the function of NS3 within the cells, but it has been identified as an NTPase. MNV NS3 could affect MHC class I proteins directly, or indirectly through the association with other host proteins. NS3 has been shown to interact with the microtubule network as well as the microtubule associated GEF, GEF-H1 (Chapter 5, Figure 23 and (Cotton, Hyde *et al*. 2016)). The microtubules form an important transport network within the cell which could be utilised by NS3 to cause the redistribution of MHC class I proteins to the lysosome or proteasome degradation pathway.

Having identified MHC class I proteins as a target of MNV NS3 raises the question if either NS3 or MHC class I can be manipulated to impede viral replication, lessen disease severity and inhibit spread. Unfortunately, the lack of information about NoV NS3 makes it difficult to speculate on the effectiveness of targeting NS3. Proteasomal inhibitors on the other hand are studied widely and are mostly used in cancer treatment, such as VELCADE® (Bortezomib) (Richardson, Hideshima *et al*. 2003, Adams and Kauffman 2004). Due to the multifunctional role of the proteasome, the inhibition of the complex could lead to severe side effects and renders a

proteasome inhibitor a less likely NoV antiviral candidate. Additionally, studies have shown that IFNγ is crucial for the induction of MHC class I synthesis and could be used to boost MHC class I expression (Blanar, Baldwin Jr *et al*. 1989, Driggers, Ennist *et al*. 1990). It has been tested in several autoimmune diseases, osteoporosis and cancer (Aulitzky, Gastl *et al*. 1989, Key Jr, Rodriguiz *et al*. 1995), but similar to proteasome inhibitors it bears side effects that might be more severe than the actual infection with MNV and would therefore be more detrimental than beneficial to the patient.

Even though the cause for the reduced levels of intracellular MHC class I remains to be fully elucidated, there is clear evidence that the reduced intracellular levels lead to a decreased surface expression of MHC class I. From a virus point of view, this is an excellent way to avoid immune detection and dampen the immune response. MHC class I antigens are usually recognised by CD8+ T cells. If no or only a few viral antigens are presented on the cell surface CD8+ T cells are inhibited in their cytotoxic activity against infected cells. This allows the virus to replicate unconstrained and facilitates spread. Although NoV infections are acute and are generally cleared within 24-48 h after the onset of symptoms, shedding of the virus has been reported to occur for up to several weeks. This indicates that a small reservoir of infected cells remains undetected and continues to generate viral particles, increasing infectivity and spread. The ability of the virus to remain in the host for several weeks might be associated with its ability to hide from the host immune response. Studies comparing acute strains of MNV with persistent strains have indicated that the CD8+ T cell response is reduced in persistent strains compared to acute strains (Tomov, Osborne *et al*. 2013). Considering that an acute strain has been used

throughout this study, it will be a crucial aim in future experiments to uncover a potential effect of the reduced MHC class I surface expression on the CD8+ T cell response.

CD8+ T cell activation leads to clonal expansion of viral antigen specific CD8+ T cells and the cytotoxic activity against viral antigen presenting cells. Additionally, a small amount of activated CD8+ T cells turns into CD8+ memory T cells, which are activated upon secondary exposure to the viral antigen and cause a stronger and faster response. Considering that a primary exposure to MNV and HuNoV does not necessarily lead to a prolonged secondary exposure protection, it is interesting to speculate that the reduced surface expression of MHC class I proteins might dampen the CD8+ T cell response and affect CD8+ T cell memory. If this hypothesis proves to be true, these findings could not only help understand the impact of the CD8+ T cell response in combatting and clearing infection, but could also have a major impact on vaccine development.

We further investigated the secretion of cytokines as a tool to analyse the efficiency and effectiveness of antiviral signalling during MNV infection. The release of cytokines such as type I IFN and TNFα is crucial to activate the innate immune response and reduce spread. Secreted cytokines can stimulate the infected cell through surface receptors to induce an antiviral response and alert surrounding cells. Depending on the cell type, surrounding cells either induce an antiviral state to protect themselves from the infection or become activated in the case of immune cells, like NK cells. We have shown that cytokine secretion is diminished in MNV infected cells despite an increased transcription of cytokine genes (Chapter 4, Figure 16 and Figure 18). This lack in cytokine secretion is not based on an inhibition of secretion, but due to reduced translation of host cell proteins (Chapter 4, Figure 19 and Figure 20).

The inability of MNV infected cells to release cytokines is highly detrimental for the host. Without the release of cytokines and other signalling molecules, surrounding cells cannot be alerted and the virus can replicate mostly unimpeded, at least during early stages of infection. NoV infections are usually associated with short incubation periods, which could be caused by the dampened immune response and impeded antiviral signalling. Even though the innate immune signalling is inhibited during the first round of replication, the host immune response is able to fight the infection and lead to the reduction of symptoms after only 24-48 h. The immune system seems be activated during the course of infection through other stress factors, like increased cell death, which is seems to be unimpeded by the virus.

We aimed to interrogate the effect of MNV on cytokine synthesis and secretion and uncovered that the lack in cytokine release is due to a shutdown of the host cell translation machinery. It is important to note that this is not only affecting cytokine translation and the subsequent secretion, but also other cellular proteins which are usually induced during viral infections. In addition to the reduced cytokine synthesis, we also observed a reduction in intracellular levels of MHC class I proteins in MNV infected cells (Chapter 3, Figure 13) and changes in abundance and location for GEF-H1 (Chapter 5, Figure 25 and Figure 28), which has been shown to promote and amplify the innate immune response to dsRNA within the cell. Both findings could be based on or connected to the reduction in host cell translation. It is important to mention that in both cases MNV NS3 seems to play a crucial role, raising the question if it might be involved in the inhibition of host cell translation. For future experiments, it would be interesting to investigate if separately expressed NS3 can induce the inhibition of host cell translation or if viral replication and the formation of dsRNA is needed to induce the shutdown.

Based on our observations so far, we hypothesise that the inhibition of host cell translation is initiated through the activation of PKR and the subsequent phosphorylation of eIF2α (Chapter 4, Figure 21). The activation of PKR is an antiviral response to infection which is induced by dsRNA, an intermediate of viral replication. In this case, activation of PKR and the subsequent inhibition of host cell translation would be an indirect effect of viral replication. Studies by Emmott *et al* propose another, more targeted kind of host translation inhibition through PABP. PABP is essential for binding of the poly-A tail of mRNAs and required for successful translation. They show that the viral protease MNV NS6 is able to cleave PABP and that expression of MNV NS6 leads to a 30% reduction in host translation (Emmott, Sorgeloos *et al*. 2017). Even though both observations, the activation of PKR and the cleavage of PABP, lead to the inhibition of host cell translation, it is critical to consider if both or only one of them is essential for the shutdown of host cell translation. From an evolutionary point of view, the additional targeting of PABP by MNV comes at a fitness cost that would only be beneficial for the virus if the activation of PKR alone is not sufficient for the shutdown of host cell translation. To be able to recover from PKR activation and subsequent phosphorylation of eIF2α as well as to induce the expression and translation of antiviral proteins during an infection, the host cell can activate GADD34. GADD34 activity is regulated through ATF4 and it can revert the phosphorylation of eIF2α (Clavarino, Cláudio *et al*. 2012). Since we were not able to detect the translation and secretion of antiviral proteins such as IFNβ and TNFα, we can speculate that either GADD34 is inhibited during MNV infection, or translation of these proteins is additionally inhibited by the cleavage of PABP through MNV NS6. We aim to investigate the role of GADD34 during MNV infection in future experiments to further clarify the effect of PKR/eIF2α and PABP/NS6 on host cell translation.

As previously mentioned, the inhibition of host cell translation is a known protective mechanism of infected cells to impede viral protein synthesis, which is dependent on host cell translation, and inhibit viral replication. This raises the question why MNV would either not counteract the activation of PKR, or specifically promote PABP cleavage, considering that viral replication is dependent on the host cell translation machinery. Unlike host cell mRNA, the 5'-end of MNV mRNA is non-capped and instead bound to the viral protein NS5. HuNoV VPg and MNV NS5 have both been shown to bind to the translation factors eIF4G and eIF4E (Daughenbaugh, Fraser *et al*. 2003, Daughenbaugh, Wobus *et al*. 2006) and studies on MNV NS5 have demonstrated a crucial role of the protein for viral translation and replication (Chung, Bailey *et al*. 2014). It has therefore been proposed that MNV proteins can be translated in a cap-independent manner and would be unimpeded by the inhibition of cap-dependent host mRNA translation. The ability of the virus to efficiently replicate while inhibiting the host cell translation and the expression of antiviral proteins is an efficient way to exploit the innate immune response to promote viral infection.

