Investigation of viral and host factors contributing to the pathogenesis of human metapneumovirus infection and development of a subunit vaccine

Thèse

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Résumé

Le métapneumovirus humain (HMPV) est un virus responsable des infections aiguës des voies respiratoires chez l'enfant ainsi que chez l'adulte. Dans les populations à risque élevée, comme les jeunes enfants, les personnes âgées et les patients immuno-supprimés, le HMPV entraîne une morbidité et une mortalité importante. La pathogenèse de l'infection par le HMPV reste largement inconnue, mais des modèles animaux qui permettent l'étude de cette pathogenèse ont été développés. En effet, le modèle des souris BALB/c a déjà été développé dans le laboratoire du Dr Guy Boivin. Ce modèle murin nous a permis de démontrer que la protéine de fusion (F) contribue à la capacité réplicative du virus, mais qu'elle n'est pas la seule protéine responsable de la virulence de ce virus. De plus, nous avons démontré que le récepteur activé par les protéases (PAR1) contribue de manière significative à la pathogenèse du HMPV.

Actuellement, aucun vaccin ou modalité thérapeutique spécifique contre le HMPV sont disponibles. Trois facteurs principaux ont entravé le développement d'un vaccin sûr et efficace contre le HMPV; 1) Le précédent d'une maladie accrue associée à l'utilisation de vaccins inactivées contre la rougeole et le virus respiratoire syncytial (HRSV) et la démonstration d'une maladie accrue suite à une vaccination avec le HMPV inactivé chez les animaux. 2) Le manque de protection à long terme induite par les vaccins contre le HMPV chez les animaux. 3) Notre compréhension limitée des contributions individuelles des protéines virales dans le développement de l'immunité contre le HMPV. En développant un vaccin sous-unitaire comprenant de la protéine F et la protéine de la matrice (M), nous avons démontré que la protéine M contribue de manière significative à la protection chez la souris immunisée.

Summary

Human metapneumovirus (HMPV) is an important etiological agent of acute respiratory tract infection in both children and adults. In high-risk populations, such as infants, elderly individuals and immunocompromised patients, HMPV causes significant morbidity and mortality. The pathogenesis of HMPV infection remains largely unknown, but animal models of HMPV infection have been developed that allow for the study of such pathogenesis. Indeed, the BALB/c mouse model of HMPV infection was previously developed in Dr Boivin's laboratory. Using this murine model, we showed that the HMPV fusion (F) protein contributes to, but is not solely responsible for the replicative capacity and virulence of the virus and we demonstrated that the cellular protease-activated receptor-1 (PAR1) significantly contributes to HMPV pathogenesis.

To date, no HMPV-specific vaccines or therapeutic modalities are available. Three major factors have haltered the development of a safe and effective HMPV vaccine; 1) the precedent of vaccine-enhanced disease associated with the use of HRSV and measles vaccines and the demonstration of vaccine-enhanced disease upon inactivated HMPV vaccination in animal models. 2) The lack of long-term protection induced by HMPV vaccines in animals. 3) Our limited understanding of the individual contributions of HMPV proteins to host immunity. By developing a subunit vaccine consisting of the HMPV F and matrix (M) protein, we demonstrate that the HMPV M protein significantly contributes to vaccine-induced protection in mice.

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AA	amino acid
AM	alveolar macrophage
AMPV	avian metapneumovirus
AMPV-C	avian metapneumovirus subtype C
APC	antigen presenting cell
ARTI	acute respiratory tract infection
Asn	asparagine
BCG	Bacillus Calmette-Guérin
BHK	Baby Hamster kidney cells
BSR	a clone from BHK-21
BRSV	bovine respiratory syncytial virus
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
COS cells	immortalized monkey kidney cell-line (**C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic material)
CPE	cytopathic effect
CR	conserved region
CRI	conserved region I
CRIII	conserved region III
CRIV	conserved region IV
cRNA	complementary RNA
CS	cytosolic
CTL	cytolytic T-lymphocyte
DI, DII, DIII	domain I, II, III
DC	dendritic cell
DFA	direct immunofluorescence assay
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DsiRNA	Dicer-substrate RNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immune-spot
ER	endoplasmic reticulum
F protein	fusion glycoprotein
FDA	Food and Drug administration
FI-HRSV	formalin-inactivated human respiratory syncytial virus
FP	fusion peptide
mRNA	messenger ribonucleic acid
G protein	attachment glycoprotein
GE	gene end
GFP	green fluorescent protein

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GS	gene start
HA	hemagglutinin
HIV	human immunodeficiency virus
HMPV	human metapneumovirus
HN	Hemagglutinin-neuraminidase
HRA/HRB	heptad repeat A and B
HRSV	human respiratory syncytial virus
HSCT	Hematopoietic stem cell transplantation
ICS	intra-cellular staining
IFN	interferon
IG	intergenic region
IL	interleukin
IP-10	Interferon gamma-induced protein 10
IRF	Interferon regulatory factor
IVIG	Intravenous immunoglobulin
LLC-MK2	rhesus mokey kidney cells
LTCF	long term care facility
KC	keratinocyte-derived chemokine
KO-mice	Knock-out mice
L protein	Large polymerase protein
LRT	lower respiratory tract
LRTI	lower respiratory tract infection
LTCF	long-term care facility
M protein	matrix protein
Ma	macrophage
MAb	monoclonal antibody
MAVS	Mitochondrial antiviral-signaling protein
MCP-1	Monocyte chemoattractant protein-1
MDA-5	Melanoma Differentiation-Associated protein 5
MERS	Middle East Respiratory Syndrome
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP-1	Matrix metalloproteinase-1
mRNA	messenger ribonucleic acid
Mtase	methyltransferase
N protein	nucleoprotein
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
NS1/2	non-structural proteins 1/2
ORF	open readingframe
PAMPs	pathogen associated molecular patterns

P protein	phosphoprotein
PAR	protease-activated receptor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFUPIV	para-influenza virus
PVM	pneumonia virus of mice
RANTES	Regulated upon activation normal T cell expressed and presumably secreted (CCL5)
RdRP	RNA-dependend RNA polymerase
RGD	arginine-glycine-aspartate motif
RIG-I	retinoic acid-inducible gene 1
RNA	ribonucleic acid
RNP	ribonucleoprotein complex
RP	replicon particles
RTCA	real-time cell analysis
RT-PCR	Real-time polymerase chain reaction
SARS	Severe acute respiratory syndrome
SH protein	small hydrophobic protein
siRNA	small interfering ribonucleic acid
SOT	solid organ transplant
SP	signal peptide
SV5	simian virus 5
$TCID_{50}$	median tissue culture infective dose
TCR	T-cell receptor
TLR	toll-like receptor
TM	transmembrane
tMK	tertiary monkey kidney cells
TNF-α	Tumor necrosis factors-α
ts	temperature sensitive
URT	upper respiratory tract
URTI	upper respiratory tract infection
VLP	virus-like particle
vRNA	viral ribonucleic acid
VSV	Vesicular stomatitis virus
WHO	World Health Organisation

This manuscript is lovingly dedicated to the memory of Frank Aerts

Acknowledgments

First and foremost, I would like to thank Dr Boivin for the opportunities he has given me. Not only did he entrust these projects to me, he gave me the possibility and freedom to develop many different skills using the very vast array of techniques available in his laboratory. Furthermore, he has encouraged me to present my data at both local and international conferences, which have all been very valuable experiences. His patience, diligence and work ethic will remain an example I will aspire to.

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Chapter 1: General introduction

1. Acute respiratory tract infections (ARTI)

Acute respiratory tract infections occur most commonly in fall and winter months, possibly because of the return to school and intensifications in indoor crowding due to colder temperatures [75]. ARTI are classified into upper (URTI) and lower (LRTI) respiratory tract infections. The URT consists of the airways from nostrils to vocal cords, including sinuses and middle ear [298]. Most URTI are of viral etiology. The virus gains entry into the respiratory tract by inhalation and invades the mucosa [75]. Most URTI are self-limiting but aspiration of infectious secretions can lead to LRTI [298]. Complications are rare, but sinusitis and otitis media can follow [75]. The LRT starts at the trachea down to the bronchi, bronchioles and alveoli. LRTI consist of bronchitis, bronchiolitis and pneumonia and can be of both bacterial and viral etiology although other pathogens such as fungi can cause atypical pneumonias as well [298]. The pathogen gains entry through inhalation, aspiration or by blood. The pathogen then multiplies in or on the airway epithelium, causing inflammation and increased mucus production which can impair muco-ciliary functions. In the case of bronchiolitis, which predominantly occurs in the first year of life, inflammation and necrosis of epithelial cells can lead to airway obstruction [75].

Acute respiratory tract infections are the most common cause of illness and mortality in children under the age of 5 years. Such children have an average of 3 to 6 ARTI episodes per year regardless of their economic situation although more severe episodes occur in developing countries [298]. In 2010, an estimated 15 million hospitalizations worldwide due to severe or very severe ARTI occurred in this age group [231]. Sixty-two percent of children with severe ARTI were estimated to have been hospitalized that year, but 81% of deaths occurred outside of hospitals.

According to the WHO, an estimated 450 million cases of pneumonia occur every year; around 4 million people die from pneumonia each year, with the highest incidences in children younger than 5 years and in adults older than 75 years [280]. Long term care facility (LTCF)-acquired pneumonia is the leading cause of morbidity and of transfer to acute care facilities with an estimate of 33–114 cases per 1000 cases of hospitalization per year [96]. Thus, ARTI account for a significant burden on health care in this population as well.

Due to the use of chemotherapy regimens to fight cancer and the availability of new immunosuppressive and immunomodulating agents that increase the survival of solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients, the number of immunocompromised individuals has grown extensively in recent decades [151, 161]. All of these therapies can have lasting effects on immune and mucosal barrier function. Taken together, patients with underlying conditions who receive such therapies are at an increased risk for nosocomial ARTIs. This increased risk is probably both due to their immunocompromised state and the fact that they spend more time in hospitals [156].

2. HMPV discovery and taxonomy

2.1. Recent discoveries

The past 2 decades have led to the discovery of many new pathogens (table 1). Due to globalized travel, changes in demographics (such as increases in live animal markets and expansion of human populations into wildlife habitats) and increased susceptibility to opportunistic organisms associated with immunosuppression, new pathogens have emerged [60, 192]. On the other hand, new and improved molecular technologies have enabled more efficient microbial surveillance and discovery. Databases used to recognize sequences as host or microbial have greatly improved and sample collection has become more sophisticated and comprehensive [192]. However, discovering

a new micro-organism does not mean it is the etiological agent of a disease. Koch's postulate (1891) states that the criteria defining a causal relationship between an agent and a disease are: 1) the agent is present in every case of a disease 2) it is specific for that disease 3) and it can be propagated in culture and inoculated into a naïve host to cause the same disease. Yet, we now know that different pathogens can cause similar symptoms, infection can lead to different signs depending on genetic background, age, nutrition, previous exposure to similar agents or underlying conditions. Some pathogens are very difficult to culture and there may not always be an animal model [192]. In addition, many micro-organisms are commensal and part of the normal human microbiome. Consequently, although recent technological and scientifically advances have facilitated the discovery of new micro-organisms, clearly establishing their precise role in human infection and disease has become more complex. Nevertheless, table 1 gives an overview of viruses that cause ARTI that have been discovered within the last 20 years.