MNV is not only dependent on the cellular translation machinery, but also on a functional microtubule network to establish its RC (Hyde, Gillespie *et al*. 2012). Recent advances have shown that MNV NS3 is associated with the microtubules and uses the microtubule network to transport vesicles within the cell (Cotton, Hyde *et al*. 2016). Successful infection is dependent on a functional microtubule network and leads to the formation of thick microtubule bundles within the cell. The observed phenotype is strikingly similar to the expression of the protein GEF-H1, which is associated with the microtubules. Apart from regulatory functions within the cell such as vesicle transport and the regulation of tight junctions, GEF-H1 plays an important role in the

innate immune response against IAV (Chiang, Zhao *et al*. 2014). Without GEF-H1, IAV infection leads to a reduced induction of IFNβ through the RLR/MAVS pathway and higher levels of viral replication. Based on the phenotypic similarity between the microtubule network of MNV infected cells and GEF-H1 expressing cells, we investigated the effect of GEF-H1 on MNV infection. We observed an interaction between GEF-H1 and its mutants with MNV NS3. Both proteins co-localised with each other and affected each other's location within the cell (Chapter 5, Figure 25). Co-expression of NS3 and GEF-H1 also lead to a reduced fibre formation of the microtubules. This could be either due to an inhibition of GEF-H1 through NS3 or caused by a reduced expression of GEF-H1 in NS3 co-transfected cells.

The transport of NS3 positive vesicles has been shown to be dependent on the microtubule network and the fact that GEF-H1 is a regulator of vesicle trafficking makes it intriguing to speculate a possible interaction of GEF-H1 and NS3. This interaction might not affect its function in the innate immune response pathway, but might play a role in the trafficking of viral proteins and the establishment of the RC during MNV infection. This hypothesis is supported by the fact that the overexpression of GEF-H1 and mutants which are associated with the microtubules cause the redistribution of the MNV RC (Chapter 5, Figure 24). In general, it is also important to acknowledge the multifunctional role of a GEF. Its activation might lead to the activation of several GTPases within the cell which could have a positive or adverse effect on the outcome of infection, which needs to be addressed in future studies.

Despite the interaction of GEF-H1 and MNV NS3, we were unable to detect any changes during MNV infection when GEF-H1 expression was silenced via siRNA treatment (Chapter 5, Figure 32, Figure 33 and Figure 34). These observations raised the question if GEF-H1 was playing

a role during MNV infection at all or if its function would be inhibited by MNV. Even though we undertook numerous efforts to answer this question, we were not able to overcome the technical issues involved in the overexpression of GEF-H1 in RAW264.7 cells or any other MNV susceptible cell line (e.g. BMM, DC 2.4). Therefore we can only speculate about the role of GEF-H1 during MNV infection. A major downstream effect of GEF-H1 activity shown during IAV infection is the induction of IFNβ, but studies on IFNβ transcription in GEF-H1 knockdown cells revealed an uninhibited induction of the cytokine (Chapter 5, Figure 33 A). Additionally, we tested the immune response of GEF-H1 knockdown cells to IAV infection and in contrast to previous reports did not observe a detrimental effect on the induction of type I IFN (Figure 34). This would support the hypothesis that the role of GEF-H1 during MNV infection as well as IAV is not as crucial as it has been proposed. It is important to note here that the previous studies by Chiang *et al* have been conducted with the IAV strain A/PuertoRico/8/1934 (PR8), whereas the A/X-31 strain was used in this study. The only difference between both viruses is the HA and NA profile, with PR8 displaying H1N1 and X-31 displaying H3N2 on the cell surface. Nevertheless, this difference in IAV strains might explain the different effect of GEF-H1 on the innate immune sensing pathway.

In conclusion, this study enhanced the current knowledge about the host immune response to MNV infection and how the virus is manipulating the host cell to ensure efficient replication. MNV is able to replicate within the host cell by employing the hosts translation machinery. Whilst exploiting host cell translation MNV is capable of inhibiting the translation of host cell proteins, causing the inhibition of innate immune responses. All immune response proteins investigated in this study, cytokines, MHC class I proteins and GEF-H1, have been shown to either be reduced or ineffective during MNV infection. This highlights how efficiently the virus

evades the host immune response, even though it does not seem to entirely prevent the recognition through the immune system. Symptoms are reduced in healthy individuals after 24-48 h, which poses the questions how and when the innate immune response is able to interfere with and counteract the infection. Lastly, observations in this study have drawn attention to MNV NS3 which seems to be a very versatile and effective protein. Unfortunately, we know little about its function so far, emphasising the need to investigate its role and function further, to potentially be able to use NS3 as a target in antiviral strategies against NoVs.

Bibliography

Adams, J. and M. Kauffman (2004). "Development of the proteasome inhibitor Velcade™(Bortezomib)." Cancer investigation **22**(2): 304-311.

Ahn, K., A. Gruhler, B. Galocha, T. R. Jones, E. J. Wiertz, H. L. Ploegh, P. A. Peterson, Y. Yang and K. Früh (1997). "The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP." Immunity **6**(5): 613-621.

Ahn, K., T. H. Meyer, S. Uebel, P. Sempé, H. Djaballah, Y. Yang, P. A. Peterson, K. Frueh and R. Tampé (1996). "Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47." The EMBO Journal **15**(13): 3247.

Alexopoulou, L., A. C. Holt, R. Medzhitov and R. A. Flavell (2001). "Recognition of double-stranded RNA and activation of NF-[kappa]B by Toll-like receptor 3." Nature **413**(6857): 732-738.

Angulo, F. J., M. D. Kirk, I. McKay, G. V. Hall, C. B. Dalton, R. Stafford, L. Unicomb and J. Gregory (2008). "Foodborne disease in Australia: the OzFoodNet experience." Clinical Infectious Diseases **47**(3): 392-400.

Atmar, R. L., D. I. Bernstein, C. D. Harro, M. S. Al-Ibrahim, W. H. Chen, J. Ferreira, M. K. Estes, D. Y. Graham, A. R. Opekun and C. Richardson (2011). "Norovirus vaccine against experimental human Norwalk Virus illness." New England Journal of Medicine **365**(23): 2178-2187.

Atmar, R. L. and M. K. Estes (2012). "Norovirus vaccine development: next steps." Expert Review of Vaccines **11**(9): 1023-1025.

Au, W. C., P. A. Moore, W. Lowther, Y. T. Juang and P. M. Pitha (1995). "Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes." Proceedings of the National Academy of Sciences of the United States of America **92**(25): 11657-11661.

Aulitzky, W., G. Gastl, W. Aulitzky, M. Herold, J. Kemmler, B. Mull, J. Frick and C. Huber (1989). "Successful treatment of metastatic renal cell carcinoma with a biologically active dose of recombinant interferon-gamma." Journal of Clinical Oncology **7**(12): 1875-1884.

Bailey, D., I. Karakasiliotis, S. Vashist, L. M. W. Chung, J. Reese, N. McFadden, A. Benson, F. Yarovinsky, P. Simmonds and I. Goodfellow (2010). "Functional analysis of RNA structures present at the 3' extremity of the murine norovirus genome: the variable polypyrimidine tract plays a role in viral virulence." Journal of virology **84**(6): 2859-2870.

Barcellini, W., G. P. Rizzardi, J. B. Marriott, C. Fain, R. J. Shattock, P. L. Meroni, G. Poli and A. G. Dalgleish (1996). "Interleukin-10-induced HIV-1 expression is mediated by induction of both membrane-bound tumour necrosis factor (TNF)-alpha and TNF receptor type 1 in a promonocytic cell line." AIDS (London, England) **10**(8): 835-842.

Bargatze, R., P. Mendelman, A. Borkowski, J. Cramer, R. Goodwin and F. Baehner (2016). "Clinical Development of the Takeda Norovirus Virus-Like Particles Vaccine Candidate." Open Forum Infectious Diseases **3**(suppl 1).

Barker, J., I. Vipond and S. Bloomfield (2004). "Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces." Journal of Hospital Infection **58**(1): 42-49.

Bartee, E., M. Mansouri, B. T. H. Nerenberg, K. Gouveia and K. Früh (2004). "Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins." Journal of virology **78**(3): 1109-1120.

Bartsch, S. M., B. A. Lopman, S. Ozawa, A. J. Hall and B. Y. Lee (2016). "Global Economic Burden of Norovirus Gastroenteritis." PLoS ONE **11**(4): e0151219.

Belov, G. A. and F. J. van Kuppeveld (2012). "(+) RNA viruses rewire cellular pathways to build replication organelles." Current opinion in virology **2**(6): 740-747.

Bernstein, D. I. (2009). "Rotavirus overview." The Pediatric infectious disease journal **28**(3): S50-S53.

Bertolino, P. and C. Rabourdin-Combe (1995). "The MHC class II-associated invariant chain: a molecule with multiple roles in MHC class II biosynthesis and antigen presentation to CD4+ T cells." Critical reviews in immunology **16**(4): 359-379.

Bertolotti-Ciarlet, A., S. E. Crawford, A. M. Hutson and M. K. Estes (2003). "The 3' End of Norwalk Virus mRNA Contains Determinants That Regulate the Expression and Stability of the Viral Capsid Protein VP1: a Novel Function for the VP2 Protein." Journal of Virology **77**(21): 11603-11615.

Biron, C. A. (1997). "Activation and function of natural killer cell responses during viral infections." Current Opinion in Immunology **9**(1): 24-34.

Biron, C. A. (1998). Role of early cytokines, including alpha and beta interferons (IFN-α\ β), in innate and adaptive immune responses to viral infections. Seminars in immunology, Elsevier.

Blanar, M., A. Baldwin Jr, R. Flavell and P. Sharp (1989). "A gamma-interferon-induced factor that binds the interferon response sequence of the MHC class I gene, H-2Kb." The EMBO journal **8**(4): 1139.

Bok , K. and K. Y. Green (2012). "Norovirus Gastroenteritis in Immunocompromised Patients." New England Journal of Medicine **367**(22): 2126-2132.

Boname, J. M. and P. G. Stevenson (2001). "MHC class I ubiquitination by a viral PHD/LAP finger protein." Immunity **15**(4): 627-636.

Cahalan, M. D. and K. G. Chandy (2009). "The functional network of ion channels in T lymphocytes." Immunological Reviews **231**(1): 59-87.

Callow, M. G., S. Zozulya, M. L. Gishizky, B. Jallal and T. Smeal (2005). "PAK4 mediates morphological changes through the regulation of GEF-H1." Journal of Cell Science **118**(9): 1861-1872.

Chang, K.-O. and D. W. George (2007). "Interferons and Ribavirin Effectively Inhibit Norwalk Virus Replication in Replicon-Bearing Cells." Journal of Virology **81**(22): 12111-12118.

Chang, Y.-C., P. Nalbant, J. Birkenfeld, Z.-F. Chang and G. M. Bokoch (2008). "GEF-H1 Couples Nocodazole-induced Microtubule Disassembly to Cell Contractility via RhoA." Molecular Biology of the Cell **19**(5): 2147-2153.

Changotra, H., Y. Jia, T. N. Moore, G. Liu, S. M. Kahan, S. V. Sosnovtsev and S. M. Karst (2009). "Type I and type II interferons inhibit the translation of murine norovirus proteins." Journal of virology **83**(11): 5683-5692.

Chaudhry, Y., A. Nayak, M.-E. Bordeleau, J. Tanaka, J. Pelletier, G. J. Belsham, L. O. Roberts and I. G. Goodfellow (2006). "Caliciviruses differ in their functional requirements for eIF4F components." Journal of Biological Chemistry **281**(35): 25315-25325.

Chen, G. and D. V. Goeddel (2002). "TNF-R1 Signaling: A Beautiful Pathway." Science **296**(5573): 1634-1635.

Chiang, H.-S., Y. Zhao, J.-H. Song, S. Liu, N. Wang, C. Terhorst, A. H. Sharpe, M. Basavappa, K. L. Jeffrey and H.-C. Reinecker (2014). "GEF-H1 controls microtubule-dependent sensing of nucleic acids for antiviral host defenses." Nature immunology **15**(1): 63-71.

Chung, L., D. Bailey, E. N. Leen, E. P. Emmott, Y. Chaudhry, L. O. Roberts, S. Curry, N. Locker and I. G. Goodfellow (2014). "Norovirus translation requires an interaction between the C terminus of the genome-linked viral protein VPg and eukaryotic translation initiation factor 4G." Journal of Biological Chemistry **289**(31): 21738-21750.

Clavarino, G., N. Cláudio, T. Couderc, A. Dalet, D. Judith, V. Camosseto, E. K. Schmidt, T. Wenger, M. Lecuit, E. Gatti and P. Pierre (2012). "Induction of GADD34 Is Necessary for dsRNA-Dependent Interferon-β Production and Participates in the Control of Chikungunya Virus Infection." PLoS Pathogens **8**(5): 1-17.

Clemens, M. J. (1997). "PKR—A protein kinase regulated by double-stranded RNA." The International Journal of Biochemistry & Cell Biology **29**(7): 945-949.

Collart, M. A., P. Baeuerle and P. Vassalli (1990). "Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B." Molecular and Cellular Biology **10**(4): 1498-1506.

Control, C. f. D. and Prevention (2013). "Emergence of new norovirus strain GII. 4 Sydney--United States, 2012." MMWR. Morbidity and mortality weekly report **62**(3): 55.

Corr, M., A. E. Slanetz, L. F. Boyd, M. T. Jelonek, S. Khilko, B. K. Al-Ramadi, Y. S. Kim, S. E. Maher, A. L. Bothwell and D. H. Margulies (1994). "T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity." SCIENCE-NEW YORK THEN WASHINGTON-: 946-946.

Cotton, B., J. L. Hyde, S. T. Sarvestani, S. V. Sosnovtsev, K. Y. Green, P. A. White and J. M. Mackenzie (2016). "The Norovirus NS3 protein is a dynamic lipid- and microtubule-associated protein involved in viral RNA replication." Journal of Virology.

Cousens, L. P., J. S. Orange, H. C. Su and C. A. Biron (1997). "Interferon-α/β inhibition of interleukin 12 and interferon-γ production in vitro and endogenously during viral infection." Proceedings of the National Academy of Sciences **94**(2): 634-639.

Daughenbaugh, K. F., C. S. Fraser, J. W. Hershey and M. E. Hardy (2003). "The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment." The EMBO journal **22**(11): 2852-2859.

Daughenbaugh, K. F., C. E. Wobus and M. E. Hardy (2006). "VPg of murine norovirus binds translation initiation factors in infected cells." Virology journal **3**(1): 1.

De Gassart, A., V. Camosseto, J. Thibodeau, M. Ceppi, N. Catalan, P. Pierre and E. Gatti (2008). "MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation." Proceedings of the National Academy of Sciences **105**(9): 3491-3496.

de Graaf, M., J. van Beek and M. P. G. Koopmans (2016). "Human norovirus transmission and evolution in a changing world." Nat Rev Micro **14**(7): 421-433.

Decker, T., M. L. Lohmann-Matthes and G. E. Gifford (1987). "Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages." The Journal of Immunology **138**(3): 957-962.

Deitz, S. B., D. A. Dodd, S. Cooper, P. Parham and K. Kirkegaard (2000). "MHC I-dependent antigen presentation is inhibited by poliovirus protein 3A." Proceedings of the National Academy of Sciences **97**(25): 13790-13795.

Der, S. D., A. Zhou, B. R. G. Williams and R. H. Silverman (1998). "Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays." Proceedings of the National Academy of Sciences **95**(26): 15623-15628.

DeWaal, C. S. and F. Bhuiya (2007). Outbreaks by the numbers: fruits and vegetables 1990–2005. Proceedings of 2007 Annual Meeting of the International Association for Food Protection, Lake Buena Vista, FL, USA.

Dodd, D. A., T. H. Giddings and K. Kirkegaard (2001). "Poliovirus 3A protein limits interleukin-6 (IL-6), IL-8, and beta interferon secretion during viral infection." Journal of virology **75**(17): 8158-8165.

Doedens, J. R., T. H. Giddings and K. Kirkegaard (1997). "Inhibition of endoplasmic reticulum-to-Golgi traffic by poliovirus protein 3A: genetic and ultrastructural analysis." Journal of virology **71**(12): 9054-9064.

Doedens, J. R. and K. Kirkegaard (1995). "Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A." The EMBO journal **14**(5): 894.

Dong, B. and R. H. Silverman (1999). "Alternative function of a protein kinase homology domain in 2', 5'-oligoadenylate dependent RNase L." Nucleic acids research **27**(2): 439-445.

Driggers, P. H., D. L. Ennist, S. L. Gleason, W.-H. Mak, M. S. Marks, B.-Z. Levi, J. R. Flanagan, E. Appella and K. Ozato (1990). "An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes." Proceedings of the National Academy of Sciences **87**(10): 3743-3747.

Eden, J.-S., R. A. Bull, E. Tu, C. J. McIver, M. J. Lyon, J. A. Marshall, D. W. Smith, J. Musto, W. D. Rawlinson and P. A. White (2010). "Norovirus GII. 4 variant 2006b caused epidemics of acute gastroenteritis in Australia during 2007 and 2008." Journal of Clinical Virology **49**(4): 265-271.

El-Kamary, S. S., M. F. Pasetti, P. M. Mendelman, S. E. Frey, D. I. Bernstein, J. J. Treanor, J. Ferreira, W. H. Chen, R. Sublett and C. Richardson (2010). "Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues." Journal of Infectious Diseases **202**(11): 1649-1658.

Eltahla, A. A., K. L. Lim, J.-S. Eden, A. G. Kelly, J. M. Mackenzie and P. A. White (2014). "Nonnucleoside Inhibitors of Norovirus RNA Polymerase: Scaffolds for Rational Drug Design." Antimicrobial Agents and Chemotherapy **58**(6): 3115-3123.