Table 1: Respiratory viruses that emerged over the last 2 decades (adapted from [9])

Emerging respiratory pathogens	Year of discovery	Region	Reference publication
sin nombre virus (Hantavirus pulmonary syndrome)	1993	USA	[234]
Influenza A H5N1	1997	Hong Kong	[307]
Influenza A H9N2	1999	Hong Kong	[255]
Human metapneumovirus	2001	Netherlands	[326]
SARS coronavirus	2003	Hong Kong	[172, 253]
Human coronavirus NL63	2004	Netherlands	[331]
Influenza A H7N7	2004	Netherlands	[170]
Human coronavirus HKU1	2005	China	[352]
Influenza A, H1 triple re-assortant	2005	USA	[232, 297]
Triple re-assortant H3N2 influenza A viruses	2005	Canada	[243]
Bocavirus	2005	Sweden	[12]
Influenza A H1N1 pdm09	2009	Mexico	[239]
Adenovirus 14p1	2010	USA	[155]
MERS-coronavirus	2012	Saudi Arabia	[364]
Influenza A H7N9	2013	China	[109]

2.2. HMPV discovery

In 2001, a new virus was isolated from clinical samples of 28 children under the age of 5 with ARTI symptoms similar to those caused by the well-known human respiratory syncytial virus (HRSV), but for whom no etiological agent had been previously identified [326]. Viral isolates grew slowly on tertiary monkey kidney cells (tMKa). Ferrets and guinea pigs, intranasally infected with the unknown viruses, did not present any clinical signs of illness and sera obtained from infected animals did not react with known human viruses, yet they reacted with each of the 28 unknown viral isolates, indicating that they were all serologically related [326]. Following inoculation of young turkeys, chickens and cynomolgus macaques with one of the unknown viruses, viral replication could not be found in throat swabs of birds but viral replication was detected in the respiratory tract of infected primates between days 2 and 8 post-infection (pi).

2.3. HMPV taxonomy and viral evolution

RNA extracted from tMK infected with the unknown virus could not be amplified by reverse-transcription-PCR (RT-PCR) using primers to known respiratory viruses. However, using random primers, bands were amplified that partially matched the avian metapneumovirus, indicating a close relatedness between both viruses [326]. Further phylogenetic investigation classified the virus as the first mammalian member of the metapneumovirus genus. Hence, the virus was called human metapneumovirus (HMPV) [326]. A few months later the same virus was identified in clinical specimens obtained from patients with unidentified ARTI, here in Dr Boivin's laboratory in Quebec, Canada [39, 258].

Subsequent genetic, biochemical and morphological analyses confirmed HMPV to be classified as a member of the order of the Mononegavirales, the family of the *Paramyxoviridae*, sub-family of

the *Pneumovirinae* and the *Metapneumovirus* genus (figure 1). Furthermore, HMPV isolates can be separated into two major groups (A and B) and at least 4 subgroups (A1, A2, B1, and B2) based mainly on sequence analysis of the fusion (F) and attachment (G) genes [34, 39, 42, 150, 250, 258, 328]. However, it remains unclear whether these genotypes represent different antigenic groups [205, 300, 327, 328]. AMPV subgroup C is more closely related to HMPV than to any other AMPV subgroup. This suggests that HMPV has emerged from AMPV-C upon zoonosis [77]. Molecular evolution studies modeling the current genetic diversity of HMPV suggest that the virus has appeared about two centuries ago, with the 2 main groups appearing about 50 years ago and the subgroups appearing less than 30 years ago [47, 254, 255]. Serological studies using stored sera indicated that HMPV was circulating in humans in the 1950's [326].

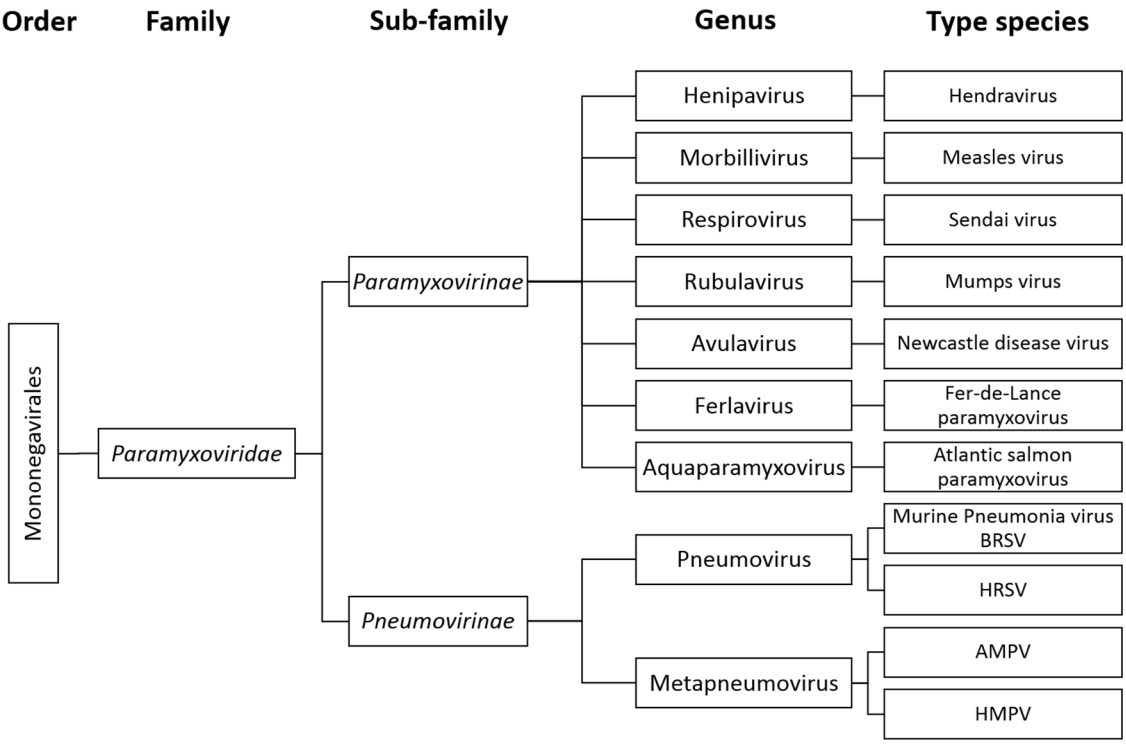

Figure 1 : Classification of the *paramyxoviridae* family.
The *paramyxoviridae* family contains 2 sub-families (*paramyxovirinae* and *pneumoviriniae*). The *paramyxovirinae* sub-family contains 7 geni, whereas the *pneumoviriniae* sub-family contains only 2 (pneumovirus and metapneumovirus). For each genus, a type species is represented, but for the *pneumovirinae* the most prominent non-human viruses are also included. Unclassified paramyxoviruses have not been included in this overview.

3. Virological features

Electron microscopy revealed pleomorphic particles measuring 150 to 600 nm, with short envelope projections of 13 to 17 nm [326]. Similarly to other pneumoviruses, HMPV virions are made up out of a lipid membrane containing three transmembrane surface glycoproteins the fusion (F), attachment (G), and small hydrophobic (SH) proteins. The matrix (M) protein lines the inner leaflet of the lipid membrane and encased within the envelope lies a helical ribonucleoprotein (RNP) complex, consisting of nucleoproteins (N), phosphoproteins (P), large polymerase proteins (L) and matrix 2 (M2) proteins, as well as the non-segmented single-stranded negative-sense RNA genome (figure 2).

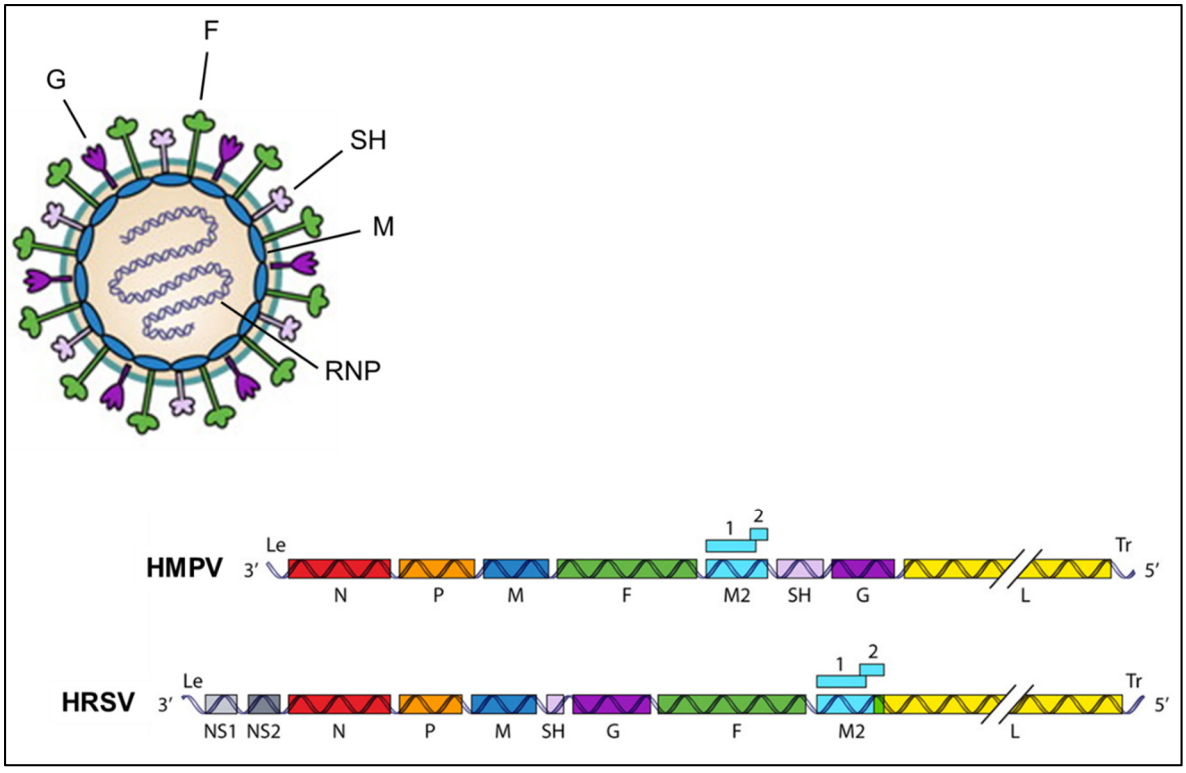

Figure 2: HMPV virion and genome (adapted from [290]):
Representation of the HMPV virion including envelope with glycoproteins (F, SH, G), the matrix (M) proteins and the ribonucleprotein (RNP) complex. A schematic representation of the HMPV genome as well as the HRSV genome are also included.

3.1. Genomic organisation

The HMPV genome size is around 13,3 kb. It contains 8 genes and 9 open reading frames (ORFs). The M2 gene contains 2 ORFs from which the M2-1 and M2-2 proteins are expressed. Between each ORF the genome of HMPV contains noncoding regions that range in size from 23 to 209 nucleotides and contain gene end signals, intergenic regions and gene start signals. There is little sequence identity with the noncoding regions of HRSV and AMPV [34, 325]. The genomic organisation resembles that of other pneumoviruses such as HRSV. However, unlike HRSV, the HMPV genome lacks the non-structural genes (NS1 and NS2) and the order of genes between M and L is different (in HRSV, the order is SH-G-F-M2, in HMPV the order is F-M2-SH-G) (Figure 2).

3.2. Viral proteins

3.2.1. Surface glycoproteins

3.2.1.1. Fusion (F) protein

Of the 3 surface glycoproteins (F, G and SH), the F protein is the most conserved between HMPV and HRSV [325] as well as within the species [34, 360]. The amino acid identity within as well as between HMPV clades, is higher than the nucleotide identity [39]. This suggests structural and/or functional restraints for the F protein [250]. Indeed, there is no evidence of major drift for the F gene over a 20 year period [360]. The F gene contains 1620 nucleotides encoding 539 amino acids (AA) resulting in a trimeric type I membrane glycoprotein with an extracellular N-terminus and a cytosolic C-terminus with a molecular mass of around 58 kDa.

Each monomer is produced as an inactive precursor protein (F_0). F_0 is cleaved by host protease at a monobasic consensus-site (RQSR), resulting in 2 disulfide-linked subunits (F_1 and F_2). Cleavage reveals a hydrophobic fusion peptide (FP) at the N-terminus of the F_1 subunit (Figure 3A), which initiates membrane fusion. The F1 subunit also contains 2 heptad repeats (HRA and HRB) and a conserved RGD motif at position 329-331. Three Asn residues are glycosylated (N_{57}, N_{172} and N_{353}). The structure of the HMPV F protein has only partially been solved (figure 3B) [69, 340].