Emmott, E., F. Sorgeloos, S. L. Caddy, S. Vashist, S. Sosnovtsev, R. Lloyd, K. Heesom, N. Locker and I. Goodfellow (2017). "Norovirus-Mediated Modification of the Translational Landscape via Virus

and Host-Induced Cleavage of Translation Initiation Factors." Molecular & Cellular Proteomics **16**(4 suppl 1): S215-S229.

English, L., M. Chemali, J. Duron, C. Rondeau, A. Laplante, D. Gingras, D. Alexander, D. Leib, C. Norbury and R. Lippé (2009). "Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection." Nature immunology **10**(5): 480-487.

Etchison, D., S. Milburn, I. Edery, N. Sonenberg and J. Hershey (1982). "Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex." Journal of Biological Chemistry **257**(24): 14806-14810.

Ettayebi, K., S. E. Crawford, K. Murakami, J. R. Broughman, U. Karandikar, V. R. Tenge, F. H. Neill, S. E. Blutt, X.-L. Zeng and L. Qu (2016). "Replication of human noroviruses in stem cell–derived human enteroids." Science **353**(6306): 1387-1393.

Fang, H., M. Tan, M. Xia, L. Wang and X. Jiang (2013). "Norovirus P particle efficiently elicits innate, humoral and cellular immunity." PloS one **8**(4): e63269.

Fankhauser, R. L., S. S. Monroe, J. S. Noel, C. D. Humphrey, J. S. Bresee, U. D. Parashar, T. Ando and R. I. Glass (2002). "Epidemiologic and Molecular Trends of “Norwalk-like Viruses” Associated with Outbreaks of Gastroenteritis in the United States." Journal of Infectious Diseases **186**(1): 1-7.

Farrell, H., M. Degli-Esposti, E. Densley, E. Cretney, M. Smyth and N. Davis-Poynter (2000). "Cytomegalovirus MHC class I homologues and natural killer cells: an overview." Microbes and infection **2**(5): 521-532.

Fernandez-Sesma, A., S. Marukian, B. J. Ebersole, D. Kaminski, M.-S. Park, T. Yuen, S. C. Sealfon, A. García-Sastre and T. M. Moran (2006). "Influenza virus evades innate and adaptive immunity via the NS1 protein." Journal of virology **80**(13): 6295-6304.

Fernandez-Vega, V., S. V. Sosnovtsev, G. Belliot, A. D. King, T. Mitra, A. Gorbalenya and K. Y. Green (2004). "Norwalk virus N-terminal nonstructural protein is associated with disassembly of the Golgi complex in transfected cells." Journal of virology **78**(9): 4827-4837.

Fitzgerald, K. A. (2011). "The interferon inducible gene: Viperin." Journal of Interferon & Cytokine Research **31**(1): 131-135.

Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S.-M. Liao and T. Maniatis (2003). "IKK[epsi] and TBK1 are essential components of the IRF3 signaling pathway." Nat Immunol **4**(5): 491-496.

Fukazawa, A., C. Alonso, K. Kurachi, S. Gupta, C. F. Lesser, B. A. McCormick and H.-C. Reinecker (2008). "GEF-H1 mediated control of NOD1 dependent NF-κB activation by Shigella effectors." PLoS Pathog **4**(11): e1000228.

Génin, P., M. Algarté, P. Roof, R. Lin and J. Hiscott (2000). "Regulation of RANTES Chemokine Gene Expression Requires Cooperativity Between NF-κB and IFN-Regulatory Factor Transcription Factors." The Journal of Immunology **164**(10): 5352-5361.

Goldfeld, A. E., C. Doyle and T. Maniatis (1990). "Human tumor necrosis factor alpha gene regulation by virus and lipopolysaccharide." Proceedings of the National Academy of Sciences **87**(24): 9769-9773.

Goodfellow, I., Y. Chaudhry, I. Gioldasi, A. Gerondopoulos, A. Natoni, L. Labrie, J. F. Laliberté and L. Roberts (2005). "Calicivirus translation initiation requires an interaction between VPg and eIF4E." EMBO reports **6**(10): 968-972.

Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore and M. K. Estes (1994). "Norwalk Virus Infection of Volunteers: New Insights Based on Improved Assays." Journal of Infectious Diseases **170**(1): 34-43.

Grandvaux, N., M. J. Servant, G. C. Sen, S. Balachandran, G. N. Barber, R. Lin and J. Hiscott (2002). "Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes." Journal of virology **76**(11): 5532-5539.

Guilluy, C., V. Swaminathan, R. Garcia-Mata, E. Timothy O/'Brien, R. Superfine and K. Burridge (2011). "The Rho GEFs LARG and GEF-H1 regulate the mechanical response to force on integrins." Nat Cell Biol **13**(6): 722-727.

Guix, S., M. Asanaka, K. Katayama, S. E. Crawford, F. H. Neill, R. L. Atmar and M. K. Estes (2007). "Norwalk virus RNA is infectious in mammalian cells." Journal of virology **81**(22): 12238-12248.

Gutiérrez-Escolano, A. L., Z. U. Brito, R. M. del Angel and X. Jiang (2000). "Interaction of cellular proteins with the 5’ end of Norwalk virus genomic RNA." Journal of virology **74**(18): 8558-8562.

Haga, K., A. Fujimoto, R. Takai-Todaka, M. Miki, Y. H. Doan, K. Murakami, M. Yokoyama, K. Murata, A. Nakanishi and K. Katayama (2016). "Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells." Proceedings of the National Academy of Sciences **113**(41): E6248-E6255.

Hall, A. J., J. Vinjé, B. Lopman, G. W. Park, C. Yen, N. Gregoricus and U. Parashar (2011). "Updated norovirus outbreak management and disease prevention guidelines." MMWR Recomm Rep **60**(3).

Haller, O. and G. Kochs (2011). "Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity." Journal of Interferon & Cytokine Research **31**(1): 79-87.

Hanson, P. J., H. M. Zhang, M. G. Hemida, X. Ye, Y. Qiu and D. Yang (2012). "IRES-Dependent Translational Control during Virus-Induced Endoplasmic Reticulum Stress and Apoptosis." Frontiers in Microbiology **3**: 92.

Hart, M. J., A. Eva, D. Zangrilli, S. A. Aaronson, T. Evans, R. A. Cerione and Y. Zheng (1994). "Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product." Journal of Biological Chemistry **269**(1): 62-65.

Herbert, T., I. Brierley and T. Brown (1997). "Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation." Journal of General Virology **78**(5): 1033-1040.

Hewitt, E. W., S. S. Gupta and P. J. Lehner (2001). "The human cytomegalovirus gene product US6 inhibits ATP binding by TAP." The EMBO Journal **20**(3): 387-396.

Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh and D. Johnson (1995). "Herpes simplex virus turns off the TAP to evade host immunity." Nature **375**(6530): 411-415.

Hinkula, J., J. M. Ball, S. Löfgren, M. K. Estes and L. Svensson (1995). "Antibody prevalence and immunoglobulin IgG subclass pattern to Norwalk virus in Sweden." Journal of medical virology **47**(1): 52-57.

Hiscott, J., P. Pitha, P. Genin, H. Nguyen, C. Heylbroeck, Y. Mamane, M. Algarte and R. Lin (1999). "Triggering the interferon response: the role of IRF-3 transcription factor." Journal of interferon & cytokine research **19**(1): 1-13.

Hudson, A. W., P. M. Howley and H. L. Ploegh (2001). "A human herpesvirus 7 glycoprotein, U21, diverts major histocompatibility complex class I molecules to lysosomes." Journal of virology **75**(24): 12347-12358.

Hunter, C. A., C. S. Subauste, V. Van Cleave and J. S. Remington (1994). "Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha." Infection and immunity **62**(7): 2818-2824.

Hutson, A. M., R. L. Atmar and M. K. Estes (2004). "Norovirus disease: changing epidemiology and host susceptibility factors." Trends in Microbiology **12**(6): 279-287.

Hutson, A. M., R. L. Atmar, D. M. Marcus and M. K. Estes (2003). "Norwalk Virus-Like Particle Hemagglutination by Binding to H Histo-Blood Group Antigens." Journal of Virology **77**(1): 405-415.

Hyde, J. L., L. K. Gillespie and J. M. Mackenzie (2012). "Mouse norovirus 1 utilizes the cytoskeleton network to establish localization of the replication complex proximal to the microtubule organizing center." Journal of virology **86**(8): 4110-4122.

Hyde, J. L. and J. M. Mackenzie (2010). "Subcellular localization of the MNV-1 ORF1 proteins and their potential roles in the formation of the MNV-1 replication complex." Virology **406**(1): 138-148.

Hyde, J. L., S. V. Sosnovtsev, K. Y. Green, C. Wobus, H. W. Virgin and J. M. Mackenzie (2009). "Mouse norovirus replication is associated with virus-induced vesicle clusters originating from membranes derived from the secretory pathway." Journal of virology **83**(19): 9709-9719.

Idriss, H. T. and J. H. Naismith (2000). "TNFalpha and the TNF Receptor Superfamily: Structure-Function Relationship (s)." Microscopy research and technique **50**(3): 184-195.

Israël, A., O. Le Bail, D. Hatat, J. Piette, M. Kieran, F. Logeat, D. Wallach, M. Fellous and P. Kourilsky (1989). "TNF stimulates expression of mouse MHC class I genes by inducing an NF kappa B-like enhancer binding activity which displaces constitutive factors." The EMBO Journal **8**(12): 3793-3800.

Johnson, P. C., J. J. Mathewson, H. L. DuPont and H. B. Greenberg (1990). "Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults." Journal of Infectious Diseases **161**(1): 18-21.

Jones, M. K., M. Watanabe, S. Zhu, C. L. Graves, L. R. Keyes, K. R. Grau, M. B. Gonzalez-Hernandez, N. M. Iovine, C. E. Wobus and J. Vinjé (2014). "Enteric bacteria promote human and mouse norovirus infection of B cells." Science **346**(6210): 755-759.

Kalvakolanu, D. V. and E. C. Borden (1996). "An overview of the interferon system: signal transduction and mechanisms of action." Cancer investigation **14**(1): 25-53.

Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica and R. M. Chanock (1972). "Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis." Journal of virology **10**(5): 1075-1081.

Karin, M. and A. Lin (2002). "NF-[kappa]B at the crossroads of life and death." Nat Immunol **3**(3): 221-227.

Karst, S. M., C. E. Wobus, M. Lay, J. Davidson and H. W. Virgin (2003). "STAT1-dependent innate immunity to a Norwalk-like virus." Science **299**(5612): 1575-1578.

Kerekatte, V., B. D. Keiper, C. Badorff, A. Cai, K. U. Knowlton and R. E. Rhoads (1999). "Cleavage of Poly(A)-Binding Protein by Coxsackievirus 2A Protease In Vitro and In Vivo: Another Mechanism for Host Protein Synthesis Shutoff?" Journal of Virology **73**(1): 709-717.

Key Jr, L. L., R. M. Rodriguiz, S. M. Willi, N. M. Wright, H. C. Hatcher, D. R. Eyre, J. K. Cure, P. P. Griffin and W. L. Ries (1995). "Long-term treatment of osteopetrosis with recombinant human interferon gamma." New England Journal of Medicine **332**(24): 1594-1599.

Kim, J. J., S. B. Lee, J. K. Park and Y. D. Yoo (2010). "TNF-[alpha]-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-XL." Cell Death Differ **17**(9): 1420-1434.

Kim, Y., S. Lovell, K.-C. Tiew, S. R. Mandadapu, K. R. Alliston, K. P. Battaile, W. C. Groutas and K.-O. Chang (2012). "Broad-Spectrum Antivirals against 3C or 3C-Like Proteases of Picornaviruses, Noroviruses, and Coronaviruses." Journal of Virology **86**(21): 11754-11762.

Koo, H. L., N. Ajami, R. L. Atmar and H. L. DuPont (2010). "Noroviruses: the principal cause of foodborne disease worldwide." Discovery medicine **10**(50): 61.

Kopp, E. B. and R. Medzhitov (1999). "The Toll-receptor family and control of innate immunity." Current Opinion in Immunology **11**(1): 13-18.

Krendel, M., F. T. Zenke and G. M. Bokoch (2002). "Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton." Nat Cell Biol **4**(4): 294-301.

Kriegler, M., C. Perez, K. DeFay, I. Albert and S. Lu (1988). "A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF." Cell **53**(1): 45-53.

Krones, E. and C. Högenauer (2012). "Diarrhea in the immunocompromised patient." Gastroenterology Clinics of North America **41**(3): 677-701.

Kropshofer, H., A. B. Vogt, G. Moldenhauer, J. Hammer, J. S. Blum and G. J. Hämmerling (1996). "Editing of the HLA-DR-peptide repertoire by HLA-DM." The EMBO Journal **15**(22): 6144.

Kumar, K. P., K. M. McBride, B. K. Weaver, C. Dingwall and N. C. Reich (2000). "Regulated Nuclear-Cytoplasmic Localization of Interferon Regulatory Factor 3, a Subunit of Double-Stranded RNA-Activated Factor 1." Molecular and Cellular Biology **20**(11): 4159-4168.

Kurts, C., B. W. Robinson and P. A. Knolle (2010). "Cross-priming in health and disease." Nature Reviews Immunology **10**(6): 403-414.

Kuyumcu-Martinez, M., G. Belliot, S. V. Sosnovtsev, K.-O. Chang, K. Y. Green and R. E. Lloyd (2004). "Calicivirus 3C-Like Proteinase Inhibits Cellular Translation by Cleavage of Poly(A)-Binding Protein." Journal of Virology **78**(15): 8172-8182.

Landry, J. and J. Huot (1995). "Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27." Biochemistry and Cell Biology **73**(9-10): 703-707.

Landsverk, O. J. B., O. Bakke and T. Gregers (2009). "MHC II and the endocytic pathway: regulation by invariant chain." Scandinavian journal of immunology **70**(3): 184-193.

Le Gall, S., L. Erdtmann, S. Benichou, C. Berlioz-Torrent, L. Liu, R. Benarous, J.-M. Heard and O. Schwartz (1998). "Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules." Immunity **8**(4): 483-495.

Lee, D. H. and A. L. Goldberg (1998). "Proteasome inhibitors: valuable new tools for cell biologists." Trends in Cell Biology **8**(10): 397-403.

Leen, E. N., K. R. Kwok, J. R. Birtley, P. J. Simpson, C. V. Subba-Reddy, Y. Chaudhry, S. V. Sosnovtsev, K. Y. Green, S. N. Prater and M. Tong (2013). "Structures of the compact helical core domains of feline calicivirus and murine norovirus VPg proteins." Journal of virology **87**(10): 5318-5330.

Lehner, P. J., J. T. Karttunen, G. W. Wilkinson and P. Cresswell (1997). "The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation." Proceedings of the National Academy of Sciences **94**(13): 6904-6909.

Lenardo, M. J., C.-M. Fan, T. Maniatis and D. Baltimore (1989). "The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction." Cell **57**(2): 287-294.

Levitskaya, J., A. Sharipo, A. Leonchiks, A. Ciechanover and M. G. Masucci (1997). "Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein–Barr virus nuclear antigen 1." Proceedings of the National Academy of Sciences **94**(23): 12616-12621.

Libermann, T. A. and D. Baltimore (1990). "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor." Molecular and cellular biology **10**(5): 2327-2334.

Limited, T. P. C. (2016). Takeda Initiates World's First Norovirus Vaccine Field Trial.

Lin, X., L. Thorne, Z. Jin, L. A. Hammad, S. Li, J. Deval, I. G. Goodfellow and C. C. Kao (2015). "Subgenomic promoter recognition by the norovirus RNA-dependent RNA polymerases." Nucleic acids research **43**(1): 446-460.

Lindesmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. LePendu and R. Baric (2003). "Human susceptibility and resistance to Norwalk virus infection." Nat Med **9**(5): 548-553.

Lindesmith, L. C., E. F. Donaldson and R. S. Baric (2011). "Norovirus GII.4 Strain Antigenic Variation." Journal of Virology **85**(1): 231-242.

Lindesmith, L. C., M. T. Ferris, C. W. Mullan, J. Ferreira, K. Debbink, J. Swanstrom, C. Richardson, R. R. Goodwin, F. Baehner and P. M. Mendelman (2015). "Broad blockade antibody responses in human volunteers after immunization with a multivalent norovirus VLP candidate vaccine: immunological analyses from a phase I clinical trial." PLoS med **12**(3): e1001807.

Lloyd, R., M. Grubman and E. Ehrenfeld (1988). "Relationship of p220 cleavage during picornavirus infection to 2A proteinase sequencing." Journal of virology **62**(11): 4216-4223.

Lloyd, R. E., H. Jense and E. Ehrenfeld (1987). "Restriction of translation of capped mRNA in vitro as a model for poliovirus-induced inhibition of host cell protein synthesis: relationship to p220 cleavage." Journal of virology **61**(8): 2480-2488.

Lochridge, V. P. and M. E. Hardy (2003). "Snow Mountain virus genome sequence and virus-like particle assembly." Virus genes **26**(1): 71-82.

Lodish H, B. A., Zipursky SL, et al. (2000). Molecular Cell Biology, New York: W. H. Freeman.

López-Manríquez, E., S. Vashist, L. Ureña, I. Goodfellow, P. Chavez, J. E. Mora-Heredia, C. Cancio-Lonches, E. Garrido and A. L. Gutiérrez-Escolano (2013). "Norovirus genome circularization and efficient replication are facilitated by binding of PCBP2 and hnRNP A1." Journal of virology **87**(21): 11371-11387.