In order for fusion to occur, the F protein needs to transition from a metastable prefusion conformation to a highly stable and irreversible postfusion conformation. To achieve this transition, the HRA and HRB fold into trimeric coiled coils to form a stable six-helix bundle. This process is not only irreversible but it is also essential to membrane fusion since peptides mimicking the HMPV heptad repeats can inhibit membrane fusion [84, 223] (See also chapter 1 section 9.2.5: Fusion inhibitors). The ability of the HMPV F protein to fuse membranes is not only of great importance for viral entry into the host cell (See also chapter 1 section 3.3.1 Attachment and fusion), it also allows for cell-cell membrane fusion of infected cells, which leads to the formation of large multinucleated cells called syncytia [293]. Not all HMPV strains induce large syncytia. The impact of an HMPV fusion protein capable of syncytia formation on viral replication and pathogenesis is the object of discussion in chapter 3 of this thesis.

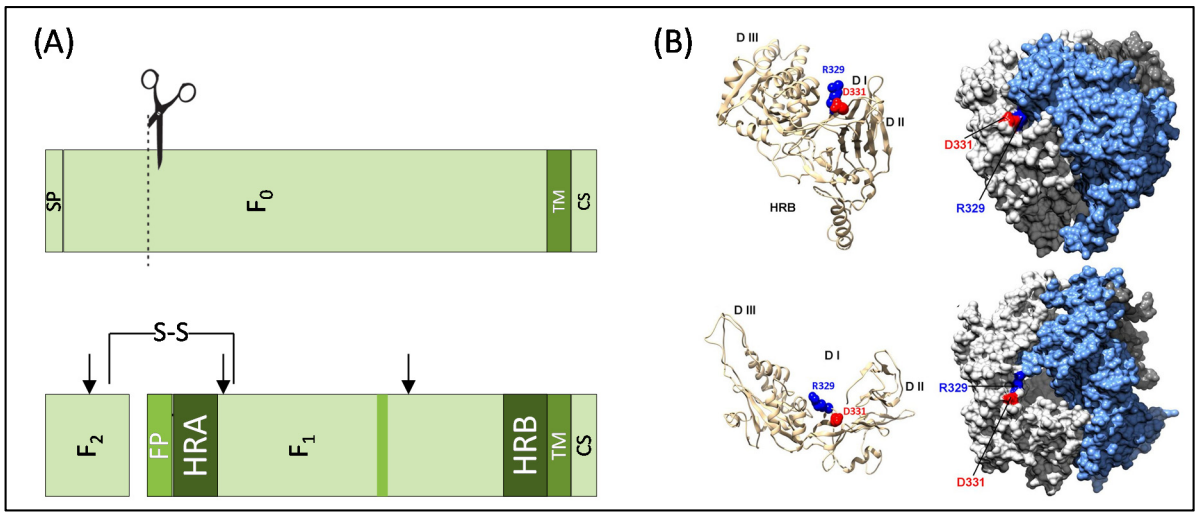

Figure 3: Representation of the HMPV fusion protein (adapted from [69] and [340]) :
(A) Schematic representation of the uncleaved F_0 precursor protein (top) which is cleaved into 2 disulfide-linked subunits (F_1 and F_2) (bottom). The 3 glycosylation-sites are indicated by the arrows. SP (signal peptide), FP (fusion peptide), HRA and HRB (heptad repeats), TM (transmembrane domain), CS (cytosolic domain), the green band in the middle of F_1 represents the conserved RGD-motif. (B) Predicted HMPV F-protein monomer (upper left) and trimer (upper right) based on the prefusion crystal structure of HRSV F protein. The RGD motives are highlighted and the surface of each monomer in the F-protein trimer is highlighted in a different color. The predicted HMPV F-protein monomer (lower left) and trimer (lower right) based on the partial structure of HMPV F protein solved in [342]. The location of the RGD motif is highlighted and the surface of each monomer in the F-protein trimer has a different color.

Glycosylation of paramyxovirus fusion proteins is of importance for both structural and functional reasons [19, 37, 54, 216, 337]. HMPV contains 3 glycosylation sites, none are shared with HRSV and two are shared with AMPV, the third appears unique to HMPV [325]. Mutating the F2 subunit glycosylation, did not alter F protein expression and function, whereas both F1 subunit glycosylation's appear critical to both F protein expression and function *in vitro* and *in vivo* [293, 363, 367].

Finally, the HMPV F protein has been shown to be the immunodominant protein, i.e. it is the only HMPV surface protein that induces the production of virus neutralizing antibodies [181,

299, 300]. This is in contrast with HRSV, for whom both anti-F and anti-G antibodies can induce protection [291].

3.2.1.2. Attachment (G) protein

Depending on the genus they belong to, different members of the *Paramyxoviridae* family, express different types of attachment proteins. Attachment proteins are called hemagglutinin (H) for morbilliviruses as they possess hemagglutination activity (the ability to cause red blood cells to cluster together *in vitro*). Rubulaviruses, respiroviruses, avulaviruses, ferlaviruses and aquaparamyxoviruses have HN (Hemagglutinin-neuraminidase) attachment proteins i.e. these have both hemagglutination and neuraminidase activity (the ability to cleave sialic acid on the surface of cells). Attachment proteins with neither hemagglutination nor neuraminidase activity are termed G (glycoprotein). HMPV, like Henipaviruses and other pneumoviruses, does not exhibit hemagglutinating activity and carries a G protein.

The G protein is the most variable among the HMPV structural proteins with around 35% amino acid identity within the species [28, 34, 257], although amino acid identity is considerably higher within subtypes [359]. Amino acid identity varies even within the protein itself, with the N-terminal cytoplasmic and transmembrane domains being more conserved than the ectodomain [359]. The G gene contains about 700 bp that encode a glycoprotein of 217-241 amino acids. The length of the G protein can vary greatly due to truncations at the C-terminus [359]. However, HMPV G proteins are shorter than the HRSV attachment protein (298 amino acids). The structural homology between both pneumovirus proteins has led to the name attachment protein for all G proteins.

The HMPV G protein has a type II membrane orientation with a single hydrophobic region, located near the N-terminus (amino acids 30 to 53), thought to serve as both signal peptide and membrane anchor whereas the C-terminal three quarters of the protein is extracellular [28, 34, 194, 325]. The HMPV G protein has a high content of serine and threonine residues (30 to 34%), which are potential acceptor sites for O-linked sugars. It contains a high level of proline residues (7 to 8.5%) and three to six potential N-linked glycosylation sites. Taken together, this suggests a heavily glycosylated mucin-like structure for the G protein [28, 257, 325]. The amount of potential glycosylation sites appears to be conserved within HMPV lineages, but the specific sites of glycosylation are not, indicating that the mucin-like character of the G protein is conserved regardless of the high level of amino acid diversity [250, 359].

Despite its name, the HMPV attachment G protein does not appear necessary for viral attachment to the host cell. In fact, recombinant HMPV lacking the attachment protein G replicates *in vitro* and is attenuated *in vivo* [33, 36] (see also chapter 1 section 9.1.2. live-attenuated vaccines). In addition, the F protein, and not the G protein of HMPV has been shown to determine host tropism [78]. This suggests that the F protein and not the G protein interacts with cellular receptors. (See also chapter 1 section 3.3.1 Attachment and fusion)

Selective immune pressure has been proposed to explain the high level of genetic variability in the HMPV G proteins based on two observations; 1) alternative start codons have been found within certain G genes, suggesting the possibility of G proteins being secreted and 2) the observations that antibodies directed against the G protein are produced in both animals and humans [359]. However, none of the alternate start codons are preceded by a signal sequence and not all HMPV lineages exhibit alternate start codons, reducing the likelihood of protein secretion

[359]. Furthermore, the antibodies directed against the G protein are non-neutralizing and do not protect against subsequent infection [225, 281, 299].

Finally, HMPV G proteins may have a role in virulence. Several studies have investigated the role of the G protein in inhibiting the innate immune response to HMPV infection [24, 25, 164, 165]. In monocyte-derived dendritic cells (moDC), the HMPV G protein inhibited Toll-like receptor 4 (TLR4) signaling [165] and, in A549 cells (an immortalized cell-line of human alveolar basal epithelial cells), the conserved N-terminus of the G protein inhibited retinoic acid-inducible gene 1 (RIG-I) signaling [25]. Both signaling pathways lead to the production of type I interferon (IFN I) and pro-inflammatory cytokines and chemokines. However, using siRNA against the G mRNA of the same viral strain, another group was unable to demonstrate increased IFN I production in A549 cells [265]. Recently, a role for HMPV G was proposed in the regulation of type III interferons (IFN-λs) [23]. Therefore, further investigation of the role of the HMPV G protein in inhibiting the innate immune response is needed.

3.2.1.3. Small hydrophobic protein

Similarly to the HMPV G protein, HMPV SH is a type II integral transmembrane glycoprotein. It is larger than the HRSV SH protein (177-183 amino acids compared to 65 amino acids for HRSV SH), but it has similar glycosylated forms; unglycosylated, N-glycosylated, and highly glycosylated [33]. The SH protein is dispensable for viral replication *in vitro* and *in vivo* [33, 36]. Nevertheless, since no clinical HMPV isolates lacking the SH protein have been reported, it is unlikely this protein is entirely expendable for viral infection *in vivo*. Conversely, *in vitro* passaging of both clinical strains and recombinant HMPV viruses in different cell lines, very quickly (after 4 passages) introduces mutations in the SH gene that result in frame-shifts leading to truncated SH proteins [32, 317].

Similarly to the HRSV SH protein, the HMPV SH protein has recently been shown to oligomerize into a viroporin structure [211]. The insertion of small, hydrophobic proteins into the membrane of an infected cell, followed by their oligomerization creates a typical hydrophilic pore with hydrophobic amino acid residues facing the phospholipid bilayer and hydrophilic residues forming part of the pore [115]. Viroporin formation modifies several cellular functions, including membrane permeability, Ca^{2+} homeostasis, membrane remodelling and glycoprotein trafficking [235]. Consequently viroporins have been proposed to be involved in virion assembly and release [115, 235]. However, to date, the HMPV SH protein has not yet been investigated in this regard.

Finally, HMPV SH has been suggested to alter NF-κB transcriptional activity, leading to enhanced secretion of proinflammatory cytokines [24], but no significant differences in expression of such genes were observed by microarray analyses [76].

3.2.2. Structural protein

3.2.2.1. Matrix protein

The HMPV M gene contains 765 nucleotides encoding a protein of 254 amino acids with a molecular mass of 28 kDa. The crystal structure of the HMPV M protein has recently been solved (Figure 4) [185]. These analyses have shown that HMPV M is a dimer and Ca^{2+} stabilizes the structure. HMPV is the only virus so far for whom a Ca^{2+} stabilized matrix protein has been demonstrated [15]. Similarly to other viral matrix proteins, the HMPV M protein self-associates into higher-order structures. This self-assembly is important for virion assembly and release, but filaments of multimeric HMPV M proteins have also been shown on the surface of infected cells [282], secreted M protein has been found in the supernatant of HMPV infected cells [20] and the HMPV M protein self-assembles into secreted virus-like particles (VLPs) [67, 68].