López, C. B., A. García-Sastre, B. R. Williams and T. M. Moran (2003). "Type I interferon induction pathway, but not released interferon, participates in the maturation of dendritic cells induced by negative-strand RNA viruses." Journal of Infectious Diseases **187**(7): 1126-1136.

Luettig, B., T. Decker and M. Lohmann-Matthes (1989). "Evidence for the existence of two forms of membrane tumor necrosis factor: an integral protein and a molecule attached to its receptor." The Journal of Immunology **143**(12): 4034-4038.

Macadam, A. J., G. Ferguson, T. Fleming, D. M. Stone, J. W. Almond and P. D. Minor (1994). "Role for poliovirus protease 2A in cap independent translation." The EMBO Journal **13**(4): 924-927.

Marshansky, V. and M. Futai (2008). "The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function." Current opinion in cell biology **20**(4): 415-426.

Matthews, J., B. Dickey, R. Miller, J. Felzer, B. Dawson, A. Lee, J. Rocks, J. Kiel, J. Montes and C. Moe (2012). "The epidemiology of published norovirus outbreaks: a review of risk factors associated with attack rate and genogroup." Epidemiology and infection **140**(07): 1161-1172.

May, J., B. Korba, A. Medvedev and P. Viswanathan (2013). "Enzyme kinetics of the human norovirus protease control virus polyprotein processing order." Virology **444**(1): 218-224.

May, J., P. Viswanathan, K. K.-S. Ng, A. Medvedev and B. Korba (2014). "The P4-P2' amino acids surrounding human norovirus polyprotein cleavage sites define the core sequence regulating self-processing order." Journal of virology **88**(18): 10738-10747.

McFadden, N., D. Bailey, G. Carrara, A. Benson, Y. Chaudhry, A. Shortland, J. Heeney, F. Yarovinsky, P. Simmonds and A. Macdonald (2011). "Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4." PLoS Pathog **7**(12): e1002413.

McInerney, G. M., N. L. Kedersha, R. J. Kaufman, P. Anderson and P. Liljeström (2005). "Importance of eIF2α Phosphorylation and Stress Granule Assembly in Alphavirus Translation Regulation." Molecular Biology of the Cell **16**(8): 3753-3763.

McWhirter, S. M., S. S. Pullen, J. M. Holton, J. J. Crute, M. R. Kehry and T. Alber (1999). "Crystallographic analysis of CD40 recognition and signaling by human TRAF2." Proceedings of the National Academy of Sciences of the United States of America **96**(15): 8408-8413.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin and R. V. Tauxe (1999). "Food-related illness and death in the United States." Emerging infectious diseases **5**(5): 607.

Meiri, D., C. B. Marshall, M. A. Greeve, B. Kim, M. Balan, F. Suarez, C. Bakal, C. Wu, J. LaRose and N. Fine (2012). "Mechanistic insight into the microtubule and actin cytoskeleton coupling through dynein-dependent RhoGEF inhibition." Molecular cell **45**(5): 642-655.

Meurs, E., K. Chong, J. Galabru, N. S. B. Thomas, I. M. Kerr, B. R. G. Williams and A. G. Hovanessian (1990). "Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon." Cell **62**(2): 379-390.

Mibayashi, M., L. Martínez-Sobrido, Y.-M. Loo, W. B. Cárdenas, M. Gale and A. García-Sastre (2007). "Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus." Journal of virology **81**(2): 514-524.

Mitchison, N. A. (2004). "T-cell-B-cell cooperation." Nat Rev Immunol **4**(4): 308-312.

Morris, G. P. and P. M. Allen (2012). "How the TCR balances sensitivity and specificity for the recognition of self and pathogens." Nat Immunol **13**(2): 121-128.

Morse, D. L., J. J. Guzewich, J. P. Hanrahan, R. Stricof, M. Shayegani, R. Deibel, J. C. Grabau, N. A. Nowak, J. E. Herrmann and G. Cukor (1986). "Widespread outbreaks of clam-and oyster-associated gastroenteritis." New England Journal of Medicine **314**(11): 678-681.

Mounts, A. W., T. Ando, M. Koopmans, J. S. Bresee, J. Noel and R. I. Glass (2000). "Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses." Journal of Infectious Diseases **181**(Supplement 2): S284-S287.

Muzio, M., J. Ni, P. Feng and V. M. Dixit (1997). "IRAK (Pelle) Family Member IRAK-2 and MyD88 as Proximal Mediators of IL-1 Signaling." Science **278**(5343): 1612-1615.

Nagae, S., W. Meng and M. Takeichi (2013). "Non-centrosomal microtubules regulate F-actin organization through the suppression of GEF-H1 activity." Genes to Cells **18**(5): 387-396.

Nanduri, S., B. W. Carpick, Y. Yang, B. R. G. Williams and J. Qin (1998). "Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular basis of its dsRNA-mediated activation." The EMBO Journal **17**(18): 5458-5465.

Neefjes, J., M. L. Jongsma, P. Paul and O. Bakke (2011). "Towards a systems understanding of MHC class I and MHC class II antigen presentation." Nature Reviews Immunology **11**(12): 823-836.

O'Donnell, T. B., J. L. Hyde, J. D. Mintern and J. M. Mackenzie (2016). "Mouse Norovirus infection promotes autophagy induction to facilitate replication but prevents final autophagosome maturation." Virology **492**: 130-139.

Oliver, S., E. Asobayire, A. Dastjerdi and J. Bridger (2006). "Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae." Virology **350**(1): 240-250.

Orchard, R. C., C. B. Wilen, J. G. Doench, M. T. Baldridge, B. T. McCune, Y.-C. J. Lee, S. Lee, S. M. Pruett-Miller, C. A. Nelson and D. H. Fremont (2016). "Discovery of a proteinaceous cellular receptor for a norovirus." Science **353**(6302): 933-936.

Pahl, H. L. (1999). "Activators and target genes of Rel/NF-κB transcription factors." Oncogene **18**(49).

Park, B., S. Lee, E. Kim, K. Cho, S. R. Riddell, S. Cho and K. Ahn (2006). "Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing." Cell **127**(2): 369-382.

Parrino , T. A., D. S. Schreiber , J. S. Trier , A. Z. Kapikian and N. R. Blacklow (1977). "Clinical Immunity in Acute Gastroenteritis Caused by Norwalk Agent." New England Journal of Medicine **297**(2): 86-89.

Paschal, B. M., H. S. Shpetner and R. B. Vallee (1987). "MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties." The Journal of cell biology **105**(3): 1273-1282.

Patel, M. M., M.-A. Widdowson, R. I. Glass, K. Akazawa, J. Vinjé and U. D. Parashar (2008). "Systematic literature review of role of noroviruses in sporadic gastroenteritis." Emerg Infect Dis **14**(8): 1224-1231.

Pathak, R., Violaine D. Delorme-Walker, Michael C. Howell, Anthony N. Anselmo, Michael A. White, Gary M. Bokoch and C. DerMardirossian (2012). "The Microtubule-Associated Rho Activating Factor GEF-H1 Interacts with Exocyst Complex to Regulate Vesicle Traffic." Developmental Cell **23**(2): 397-411.

Pathak, R. and C. DerMardirossian (2013). "GEF-H1: Orchestrating the interplay between cytoskeleton and vesicle trafficking." Small GTPases **4**(3): 174-179.

Pham, A. M., F. G. Santa Maria, T. Lahiri, E. Friedman, I. J. Marié and D. E. Levy (2016). "PKR transduces MDA5-dependent signals for type I IFN induction." PLoS Pathog **12**(3): e1005489.

Pollard, T. D. and G. G. Borisy (2003). "Cellular motility driven by assembly and disassembly of actin filaments." Cell **112**(4): 453-465.

Prasad, B., R. Rothnagel, X. Jiang and M. Estes (1994). "Three-dimensional structure of baculovirus-expressed Norwalk virus capsids." Journal of virology **68**(8): 5117-5125.

Prasad, B. V., M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann and M. K. Estes (1999). "X-ray crystallographic structure of the Norwalk virus capsid." Science **286**(5438): 287-290.

Prasad, B. V. V., D. O. Matson and A. W. Smith (1994). "Three-dimensional Structure of Calicivirus." Journal of Molecular Biology **240**(3): 256-264.

Rabinovich, E., A. Kerem, K.-U. Fröhlich, N. Diamant and S. Bar-Nun (2002). "AAA-ATPase p97/Cdc48p, a Cytosolic Chaperone Required for Endoplasmic Reticulum-Associated Protein Degradation." Molecular and Cellular Biology **22**(2): 626-634.

Reeck, A., O. Kavanagh, M. K. Estes, A. R. Opekun, M. A. Gilger, D. Y. Graham and R. L. Atmar (2010). "Serological correlate of protection against norovirus-induced gastroenteritis." Journal of Infectious Diseases **202**(8): 1212-1218.

Ren, Y., R. Li, Y. Zheng and H. Busch (1998). "Cloning and Characterization of GEF-H1, a Microtubule-associated Guanine Nucleotide Exchange Factor for Rac and Rho GTPases." Journal of Biological Chemistry **273**(52): 34954-34960.

Richardson, P. G., T. Hideshima and K. C. Anderson (2003). "Bortezomib (PS-341): a novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers." Cancer Control **10**(5): 361-369.

Rivera, C. I. and R. E. Lloyd (2008). "Modulation of enteroviral proteinase cleavage of poly(A)-binding protein (PABP) by conformation and PABP-associated factors." Virology **375**(1): 59-72.

Rocha-Pereira, J., D. Jochmans, K. Dallmeier, P. Leyssen, M. Nascimento and J. Neyts (2012). "Favipiravir (T-705) inhibits in vitro norovirus replication." Biochemical and biophysical research communications **424**(4): 777-780.

Rocha-Pereira, J., D. Jochmans, Y. Debing, E. Verbeken, M. S. J. Nascimento and J. Neyts (2013). "The Viral Polymerase Inhibitor 2'-C-Methylcytidine Inhibits Norwalk Virus Replication and

Protects against Norovirus-Induced Diarrhea and Mortality in a Mouse Model." Journal of Virology **87**(21): 11798-11805.

Rocha, N., C. Kuijl, R. van der Kant, L. Janssen, D. Houben, H. Janssen, W. Zwart and J. Neefjes (2009). "Cholesterol sensor ORP1L contacts the ER protein VAP to control Rab7–RILP–p150Glued and late endosome positioning." The Journal of cell biology **185**(7): 1209-1225.

Rockx, B., M. de Wit, H. Vennema, J. Vinjé, E. de Bruin, Y. van Duynhoven and M. Koopmans (2002). "Natural History of Human Calicivirus Infection: A Prospective Cohort Study." Clinical Infectious Diseases **35**(3): 246-253.

Roeth, J. F., M. Williams, M. R. Kasper, T. M. Filzen and K. L. Collins (2004). "HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail." The Journal of cell biology **167**(5): 903-913.

Rohayem, J., I. Robel, K. Jäger, U. Scheffler and W. Rudolph (2006). "Protein-primed and de novo initiation of RNA synthesis by norovirus 3Dpol." Journal of virology **80**(14): 7060-7069.

Ruuls, S. R., R. M. Hoek, V. N. Ngo, T. McNeil, L. A. Lucian, M. J. Janatpour, H. Körner, H. Scheerens, E. M. Hessel, J. G. Cyster, L. M. McEvoy and J. D. Sedgwick (2001). "Membrane-Bound TNF Supports Secondary Lymphoid Organ Structure but Is Subservient to Secreted TNF in Driving Autoimmune Inflammation." Immunity **15**(4): 533-543.

Ryman, K. D. and W. B. Klimstra (2008). "Host responses to alphavirus infection." Immunological Reviews **225**(1): 27-45.

Sakihama, T., A. Smolyar and E. L. Reinherz (1995). "Molecular recognition of antigen involves lattice formation between CD4, MHC class II and TCR molecules." Immunology Today **16**(12): 581-587.

Samuel, C. (1998). Reoviruses and the interferon system. Reoviruses II, Springer**:** 125-145.

Sandoval-Jaime, C. and A. L. Gutiérrez-Escolano (2009). "Cellular proteins mediate 5'–3' end contacts of Norwalk virus genomic RNA." Virology **387**(2): 322-330.

Sarvestani, S. T., B. Cotton, S. Fritzlar, T. B. O'Donnell and J. M. Mackenzie (2016). "Norovirus infection: replication, manipulation of host, and interaction with the host immune response." Journal of Interferon & Cytokine Research **36**(4): 215-225.

Sato, M., N. Tanaka, N. Hata, E. Oda and T. Taniguchi (1998). "Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-β gene." FEBS Letters **425**(1): 112-116.

Scholl, T., S. K. Mahanta and J. L. Strominger (1997). "Specific complex formation between the type II bare lymphocyte syndrome-associated transactivators CIITA and RFX5." Proceedings of the National Academy of Sciences **94**(12): 6330-6334.

Schwartz, O., V. Maréchal, S. Le Gall, F. Lemonnier and J.-M. Heard (1996). "Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein." Nature medicine **2**(3): 338-342.

Seitz, S. R., J. S. Leon, K. J. Schwab, G. M. Lyon, M. Dowd, M. McDaniels, G. Abdulhafid, M. L. Fernandez, L. C. Lindesmith, R. S. Baric and C. L. Moe (2011). "Norovirus Infectivity in Humans and Persistence in Water." Applied and Environmental Microbiology **77**(19): 6884-6888.

Shanker, S., L. Hu, S. Ramani, R. L. Atmar, M. K. Estes and B. V. Venkataram Prasad (2017). "Structural features of glycan recognition among viral pathogens." Current Opinion in Structural Biology **44**(Supplement C): 211-218.

Sharipo, A., M. Imreh, A. Leonchiks, S. Imreh and M. G. Masucci (1998). "A minimal glycine-alanine repeat prevents the interaction of ubiquitinated IκBα with the proteasome: a new mechanism for selective inhibition of proteolysis." Nature medicine **4**(8): 939-944.

Sharp, D. J., G. C. Rogers and J. M. Scholey (2000). "Microtubule motors in mitosis." Nature **407**(6800): 41-47.

Shen, L., L. J. Sigal, M. Boes and K. L. Rock (2004). "Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo." Immunity **21**(2): 155-165.

Shin, J.-S., M. Ebersold, M. Pypaert, L. Delamarre, A. Hartley and I. Mellman (2006). "Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination." Nature **444**(7115): 115-118.

Sidwell, R. W., D. L. Barnard, C. W. Day, D. F. Smee, K. W. Bailey, M.-H. Wong, J. D. Morrey and Y. Furuta (2007). "Efficacy of orally administered T-705 on lethal avian influenza A (H5N1) virus infections in mice." Antimicrobial agents and chemotherapy **51**(3): 845-851.

Sigrist, C. J. A., E. de Castro, L. Cerutti, B. A. Cuche, N. Hulo, A. Bridge, L. Bougueleret and I. Xenarios (2013). "New and continuing developments at PROSITE." Nucleic Acids Research **41**(Database issue): D344-D347.

Silverman, R. H. (1994). "Fascination with 2-5A-Dependent RNase: A Unique Enzyme That Functions in Interferon Action*." Journal of interferon research **14**(3): 101-104.

Simmonds, P., I. Karakasiliotis, D. Bailey, Y. Chaudhry, D. J. Evans and I. G. Goodfellow (2008). "Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses." Nucleic acids research **36**(8): 2530-2546.

Smith-Garvin, J. E., G. A. Koretzky and M. S. Jordan (2009). "T Cell Activation." Annual Review of Immunology **27**(1): 591-619.

Smith, D. M., N. Benaroudj and A. Goldberg (2006). "Proteasomes and their associated ATPases: A destructive combination." Journal of Structural Biology **156**(1): 72-83.

Someya, Y., N. Takeda and T. Wakita (2008). "Saturation mutagenesis reveals that GLU54 of norovirus 3C-like protease is not essential for the proteolytic activity." Journal of biochemistry **144**(6): 771-780.

Sosnovtsev, S. V., G. Belliot, K.-O. Chang, V. G. Prikhodko, L. B. Thackray, C. E. Wobus, S. M. Karst, H. W. Virgin and K. Y. Green (2006). "Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells." Journal of virology **80**(16): 7816-7831.

Stark, G. R., I. M. Kerr, B. R. G. Williams, R. H. Silverman and R. D. Schreiber (1998). "How Cells Respond to Interferons." Annual Review of Biochemistry **67**(1): 227-264.

Steimle, V., C. Siegrist, A. Mottet, B. Lisowska-Grospierre and B. Mach (1994). "Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA." Science **265**(5168): 106-109.

Stevenson, P. G., S. Efstathiou, P. C. Doherty and P. J. Lehner (2000). "Inhibition of MHC class I-restricted antigen presentation by γ2-herpesviruses." Proceedings of the National Academy of Sciences **97**(15): 8455-8460.

Strachan, T. (1987). "Molecular genetics and polymorphism of class I HLA antigens." British medical bulletin **43**(1): 1-14.

Subba-Reddy, C. V., I. Goodfellow and C. C. Kao (2011). "VPg-primed RNA synthesis of norovirus RNA-dependent RNA polymerases by using a novel cell-based assay." Journal of virology **85**(24): 13027-13037.

Subba-Reddy, C. V., M. A. Yunus, I. G. Goodfellow and C. C. Kao (2012). "Norovirus RNA synthesis is modulated by an interaction between the viral RNA-dependent RNA polymerase and the major capsid protein, VP1." Journal of virology **86**(18): 10138-10149.