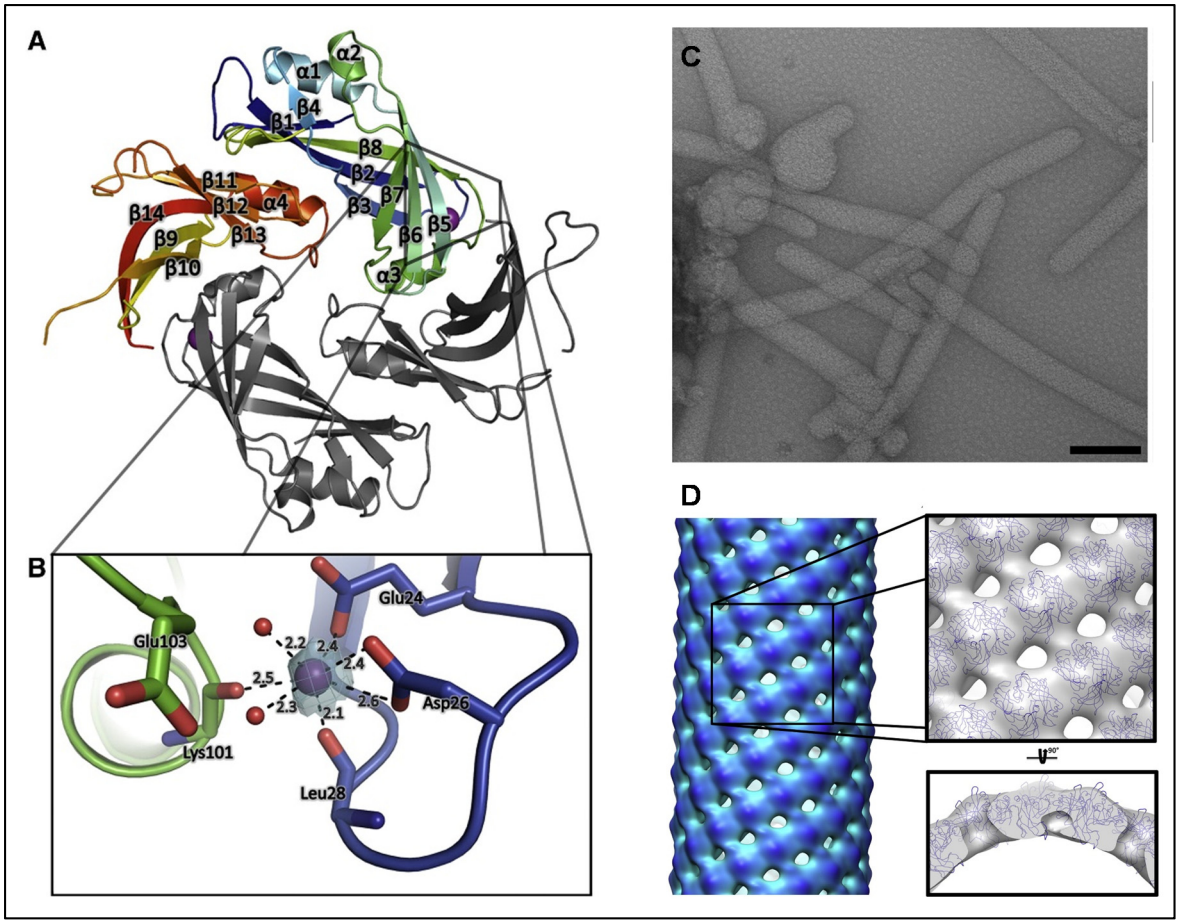

Figure 4: Structure of the HMPV matrix protein (adapted from [185]):
(A) Structure of the M dimer. One of the monomers is colored from blue (N terminus) to red (C terminus) with secondary structure elements labeled, whereas the other one is in gray. (B) Close-up of the Ca^{2+} binding site. The Ca^{2+} ion is represented as a purple sphere, Coordinating water molecules are displayed as non-bonded spheres. (C) Samples of M incubated in the presence of lipids stained with uranyl acetate revealed tubular and spherical structures with free M in the background. Scale bar = 100 nm. (D) A representation of the density map for lipid-bound M, A close-up of the map (gray transparent surface) shows the packing of the fitted M (blue) after imposing helical symmetry, viewed from above and sideways.

3.2.3. Proteins involved in viral replication

Limited data about HMPV replication is currently available; most information has been inferred from analysis done on HRSV and other members of the *paramyxoviridae* family. HMPV replication involves 5 viral proteins. The nucleoprotein (N), phosphoprotein (P) and large

polymerase protein (L) are essential to viral replication, whereas the transcription anti-termination factor (M2-1) and RNA synthesis regulatory factor (M2-2) are dispensable *in vitro*.

3.2.3.1. Nucleoprotein

The HMPV N gene contains 1185 nucleotides, and codes for a protein of 394 amino acids, similar to the size of other pneumovirus N proteins [325]. The N protein of the *paramyxoviridae* serves several functions in the viral life cycle, including encapsidating the genomic RNA and protecting it from cellular nucleases, association with the L polymerase and phosphoprotein during replication and transcription, and interaction with the M protein during virus assembly. Furthermore, the HMPV N protein is essential to viral rescue of recombinant HMPV viruses using reverse genetics (see chapter 1 section 9.2.1 live-attenuated vaccines) [140]. The N protein could also be considered a structural protein, since N proteins expressed in yeasts self-assemble into nucleocapside-like structures [259].

3.2.3.2. Large polymerase protein

The last ORF of the HMPV genome contains 6018 bp and encodes for the largest viral protein of 2005 amino acids, an RNA-dependent RNA polymerase (RdRP) [325]. However to be functional, the L protein needs to form a ribonucleoprotein complex with the viral RNA and the phosphoprotein P.

The HMPV L protein contains the same 6 conserved regions (CR) as those found in L proteins of other viruses of the mononegavirales order [325]. CRI is implicated in recruitment of N protein during Sendai virus replication and interaction with P protein, while CRII appears to play a role in template binding [187]. For all non-segmented negative-sense RNA viruses, The RdRP activity maps to CRIII as does poly-adenylation, whereas CRIV appears to be of structural importance [266].

Capping of VSV mRNA is achieved by a polyribonucelotidyltransferase, located in CRV. Finally, CRVI has been shown to function as the mRNA cap methylase in HMPV as in other viruses [369].

3.2.3.3. Phosphoprotein

The P gene contains 885 nucleotides, encoding a highly phosphorylated, 294 amino acid-long protein, which is 53 residues longer than its HRSV homologue and shares only 28% identity with the HRSV P protein [326]. Like P proteins of other members of the *paramyxoviridae* family, the HMPV P protein is an intrinsically disordered protein that forms tetramer using a central α-helical coiled-coil region. Based on studies done on other paramyxoviruses, the P protein recruits the large polymerase L to the viral N-RNA template through direct interactions with N and L. P also chaperones the newly synthesized N and sequesters it in the form of an RNA-free NP complex [183]. It appear that host cell kinases are able to phosphorylate the P protein, but the effect of these phosphorylations on viral replication remains to be determined [106].

3.2.3.4. Matrix 2 proteins

The M2 gene, containing 2 overlapping ORF's, is unique to the *pneumovirinae* sub-family. The M2-1 protein contains 187 amino acids whereas the M2-2 contains 71 amino acids [51, 325]. M2 proteins are highly conserved within this subfamily and like all other M2-1 proteins of the pneumovirus subfamily; the HMPV M2-1 protein contains a conserved Cys/His zinc finger-like motif at its N terminus.

The closely related HRSV M2-1 protein has been found to be involved in viral transcription (as an essential transcription elongation factor as well as an antiterminator) and nucleocapsid

formation (binding both viral RNA and the HRSV N protein). In addition, the HRSV M2-1 protein appears to be essential for the recovery of recombinant HRSV from transfected cDNA [51]. In contrast, the HMPV M2-1 ORF is dispensable for virus recovery and replication *in vitro* [51]. Moreover, deleting M2-1 from HMPV viruses does not affect the production of full-length mRNAs, nor does it modify the frequency of read-through mRNAs *in vitro* [51]. However, HMPV M2-1 was recently found to bind the HMPV P protein as well as viral RNA, indicating some sort of transcription regulation function [184]. In fact, i*n vivo*, HMPV lacking M2-1 could not be demonstrated to replicate in the lungs of infected hamsters, nor were there detectible levels of HMPV neutralizing antibodies present in the serum of these infected hamsters [51].

The HRSV M2-2 functions as a regulatory factor that helps switch the viral RNA synthetic program from transcription to RNA replication. HRSV lacking M2-2 was highly attenuated, immunogenic and protective in chimpanzees [318]. The deletion of M2-2 from HMPV viruses did not alter viral growth *in vitro*, yet an increase in transcription as well as in viral proteins within infected cells was observed. *In vivo*, HMPV lacking M2-2 is highly attenuated and highly immunogenic [51]. Contrary to HMPV M2-1, M2-2 has been found to bind the L protein [162]. In addition over-expression of M2-2 prior to HMPV infection reduces viral RNA synthesis, supporting a regulatory function [162].

Finally, a role in immune evasion has been suggested for HMPV M2-2, by targeting innate antiviral signaling directly but also indirectly, by inhibiting the expression of viral proteins that can act as virulence factors of HMPV [273, 274].

3.3. Viral cycle

Most of the attention in elucidating the viral cycle of HMPV has focussed on viral entry. What happens once the virus has entered the host cells has thus far been inferred from other paramyxoviruses. An overview of the proposed viral cycle is represented in figure 5.

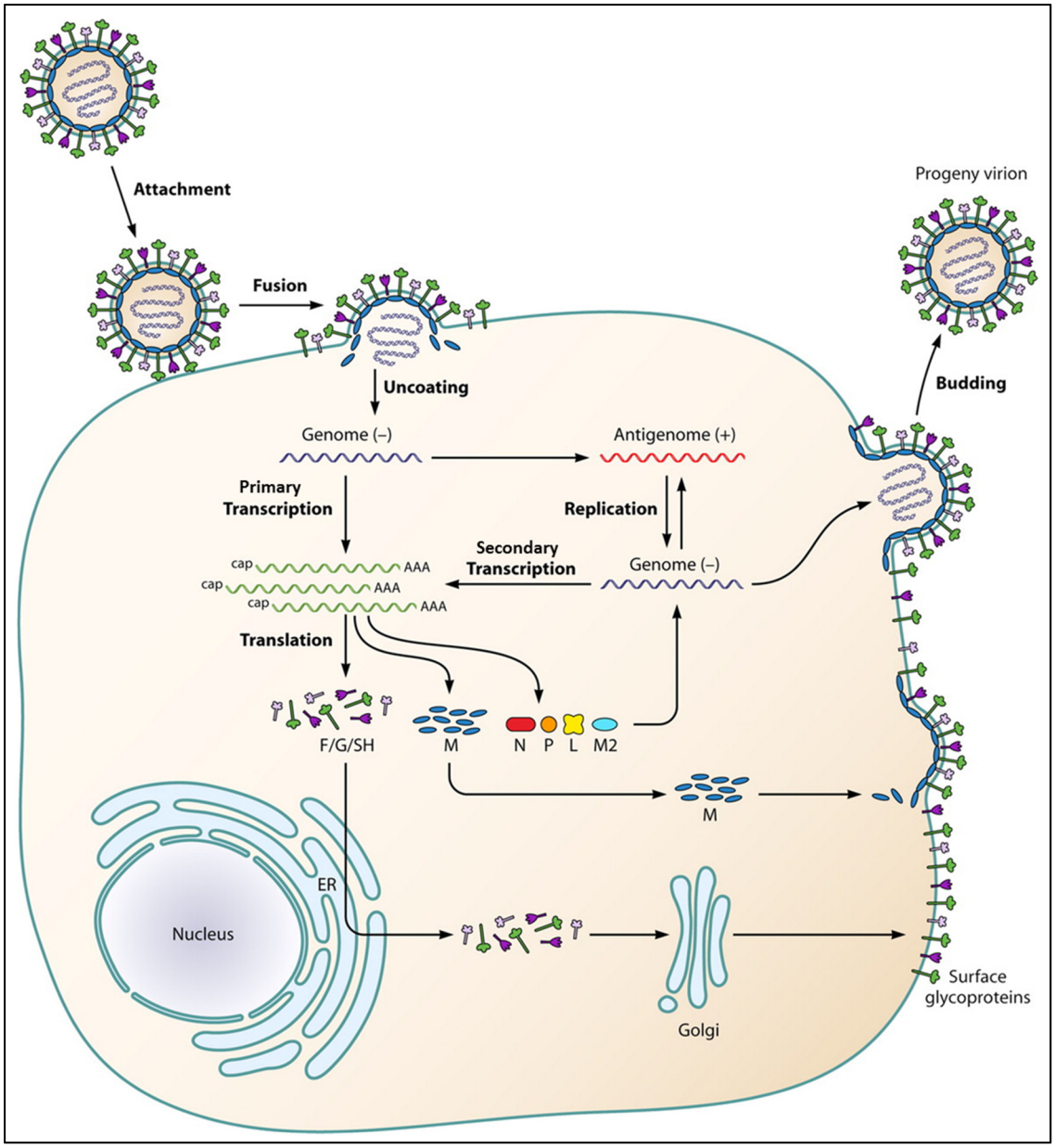

Figure 5: HMPV viral cycle (adapted from [290]):
Schematic representation of the HMPV viral cycle; After attachment of the virion to the plasma membrane, the viral and plasma membranes fuse, resulting in uncoating of the virion and release of the RNP into the cytoplasm. After primary transcription, the genome is replicated to produce the antigenome. The antigenome is used to synthesize genomic RNA, which is incorporated into progeny virions or used as a template for secondary transcription. After translation, M proteins and RNPs are transported intracellularly to the plasma membrane and the viral glycoproteins F, G and SH are transported from the endoplasmic reticulum (ER) to the Golgi apparatus and then to the plasma membrane. Finally, new virions are assembled and are subsequently released from the plasma membrane by the budding process.