Sundararajan, A., M. Y. Sangster, S. Frey, R. L. Atmar, W. H. Chen, J. Ferreira, R. Bargatze, P. M. Mendelman, J. J. Treanor and D. J. Topham (2015). "Robust mucosal-homing antibody-secreting B cell responses induced by intramuscular administration of adjuvanted bivalent human norovirus-like particle vaccine." Vaccine **33**(4): 568-576.

Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese and A. García-Sastre (2000). "Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein." Journal of virology **74**(17): 7989-7996.

Tan, M., R. S. Hegde and X. Jiang (2004). "The P domain of norovirus capsid protein forms dimer and binds to histo-blood group antigen receptors." Journal of virology **78**(12): 6233-6242.

Terenzi, F., H. Ying, N. P. Restifo, B. R. Williams and R. H. Silverman (1999). "The antiviral enzymes PKR and RNase L suppress gene expression from viral and non-viral based vectors." Nucleic acids research **27**(22): 4369-4375.

Teunis, P. F., C. L. Moe, P. Liu, S. E Miller, L. Lindesmith, R. S. Baric, J. Le Pendu and R. L. Calderon (2008). "Norwalk virus: how infectious is it?" Journal of medical virology **80**(8): 1468-1476.

Thackray, L. B., E. Duan, H. M. Lazear, A. Kambal, R. D. Schreiber, M. S. Diamond and H. W. Virgin (2012). "Critical role for interferon regulatory factor 3 (IRF-3) and IRF-7 in type I interferon-mediated control of murine norovirus replication." Journal of virology **86**(24): 13515-13523.

Thiel, H.-J. and M. König (1999). "Caliciviruses: an overview." Veterinary Microbiology **69**(1–2): 55-62.

Thomson, A. W. and M. T. Lotze (2003). The Cytokine Handbook, Two-Volume Set, Gulf Professional Publishing.

Thorne, L., D. Bailey and I. Goodfellow (2012). "High-resolution functional profiling of the norovirus genome." Journal of virology **86**(21): 11441-11456.

Thorne, L. G. and I. G. Goodfellow (2014). "Norovirus gene expression and replication." Journal of General Virology **95**(2): 278-291.

Tiwari, N., N. Garbi, T. Reinheckel, G. Moldenhauer, G. J. Hämmerling and F. Momburg (2007). "A transporter associated with antigen-processing independent vacuolar pathway for the MHC class I-mediated presentation of endogenous transmembrane proteins." The Journal of Immunology **178**(12): 7932-7942.

Tomov, V. T., L. C. Osborne, D. V. Dolfi, G. F. Sonnenberg, L. A. Monticelli, K. Mansfield, H. W. Virgin, D. Artis and E. J. Wherry (2013). "Persistent Enteric Murine Norovirus Infection Is Associated with Functionally Suboptimal Virus-Specific CD8 T Cell Responses." Journal of Virology **87**(12): 7015-7031.

Trowsdale, J. (1987). "Genetics and polymorphism: class II antigens." British medical bulletin **43**(1): 15-36.

Valderrama, F., J. M Durán, T. Babia, H. Barth, J. Renau-Piqueras and G. Egea (2001). "Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells." Traffic **2**(10): 717-726.

Vashist, S., L. Urena, Y. Chaudhry and I. Goodfellow (2012). "Identification of RNA-protein interaction networks involved in the norovirus life cycle." Journal of virology **86**(22): 11977-11990.

Vilcek, J. (1996). "Interferons and other cytokines." Fields virology.

Vinjé, J., J. Green, D. C. Lewis, C. I. Gallimore, D. W. G. Brown and M. P. G. Koopmans (2000). "Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses"." Archives of Virology **145**(2): 223-241.

Vongpunsawad, S., B. V. Venkataram Prasad and M. K. Estes (2013). "Norwalk Virus Minor Capsid Protein VP2 Associates within the VP1 Shell Domain." Journal of Virology **87**(9): 4818-4825.

Vyas, J. M., A. G. Van der Veen and H. L. Ploegh (2008). "The known unknowns of antigen processing and presentation." Nature Reviews Immunology **8**(8): 607-618.

Wang, L., F. Du and X. Wang (2008). "TNF-α Induces Two Distinct Caspase-8 Activation Pathways." Cell **133**(4): 693-703.

Wang, X., M. Li, H. Zheng, T. Muster, P. Palese, A. A. Beg and A. García-Sastre (2000). "Influenza A virus NS1 protein prevents activation of NF-κB and induction of alpha/beta interferon." Journal of virology **74**(24): 11566-11573.

Wearsch, P. A. and P. Cresswell (2008). "The quality control of MHC class I peptide loading." Current opinion in cell biology **20**(6): 624-631.

Wearsch, P. A., D. R. Peaper and P. Cresswell (2011). "Essential glycan-dependent interactions optimize MHC class I peptide loading." Proceedings of the National Academy of Sciences **108**(12): 4950-4955.

Welte, M. A. (2004). "Bidirectional Transport along Microtubules." Current Biology **14**(13): R525-R537.

Wobus, C. E., S. M. Karst, L. B. Thackray, K.-O. Chang, S. V. Sosnovtsev, G. Belliot, A. Krug, J. M. Mackenzie, K. Y. Green and H. W. Virgin IV (2004). "Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages." PLoS Biol **2**(12): e432.

Wobus, C. E., L. B. Thackray and H. W. Virgin (2006). "Murine norovirus: a model system to study norovirus biology and pathogenesis." Journal of virology **80**(11): 5104-5112.

Wyatt, R. G., R. Dolin, N. R. Blacklow, H. L. DuPont, R. F. Buscho, T. S. Thornhill, A. Z. Kapikian and R. M. Chanock (1974). "Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers." Journal of Infectious Diseases **129**(6): 709-714.

Yajima, T. and K. U. Knowlton (2009). "Viral Myocarditis." From the Perspective of the Virus **119**(19): 2615-2624.

Yang, W. and M. A. McCrae (2012). "The rotavirus enterotoxin (NSP4) promotes re-modeling of the intracellular microtubule network." Virus research **163**(1): 269-274.

Yarmolinsky, M. B. and L. Gabriel (1959). "Inhibition by puromycin of amino acid incorporation into protein." Proceedings of the National Academy of Sciences **45**(12): 1721-1729.

Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai and Y. Tashiro (1991). "Bafilomycin A1, a specific inhibitor of vacuolar-type H (+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells." Journal of Biological Chemistry **266**(26): 17707-17712.

Yoshimura, Y. and H. Miki (2011). "Dynamic regulation of GEF-H1 localization at microtubules by Par1b/MARK2." Biochemical and Biophysical Research Communications **408**(2): 322-328.

Yunus, M. A., X. Lin, D. Bailey, I. Karakasiliotis, Y. Chaudhry, S. Vashist, G. Zhang, L. Thorne, C. C. Kao and I. Goodfellow (2015). "The murine norovirus core subgenomic RNA promoter consists of a stable stem-loop that can direct accurate initiation of RNA synthesis." Journal of virology **89**(2): 1218-1229.

Yuseff, M.-I., D. Lankar and A.-M. Lennon-Duménil (2009). "Dynamics of Membrane Trafficking Downstream of B and T Cell Receptor Engagement: Impact on Immune Synapses." Traffic **10**(6): 629-636.

Zahorsky, J. (1929). "Hyperemesis hiemis or the winter vomiting disease." Arch Pediatr **46**: 391-395.

Zeitler, C. E., M. K. Estes and B. V. Prasad (2006). "X-ray crystallographic structure of the Norwalk virus protease at 1.5-Å resolution." Journal of virology **80**(10): 5050-5058.

Zhao, Y., C. Alonso, I. Ballester, J. H. Song, S. Y. Chang, B. Guleng, S. Arihiro, P. J. Murray, R. Xavier and K. S. Kobayashi (2012). "Control of NOD2 and Rip2-dependent innate immune activation by GEF-H1." Inflammatory bowel diseases **18**(4): 603-612.

Zheng, D.-P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass and S. S. Monroe (2006). "Norovirus classification and proposed strain nomenclature." Virology **346**(2): 312-323.

Zheng, Y., M. L. Wong, B. Alberts and T. Mitchison (1995). "Nucleation of microtubule assembly by a [gamma]-tubulin-containing ring complex." Nature **378**(6557): 578-583.

Zheng, Y., D. Zangrilli, R. A. Cerione and A. Eva (1996). "The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting." Journal of Biological Chemistry **271**(32): 19017-19020.

Zhou, A., S. Scoggin, R. B. Gaynor and N. S. Williams (2003). "Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference." Oncogene **22**(13): 2054-2064.

Zhu, S., D. Regev, M. Watanabe, D. Hickman, N. Moussatche, D. M. Jesus, S. M. Kahan, S. Napthine, I. Brierley and R. N. Hunter III (2013). "Identification of immune and viral correlates of norovirus protective immunity through comparative study of intra-cluster norovirus strains." PLoS pathogens **9**(9): e1003592.