3.3.1. Attachment and Fusion

Unlike other members of the paramyxovirus-family, pneumoviruses such as HMPV and HRSV do not require two surface glycoproteins (an attachment protein and a fusion protein) for viral entry *in vitro* [69].

The conserved arginine-glycine-aspartate motif at position 329-331 is unique to the HMPV F protein within the paramyxovirus family (Figure 3) [70]. The RGD motif is a recognition sequence for a specific subset of cell-surface molecules called integrins, specifically of αVβ1 integrins. Use of RGD-binding integrins as a (co-)receptor has been demonstrated for several viruses, yet so far, not for any other paramyxoviruses [70]. Inhibition of RGD-binding by integrins, using EDTA, peptides, monoclonal antibodies (mAb) and siRNA has shown to inhibit HMPV infection (but not HRSV infection) *in vitro*, still it only inhibited 50% of viral binding [68-70]. The authors conclude that although HMPV F protein interaction with cell surface integrins is necessary for HMPV attachment, it is not sufficient. In fact, another group identified heparan sulfate, a glycosaminoglycan, as a receptor for the HMPV F protein [58]. It has recently been suggested that HMPV attachment might occur in multiple phases, in which the F protein first binds to heparan sulfate before interacting with proteinaceous receptors, including RGD-binding integrins [69]. Others have found the HMPV G protein capable of binding heparan sulfates as well [320]. Although the G protein is dispensable *in vitro*, HMPV viruses lacking the G protein are attenuated *in vivo*, suggesting that while the G protein

is not necessary for membrane fusion and viral entry, G protein binding to glycosaminoglycans might be needed to concentrate virions at the cell surface in order for F protein dependent fusion and entry to occur. The importance of glycosaminoglycans in viral attachment has been demonstrated by the effect of a vast range of experimental molecules, inhibiting glycosaminoglycan-binding, on HMPV infectivity (see chapter 1 section 9.2.4: Glycosaminoglycans).

In order for the HMPV F protein to be activated, the F_0 precursor polypeptide needs to be cleaved into the fusion-competent F_1/F_2 heterodimer. For many paramyxoviruses, the F_0 protein is cleaved intracellularly by host proteases, such as furin. The minimal consensus motif for furin is R-X-X-R↓. Although the sequence at the HMPV F cleavage site (RQSR↓) matches the minimal requirements for furin recognition, all HMPV clinical isolates require the addition of extracellular proteases (in particular trypsin) to grow *in vitro*. Whether furin participates in HMPV F protein activation *in vivo* remains to be evaluated. Interestingly, The F proteins of a few HMPV strains have been shown to require a low pH pulse to be activated *in vitro*. This pH-dependency was attributed to an E294G mutation in the F gene [143], a mutation that was only found in 6% of published HMPV F gene sequences [69, 143], making this an anomalous rather than a general phenomenon.

The exact trigger of membrane fusion is currently unknown; however, studies done on AMPV F proteins and pH-dependent HMPV F proteins indicate that electrostatic repulsion between regions containing clusters of charged residues may be involved [69]. Regardless of the trigger, HMPV mediated membrane fusion is thought to follow the general model established for membrane fusion mediated by viruses of the *Paramyxoviridae* family (Figure 5). The melting of the HRB helices, while leaving the HRA in the prefusion state, may result in a reduced conformational stability in the other regions of the protein. This is thought to result in refolding of DIII (magenta region in figure 5), the coiled-coil formation of the HRA as well as translocation of the FP towards the cell-

membrane. This state is called the pre-hairpin state. Removal of the FP from the head region of the F protein results in more conformational changes that compact the head and approach HRA and HRB, finally resulting in the formation of the irreversible 6-helix bundle (6HB) and concomitant membrane fusion.

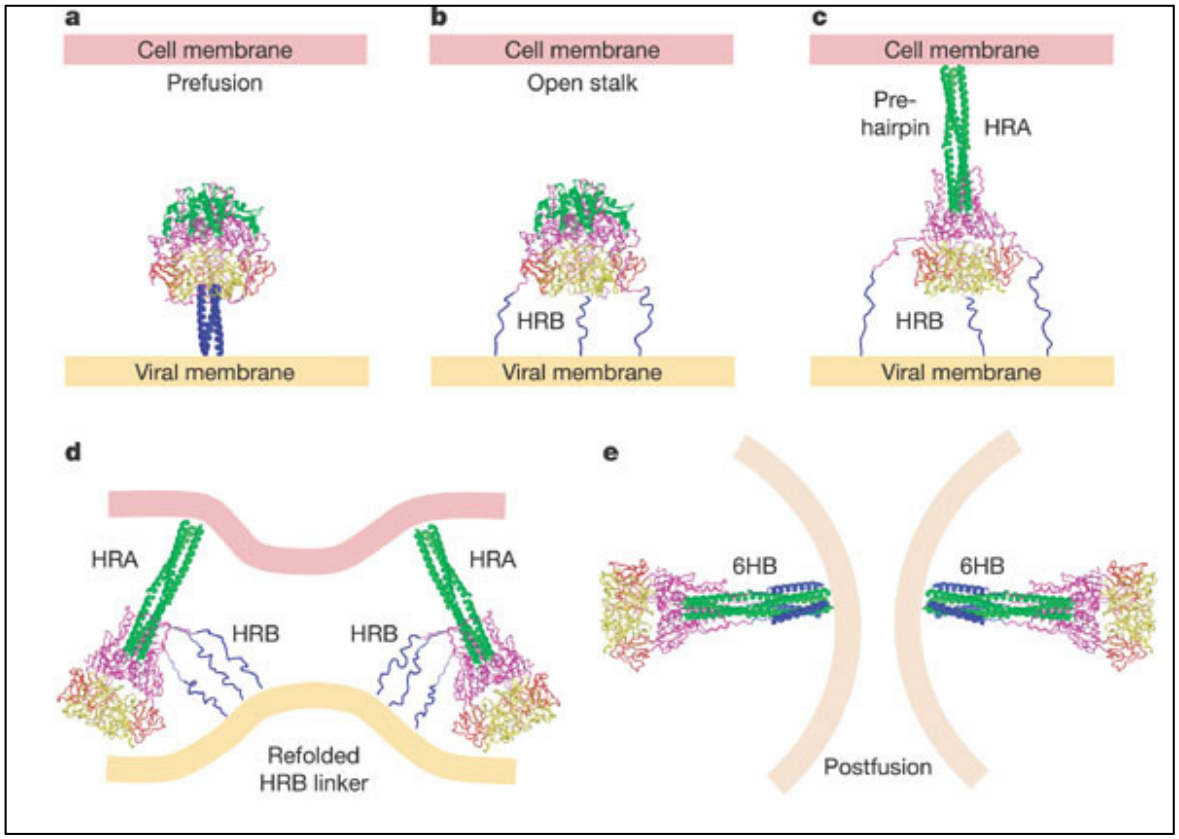

Figure 6: F-mediated membrane fusion (adapted from [362]):
HRA is green, HRB is blue and DI, DII and DIII are yellow, red and magenta, respectively. (a) Structure of the prefusion conformation. (b) 'Open stalk' conformation; the HRB stalk melts and separates from the prefusion head region. (c) A pre-hairpin intermediate is formed by refolding of DIII, facilitating formation of the HRA coiled coil and insertion of the fusion peptide into the target cell membrane. (d) Before formation of the final 6HB, folding of the HRB linker onto the newly exposed DIII core stabilizes the juxtaposition of viral and cellular membranes. (e) The formation of the postfusion 6HB is tightly linked to membrane fusion and pore formation.

Following membrane fusion, the viral RNP complex containing the negative-sense viral RNA (vRNA) genome is released into the cytoplasm, where it serves as a template for the synthesis of mRNA and antigenomic cRNA.

3.3.2. Transcription, translation and replication

The exact driving force behind the uncoating of paramyxovirus nucleocapsids that have entered a host cell is still unknown. All the same, all paramyxoviruses replicate in the cytoplasm. After uncoating, the viral RNA (vRNA) is used as a template for primary transcription by the RdRP complex, consisting of at least the L and P proteins and possibly M2-1. The RdRP enters the N protein encapsidated vRNA at the 3' entry site and starts the synthesis of mRNA until it reaches the gene end region (GE) of the first gene (the N gene). This GE region contains the transcription termination signal as well as a U tract that will sever as a template for poly-adenylation of the mRNA. The RdRP remains attached to the template and moves through the very variable intergenic region (IG) until the gene start (GS) region of the downstream gene is reached. The GS directs re-initiating of the RdRP as well as the addition of a methylated 5' guanine cap to the mRNA. This re-initiation is not perfect and polymerase falloff leads to a gradient of mRNA's with the greatest abundance of N mRNA's and the least amount of L mRNA's. Read-through can lead to polycistronic mRNA whose function is thought to be either to increase the transcription of the more distal genes or to down regulate the expression of more proximal genes (reviewed in [177]). For HMPV specifically a high level of polycistronic SH genes have been observed [36, 286]. The M2-1 protein may also serve as a transcription elongation factor, controlling RdRP activity [184]. The produced mRNAs are subsequently translated using the host cells translation machinery.

After the primary transcripts are translated and viral proteins accumulate, the RdRP uses the same vRNA template to synthesize a positive sense antigenome strand (cRNA), by ignoring all gene-junction signals. During cRNA synthesis, the newly produced RNA immediately assembles with newly synthesized N proteins. The N-associated cRNA is then used as template to create a new strand of negative sense genomic RNA (vRNA). Newly synthesized vRNA also assembles with N

proteins as soon as it leaves the RdRP complex. The newly synthesized vRNA can serve 3 functions; (1) serve as additional templates for transcription in order to increase the amount of viral proteins, (2) serve as template for more antigenomic RNA (cRNA) or (3) be encapsidated and incorporated into progeny virions (reviewed in [177]).

Several mechanisms are thought to assure the proper timing of the RdRP switch from mRNA synthesis to cRNA and vRNA synthesis. The quantity of free N proteins is one of the limiting factors that could direct the RdRP towards either transcription (low N concentration) or RNA synthesis (high N concentration). Also newly synthesized L protein is needed for cRNA and vRNA synthesis. Since the L gene is the most distal of genes to be transcribed, the presence of sufficient L protein may also be a restricting feature. Finally, the M2-2 protein has been identified as a RNA synthesis regulation factor for HRSV [31] and a similar function has been suggested for the HMPV M2-2 protein [286].

3.3.3. Assembly and budding

During replication the HMPV glycoproteins, matrix proteins and RNP complexes are synthesized at distinct sites within the host cell. The glycoproteins, on one hand, are produced in the endoplasmic reticulum where their proper folding and oligomerization occurs. They are then trafficked by means of the secretory pathway to the plasma membrane, fist passing through the Golgi apparatus, where glycosylation occurs. The viral glycoproteins are believed to cluster within lipid raft micro-domains within the cell membrane [97].

The matrix proteins and RNP's, on the other hand, are synthesized in the cytoplasm. Newly synthesized vRNA is swiftly encapsidated by the N protein into a helical RNP that subsequently associates with newly synthesized L-P and M2-1 complexes. Consequent interactions between the

RNP complex and the M protein are thought to mediate targeting of the RNP to the budding site at the plasma membrane although the exact mechanism of transport is still unknown [97].

Once all of the viral components are present at the budding site, the oligomerized M proteins, bound to the cytoplasmic tails of the glycoproteins, the RNP and the cell membrane, initiates the deformation of the membrane that leads to the budding and release of the newly formed viral particle [97].

Finally, the recent discovery of a Ca^{2+} binding site in the HMPV M protein [185], combined with the identification of the SH protein as a viroporin [211], makes it tempting to speculate that a change in intracellular Ca^{2+} concentration could play an important role in HMPV assembly and/or budding [115, 235].

4. Diagnosis

Rapid and accurate detection of HMPV is desirable in order for appropriate control measures to be put in place and particularly to limit or prevent the unnecessary use of antibiotics. Nasopharyngeal swabs or aspirates are the optimal specimens for HMPV diagnosis [114, 248]. A number of different techniques are available for HMPV diagnosis and they will be discussed in the following sections.

4.1. Cell culture

HMPV growth in cell culture is fastidious and requires regular supplementation with trypsin. This is likely to be one reason for its late discovery. Reliable cytopathic effects (CPE) have been

reported in tertiary monkey kidney (TMK), Rhesus monkey kidney cells (LLC-MK2) cells and African green monkey kidney cells (Vero cells) [83, 321, 326]. Some studies have also demonstrated the susceptibility of cell lines of human origin [27, 289]. The CPE are variable and strain-dependent; ranging from syncytia formation (i.e. the fusion of adjacent cell membranes resulting into one giant multinucleated cell) to focal rounding and cell lysis (Figure 7). CPE are usually observed after 10 to 14 days of culture.

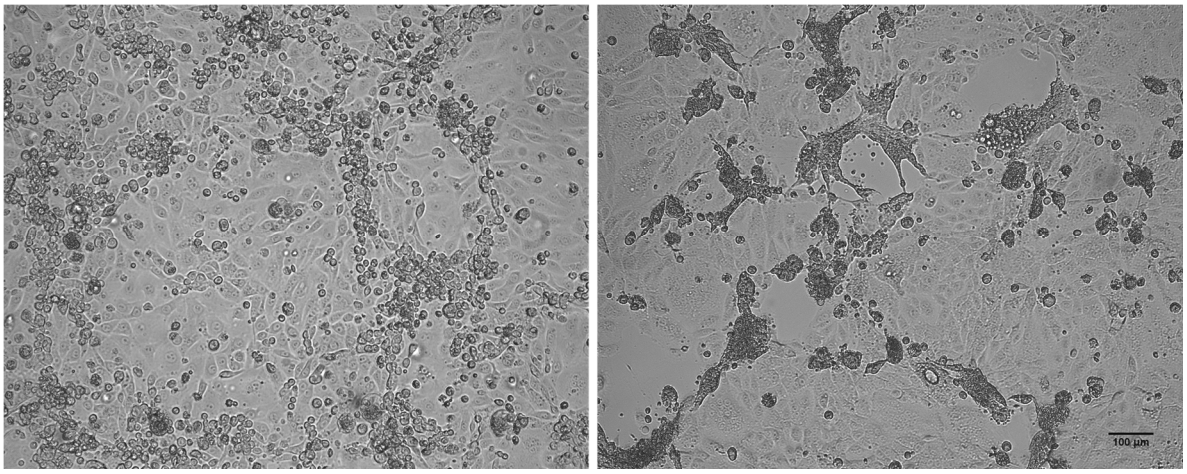

Figure 7 : Cytopathic effect (CPE) induced by HMPV:
On the left infection of LLC-Mk2 cells with HMPV leads to focal rounding of infected cells. On the right infection of LLC-MK2 cells leads to the formation of syncytia.

4.2. Immunofluorescence

The direct immunofluorescence assay (DFA) uses labeled antibodies directed against viral antigens to detect HMPV directly in patient samples without the need for amplification. This test is fast (within 2h) and can be multiplexed (using multiple antibodies directed against different respiratory pathogens) although this reduces sensitivity [290]. In addition the sensitivity of methods detecting HMPV antigens (such as DFA and enzyme linked immunosorbent assays (ELISA)) appears to vary depending on patient populations, sample type and time point within the course of the disease [248].

4.3. PCR

Real Time-PCR is considered the method of choice for the diagnosis of acute HMPV infections. Rapid and sensitive RT-PCR assays have been described allowing for the amplification of different viral genes (more frequently the L, N or F genes) and their detection within 2 h [66, 203]. The N gene seems to be a particularly attractive target capable of detecting all 4 HMPV subgroups [207]. However, to date there is no cross-laboratory standardization. Multi-parametric approaches for the identification of the causative agent of RTIs are now also available. The most common platforms, able to discriminate multiple specific pathogens simultaneously, consist of a multiplex PCR followed by hybridization of the amplified PCR products to specific capture probes. The oligonucleotide capture probes can be immobilized onto a variety of surfaces depending on the technology (reviewed in [271]). More recently, multiplex PCR has been combined with mass spectrometry technologies to detect and identify respiratory pathogens [271]. Although very accurate and fast, this technology is very expensive.

4.4. Virus quantification

Different approaches can be taken to quantify the virus in a given sample. Physical assays measure an amount of viral proteins, or nucleic acids (e.g. hemagglutination, quantitative PCR, and immunological assays such as enzyme-linked immunosorbent assays (ELISA)) [63]. As mentioned earlier, these approaches are often used to quantify viral load in clinical samples. Notably, the use of a hemagglutination assay is not possible in the case of HMPV, since the virus does not carry a HA protein at its surface.

Biological assays, on the other hand, measure infectivity and are frequently used for laboratory strains. Most biological assays are end point assays that look at infectivity at a given time

point. It is important to note that any type of quantification will be arbitrary and relative [63]. In plaque assays, commonly used for lytic viruses, cell monolayers are infected with serial dilutions of the viral culture and are subsequently overlaid with a semisolid medium (usually agar- or methylcellulose-containing mediums) preventing the spread of infection. As the virus grows, it lyses the infected cell, but spread is limited to adjacent cells only. This results in 'holes' in the cell monolayers known as plaques that can be counted. Viral concentrations are then expressed as plaque forming units (PFU). Unfortunately, HMPV is not a lytic virus and consequently does not induce plaques. However, immobilized HMPV infected monolayers can be stained using antibodies directed at viral proteins in order to detect and count areas of infection. Alternatively, confluent monolayers are infected with serial dilutions of the HMPV culture and incubated until CPE are present. The dilution that results in CPE in 50% of the inoculated replicates is considered the tissue culture infectious dose 50 ($TCID_{50}$). When CPE are difficult to assess or a virus does not induce CPE, laboratory viruses can be genetically engineered to express a reporter gene [35, 140].

Recently an electronic cell sensor array was developed, allowing for real time cell monitoring [319]. Culture wells are coated with gold micro-electrodes, which measure the electronic impedance in the well in real time. When cells are added to the wells they attach and grow, changing the impedance in the well. A parameter called cell index is used to quantify cell status based on the detected impedance (figure 8). Infecting these cells allows for real-time monitoring of virus-induced cell status changes and consequently the formation of CPE.

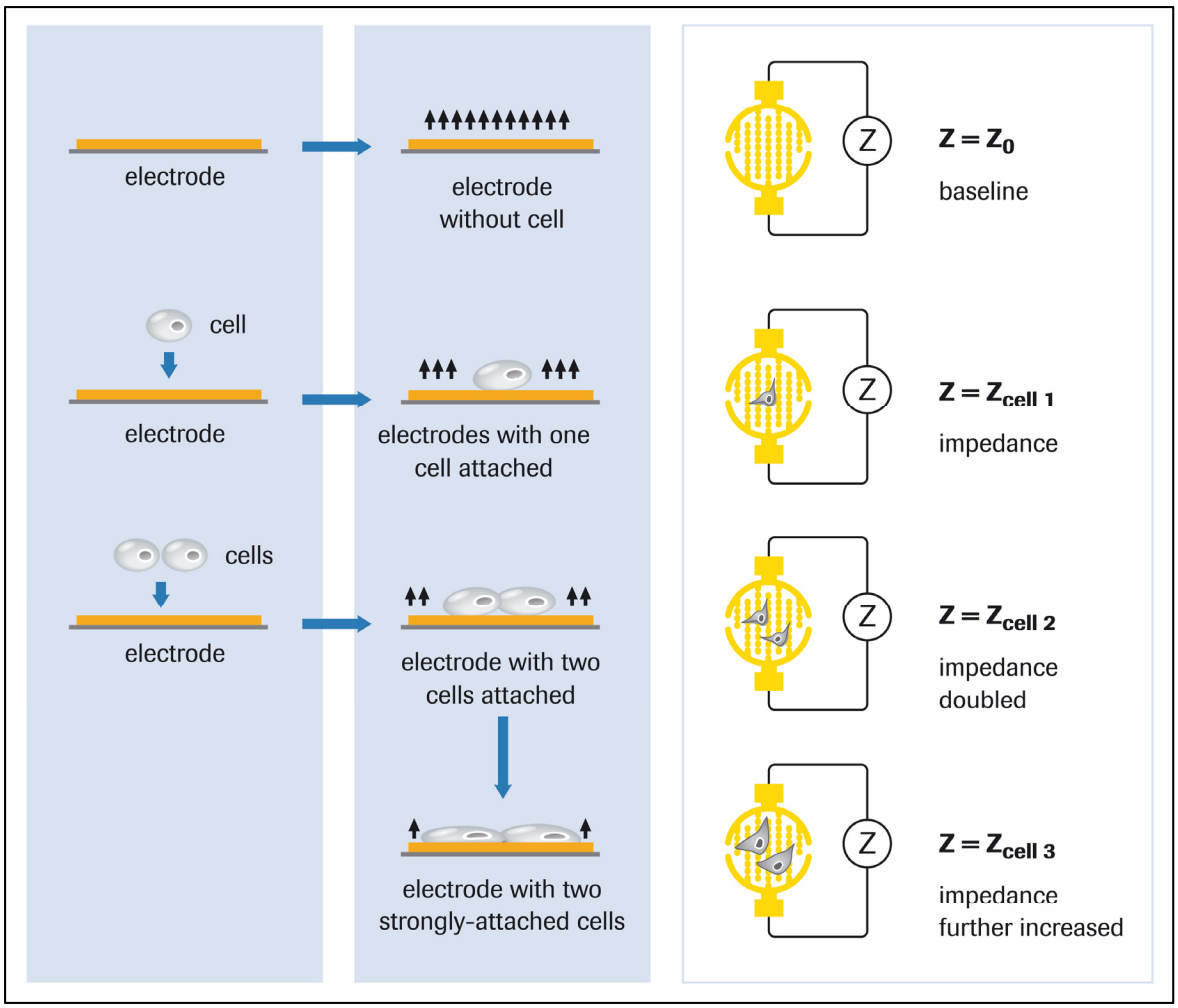

Figure 8: Real-time cell analysis (RTCA) (Source: ACEA Bioscience, Inc):
Representation of a well seeded with cells during RTCA. When only medium is present in the wells a baseline impedance (z_0) is measured (top). Cells are added to the wells and the impedance increases (z_1) (second line). As the cells proliferate (z_3) and attach to the wells (z_4) impedance increases further (bottom).

4.5. Serology

The indirect detection of anti-HMPV antibodies in patient sera can be achieved using immunofluorescence, ELISA or by determining neutralizing antibody titers. However, serology has limited diagnostic value and is usually used for epidemiological studies, for instance to study the magnitude of an outbreak [316], but could also be used to study vaccine efficacy.

5. Epidemiology and clinical features

5.1. Epidemiology

HMPV is one of the leading cause of bronchiolitis (second to HRSV) accounting for 5-10 % of hospitalization for ARTI in young children [18, 39, 40, 72, 93, 99, 105, 111, 215, 240, 251, 339, 366]. Also, HMPV has been associated with 12-15 % of consultations for LRTI and URTI in outpatient children [93, 237, 251, 346, 357]. The contribution of HMPV in respiratory syndromes of adults has not been studied as extensively, but HMPV has been identified in approximately 5-10 % of young and elderly adults with ARTI [101, 306, 338, 343] as well as in 3-5 % of adults with exacerbation of chronic obstructive pulmonary disease or community-acquired pneumonia [129, 153, 154, 371].

5.1.1. Geography and seasonality

Since its initial discovery in 2001 [326], HMPV has been found on all continents [3, 7, 22, 92, 99, 102, 103, 112, 198, 200, 215, 237, 254, 272, 322, 357]. In temperate countries, HMPV seems to have a seasonal distribution overlapping with human respiratory syncytial virus (HRSV) with most cases reported during the winter/spring months [2, 3, 10, 39, 193, 224, 245, 276]. In tropical and subtropical countries, HMPV periodicity is more variable [7, 102, 112, 206, 254]. A clear correlation between air temperature or air humidity and HMPV prevalence has only been found in a study in Nepal [212], whereas a study in China [186] and one in Brazil [112] were unable to detect a correlation between meteorological conditions and HMPV prevalence.

5.1.2. Seroprevalence

Despite its recent discovery, HMPV is not a new pathogen. Serological evidences of human infection can be traced back to the 1950's [326] and molecular evolution studies modeled the current genetic diversity of HMPV to have appeared about two centuries ago [77, 359, 360].

After birth, maternal anti-HMPV-antibody titers slowly decline until between the 3rd and 6th month of life at which point seropositivity reaches its lowest point and the risk of HMPV infection is greatest [89, 92, 181]. Practically all children have been infected with HMPV by the age of 5 to 10 years [22, 91, 103, 195, 198, 200, 202, 272, 368]. Reports of severe HMPV infections in adults [41, 56, 125, 149, 154, 188, 301, 308, 316] suggest that despite universal infection in childhood, re-infections can occur throughout life.

5.2. Clinical features

HMPV is associated with ARTI in all age groups with severe diseases occurring most frequently in young children, elderly individuals and immunocompromised hosts [39, 40, 101, 128, 330, 335].

Due to the overlapping seasonality of HMPV and HRSV, these viruses are often co-detected. Some reports have indicated that such dual infections are associated with severe bronchiolitis, raising the question whether HMPV could be a determinant of HRSV disease severity [104, 119, 169, 217, 294]. However, this association has not been found in other studies [40, 52, 99, 208, 356]. In fact it should be taken into consideration than the co-detection of viral RNA could be due to the presence of residual viral material from a previous infection.

5.2.1. HMPV in children

Compared to HRSV infections, primary HMPV infections tend to occur at an older age, i.e. between 6 to 12 months compared to 2 to 3 months [8, 248]. Also, more severe clinical outcomes may be associated with HRSV than HMPV [248]. The clinical features associated with HMPV infections in young children are very similar to those induced by HRSV (Table 2) [46, 248], comprising both symptoms of upper and lower respiratory tract infections. Most commonly, bronchiolitis with or without pneumonitis is diagnosed [248]. HMPV has repeatedly been detected in children with acute otitis media [137, 238, 287, 311, 349, 350]. Acute wheezing is common during HMPV infection, and establishing a diagnosis of asthma during infancy is difficult. Subsequently, studies on the causative role of HMPV in asthma have produced conflicting results [110, 152, 269, 288, 346, 347].

Table 2 : Symptoms of HMPV and HRSV infections in hospitalized children (adapted from [209])

	Percentage of HMPV cases	Percentage of RSV cases
Cough	46–92	97–100
Fever	50–92	45–65
Respiratory distress	43–83	36–78
Rhinitis/rhinorrhea	57–83	56–82
Respiratory crackles	57	27–72
Wheezing	45–50	45–78
Pharyngitis	19–43	45–54
Otitis media	16	31
Conjunctivitis	14	9

Children with underlying medical conditions such as prematurity, cardio-pulmonary problems and immunosuppression are more susceptible to severe HMPV disease leading to hospitalization [1, 11, 40, 86, 93, 94, 127, 163, 214, 229, 251, 256, 295, 302, 343]. Two studies have also found a higher viral load to be associated with more severe HMPV disease [47, 279].

5.2.2. HMPV in adults

HMPV has been associated with flu-like illnesses and colds in healthy adults [100, 101, 338]. Walsh et al. observed a higher rate of flu-like illnesses among HMPV-infected young adults although older adults experienced more dyspnea and wheezing [338].

HMPV-infected elderly patients may develop bronchitis and pneumonia, some of which can be fatal. The virus has been responsible for outbreaks of severe respiratory tract infection in long-term care facilities [41, 188, 199, 316] with case fatality rates ranging from 0% [199] to 10% [41] and even 30% [188].

HMPV has also been associated with fatal lower respiratory tract diseases in hematopoietic stem cell transplant recipients [53, 95, 98, 275] and severe complications in solid organ transplant recipients [145, 270, 308].

On the other hand, asymptomatic HMPV infections have also been described, in severely immunocompromised [1, 80, 81] and healthy adults [338].

6. Pathogenesis in humans and laboratory animals

Only a limited number of studies on the pathogenesis of HMPV in humans have been published. Most studies focused either on histopathology findings or cytokine levels in severe cases of HMPV in individuals with underlying conditions. Most information about HMPV pathogenesis and immune responses induced by HMPV has been determined in animal models, predominantly small rodents and non-human primates.

6.1.1. Transmission

The incubation period has been estimated to be 4 to 9 days [90, 160, 213, 254]. In a recent study, in which adults were experimentally infected with HMPV, clinical symptoms and shedding of challenge virus coincided. Viral shedding appeared later and persisted for longer than has been described for other paramyxoviruses [62, 180, 312] with viral shedding peaking on days 6-9.

HMPV is thought to be transmitted by close contact with contaminated secretions such as saliva, droplets or large particle aerosols as is the case for HRSV [126], although fomites may also be a source of contamination [228, 321].

6.2. Immune response

The primary targets of HMPV viruses are epithelial cells in the respiratory tract [173, 308]. When HMPV is introduced into the respiratory tract, like any other respiratory pathogens, it first needs to overcome the physical barrier of secreted mucus and ciliary sweeping. Once HMPV infects an epithelial cell, intracellular signaling pathways are activated which result in the activation of anti-viral and pro-inflammatory mechanisms. Resolution of HMPV infection requires both innate and acquired immune responses. In this regard, HMPV appears to induce a more robust innate immune response compared to HRSV, yet a weaker acquired immune response [88, 147]. However, some conflicting results have been obtained in various animal models (Table 3), possibly due to the use of different strains, inoculums, time points and detection techniques, and in clinical studies as well (see section 7.3 Pathogenesis in humans).

Persistent infections in animal models as well as the occurrence of reinfections in humans throughout life, suggest that HMPV is capable of impairing the immune response to infection. HMPV

lacks the NS1 and NS2 proteins [29] whose function in HRSV it is to inhibit innate immune response mechanisms [48]. It is likely that other HMPV proteins have taken over the function of NS1 and NS2.

Table 3 : Animal studies comparing the immune response of HMPV to that of HRSV

Reference publication	Aspect under investigation	conclusions
[121]	Role of IFNα during HMPV and HRSV infection in BALB/c mice	HRSV was significantly less sensitive to IFNα than HMPV. HRSV was a weaker inducer of IFNα production than HMPV. Yet when IFNα was induced prior to infection, both viruses abrogated the production of IFNα.
[122]	Lung cytokine and chemokine production by BALB/c mice infected with HMPV and HRSV	HMPV infection induced lower levels of the inflammatory cytokines IL-1, IL-6 and TNFα, but was a more potent inducer GM-CSF and triggered a more sustained production of the CXC chemokine KC compared to HRSV. HMPV was a stronger inducer of both IFNα and IFNγ than RSV. HMPV failed to induce detectable IL-10 or IL-12p70 but was a potent inducer of IL-12 p40.
[147]	HMPV and HRSV were compared for replication, pathogenesis and immune induction in BALB/c mice.	HRSV replicated to higher titers and for a longer period of time than HMPV in both URT and LRT. Clinical signs were significantly more severe after HMPV infection. Yet, there was similar recruitment of T lymphocytes with a predominance of IFN-gamma-producing CD8+ T cells. After HMPV infection, more neutrophils could be detected in the airways and there were more activated NK cells than in RSV-infected mice, which correlated with higher levels of IL-6, TNF-alpha and MCP-1.
[124]	Effect of HMPV and HRSV experimental infections on the phenotype and function of dendritic cell (DC) subsets that are recruited to the lungs of BALB/c mice.	Both HRSV and HMPV infection induced an impaired capacity of lung DC to produce IFNα as well as other cytokines. An impaired Ag-presenting function was also observed, as measured by decreased T cell proliferation following stimulation of T cells with DC from infected mice
[138]	induction of the CTL- mediated immune response and the induction of cytokines and chemokines in HMPV- and HRSV-infected BALB/c mice.	A similar pattern of cell-mediated responses was observed in HMPV- and HRSV-infected mice: T-cell immunity was associated with increased expression of Th1-type and antiviral cytokines, while a moderate increase in the expression of Th2-type cytokines was observed.
[85]	HRSV and HMPV infection in young and aged BALB/c-mice.	No differences in clinical signs among the tested groups. In young animals, NF-κB levels were elevated if infected with HMPV and HMPV+HRSV but remained low in HRSV infections In aged animals solely, HRSV-infected animals showed elevated levels of NF-κB.
[87]	The expressions of TLRs in airway epithelial cells and lungs of BALB/c mice infected by HMPV or HRSV were measured	Both HMPV and RSV infection up-regulated the expressions of TLRs and inflammatory cytokines in mice lungs. Specifically, TLR3 expression was elevated in vitro as in mouse lungs.
[167]	Involvement of alveolar macrophages (AM) in innate immune responses to HMPV and HRSV infections in BALB/c mice	HMPV-infected mice lacking AMs exhibited improved disease and reduced pulmonary viral titers compared with AM-competent mice. In contrast, AM depletion in the context of RSV infection was characterized by an increase in viral replication and worsened disease and inflammation.

6.2.1. Innate immune response

Innate immune responses provide the first line of defense during infection and influence both the quality and magnitude of the acquired immune response. Conserved structural motifs on pathogens called pathogen associated molecular patterns (PAMPs) trigger pattern recognition receptors, e.g. Toll-like receptors (TLRs) and RNA helicases, which initiate signaling cascades, activation of the transcription factors and the expression of proinflammatory and effector cytokines that will direct the acquired immune response [209].

10 members of TLRs have thus far been identified in humans and 13 in mice. Among those, TLR3, 4, 7, 8 and 9 have been shown to be more commonly involved in the innate response to viral infections [164]. The induction of cytokines and type I Interferon (IFN) by HMPV has been demonstrated to be TLR7- and TLR4-dependent [116, 165, 333]. However, TLR4 also contributes to HMPV disease pathogenesis.

The RNA helicases, retinoic acid inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA)-5, have also been shown to be implicated in the induction of cytokines and type I IFNs by HMPV (figure 9). In fact RIG-I, appears critical in reducing HMPV replication *in vitro* [189].

Several HMPV proteins have been described to antagonize these innate immune signals (Figure 9). The HMPV SH protein appears to affect NF-κB-dependent gene transcription by modulating NF-κB transcriptional activity and not by inhibiting nuclear translocation [24]; however, another group was unable to confirm this effect [76]. The HMPV G protein physically interacts with RIG-I and inhibits RIG-I-dependent IFN-β gene transcription [26], while HMPV M2-2 appears to interfere with MAVS-dependent IFN-β gene transcription [274]. However, siRNA directed against the HMPV G gene did not alter IFN-β gene transcription in infected A549 cells [265]. Surprisingly, the HMPV P protein of the subtype B strain

NL\1\99, but not of the subtype A strain NL\1\00, was found to inhibit RIG-I-dependent IFN-α/β gene transcription [116].

Dendritic cells (DC) are antigen-presenting cells (APC) that specialize in detecting pathogens and initiating both innate and adaptive immune responses. The central role of DCs in initiating and shaping the immune response together with their exposure to infectious virus makes them obvious targets for viral manipulation of the host immune response. Indeed, the HMPV G protein has been described to impair TLR4-dependent signaling in DCs [165]. On the other hand, secreted HMPV M proteins have been shown to induce maturation and cytokine production of naïve DCs [20].

Other cells of the innate immune response have received less attention. A recent study found an increase in natural killer cells (NK) in the lungs of HMPV-infected mice compared to uninfected mice. Yet, depletion of NK cells did not alter viral replication suggesting they do not play a role in the innate immune response to HMPV [341]. Interestingly, alveolar macrophages have been demonstrated to increase viral replication in mice, although the mechanism by with these innate immune cells support viral infection remains to be elucidated [167].

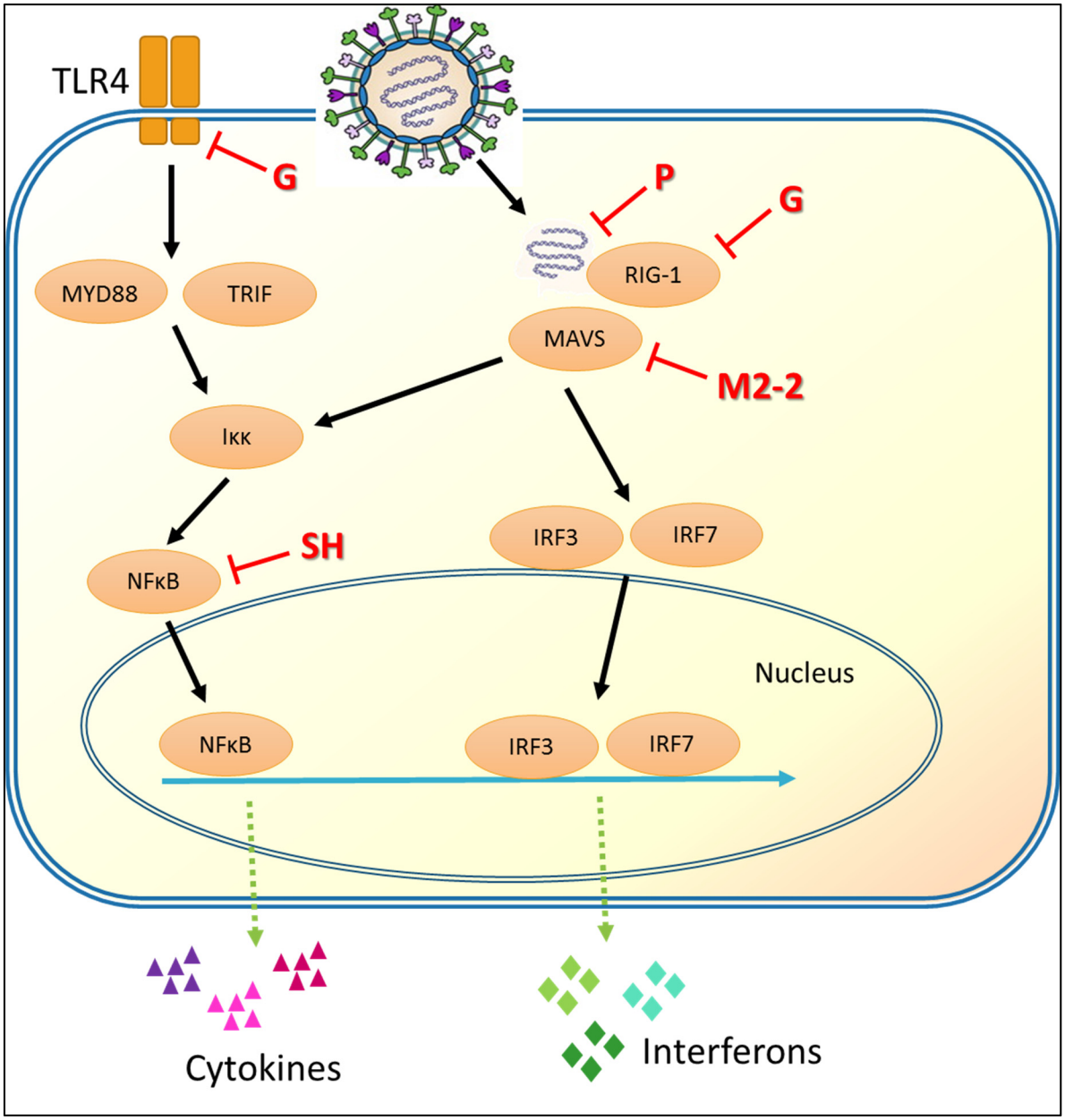

Figure 9 : The innate immune response to HMPV and ways of immune evasion (adapted from [164] and [247]): Schematic representation of signaling pathways activated during HMPV infection. Infection by HMPV leads to TLR4 activation (by an unknown mechanism) and RNA-helicase activation (by cytoplasmic viral RNA), which starts an intracellular signaling cascade that leads to the translocation of phosphorylated transcription factors NFκB and IRF-3 and -7, which in turn results in the production of pro-inflammatory cytokines and interferons. Four HMPV proteins have so far been shown to assist in immune evasion by HMPV (indicated in red).

6.2.2. Acquired immune response

As is the case for HRSV, DCs are not highly permissive for HMPV infection, but infection can occur and leads to the maturation of the DC and alterations of DC function [120, 123, 178, 179, 313]. This interaction of HMPV with dendritic cells could significantly alter the primary $CD4^+$ T-cell response to infection and might at least in part be responsible for the incomplete or transient immunity induced by HMPV and contribute to the possibility of re-infections [55].

HMPV, like most viruses, is both present extracellularly (as free virions) and intracellularly (during its replicative cycle). Therefore, both cellular (targeting infected cells) and humoral (targeting virions) acquired immune responses participate in viral clearance. In fact, all permissive animal models (see chapter 1 section 8: animal models) develop HMPV neutralizing antibodies following experimental HMPV infection [13, 135, 205, 327, 348, 354]. In addition, passive antibody transfer can protect these animals against HMPV infection [13, 324, 345]. Nevertheless, essentially all humans above the age of 5 are seropositive yet re-infection does occur. Moreover, a study in non-human primates showed that animals inoculated 3 times with HMPV over 10 weeks, demonstrated the presence of low levels of neutralizing antibodies 48 weeks after the last inoculations, but they were no longer protected against viral challenge [327], suggesting, indeed, an inefficient immunological memory response.

Mice depleted of $CD4^+$ T-cells had an impaired antibody-response to HMPV, yet following challenge these antibody-deficient mice were still protected against HMPV replication, suggesting that $CD8^+$ cytolytic T-cells (CTL) can also protect from infection [166]. Human CTL responses appear to be directed against epitopes of several HMPV proteins particularly the M protein and these CTL-epitopes seem conserved between HMPV lineages [139]. In mice, CTL-epitopes were also found in the HMPV M protein but epitopes of the N and M2-1 protein appear more dominant [219].

The immune response to HMPV infection is important in keeping viral replication in check. This is illustrated by the increased severity of HMPV infection in immunocompromised patients. On the other hand, immunopathogenic effects due to uncontrolled and unbalanced immune responses are also associated with severity of HMPV infection. So, achieving an immune response that is sufficient to clear the virus, but controlled enough to not cause excessive damage appears to be a great challenge.

6.3. Pathogenesis in humans

Histopathological analysis of lung tissue samples obtained from patients with severe cases of HMPV infection suggested that this virus is capable of causing acute and organizing lung injury in a pattern of diffuse alveolar damage with hyaline membrane formation [308]. Also, HMPV seems to induce the formation of prominent smudge cells not found in other paramyxovirus infections, but typical for CMV and adenovirus infections [308]. However, HMPV was detected in alveolar and airway epithelial cells and not in smudge cells. Lung biopsy specimens collected at least 1 month after diagnosis, revealed chronic inflammatory changes of the airways such as intra-alveolar foamy and hemosiderin-laden macrophages [332].

Nasal secretions of children admitted to hospital for acute expiratory wheezing, showed lower concentrations of RANTES but higher concentrations of IL-8 in children infected with HMPV than in those infected with HRSV [152]. A study in children with primary HMPV infections showed nasal IL-12, TNF-α and IL-1β levels lower than those in HRSV-infected children, but similar to those in influenza-infected children. In addition, no statistical difference in nasal inflammatory cytokine levels were observed between HMPV-infected hospitalized children and outpatients [175]. The same group later found higher IFN-γ- and IL-4-levels in influenza-infected children compared to HMPV- or HRSV-infected children, whereas no difference in IL-13 was found between any of the groups [218]. However, IFN-γ/IL-4 ratios

were significantly lower in infants infected with HMPV than in children in the two control groups, indicating a Th2 bias in cytokine-levels of HMPV-infected children [218].

Recently, 21 adults were experimentally infected with a wild-type-like HMPV virus [312]. Only 43% were considered to have been infected, defined as viral shedding and/or a ≥4-fold rise in serum antibodies to HMPV. Within this group, increases in IL-10 and IFN-γ generally coincided with the peak of viral replication as well as clinical symptoms. Increases in other cytokines, such as IL-2, IL-4, IL-5, IL-8, IL-13, and granulocyte-macrophage colony-stimulating factor tended to occur earlier. There was a large subject-to-subject heterogeneity in cytokine pattern, with regards to the specific cytokines that were induced, their expression-level and the temporal pattern. However, only 9 HMPV-infected subjects were evaluated [312].

7. Animal models

Permissive animal models have been developed in small animals (hamsters, cotton rats, mice, ferrets) as well as non-human primates (chimpanzees, cynomolgus and rhesus macaques and African green monkeys) in order to characterize both the pathogenesis associated with HMPV infection and mechanisms of protection against HMPV infection [13, 135, 205, 348]. In each of the permissive animal models, viral replication is restricted to the upper and lower respiratory tract with peak viral replication in lungs occurring on days 3-5 post-infection. Clinical symptoms of HMPV disease have only been observed in chimpanzees, cynomolgus macaques and BALB/c mice [13, 135, 205, 348].

7.1. Rodents

In the BALB/c mouse model, HMPV infection elicits transient weight loss and dyspnea when a high intranasal inoculum is used [13, 135, 303]. Acute HMPV infection is also associated with long-term pulmonary inflammation, airways obstruction and hyper-responsiveness [134]. Two groups showed that

viral RNA could still be recovered from mouse lungs >150 days after infection [13, 134]. One study describes that HMPV mediates biphasic replication in respiratory epithelial cells of BALB/c mice, after which infection migrated to neuronal processes that innervate the lungs where the virus persisted with no detectable infection in epithelial cells. After glucocorticoid treatment, the virus was found to be reactivated from neural fibers and re-infected epithelial cells [197].

Histopathological changes following HMPV infection in mice, are mostly characterized by alveolitis and interstitial inflammation [13, 135, 166] and by increased peribronchiolitis in cotton rats [135, 348]. These inflammatory changes persisted more than 21 days after viral challenge in mice [134, 135]. An increase in IL-2, IL-4, IL-8/KC, IFN-γ and α, macrophage inflammatory protein 1α (MIP-1α), and monocyte chemotactic protein (MCP-1) were found in lungs or bronchoalveolar lavages of HMPV-infected mice and cotton rats compared with uninfected controls [14, 73, 122, 135].

The murine model has also been used to investigate sequential viral-bacterial infections. Initial infection by HMPV increased pneumococcus lung bacterial titers in mice [174, 176]. Yet, unlike influenza A, HMPV did not induce long-term impairment of pneumococcus lung clearance [201]. Interestingly, in a clinical study, 14 out of 15 (93%) children frequently exposed to *S. pneumonia* (but not those frequently exposed to *H. influenzae*, *M. catarrhalis* or *S. aureus*) had seroconverted to HMPV at 24 months compared to 22 of 37 (59%) of children exposed one time or less to *S. pneumonia* [334]. Furthermore, in children who remained seronegative for HMPV during the first 2 years of life, only 1 of 16 (7%) was frequently exposed to *S. pneumoniae*. These results, combined with the findings that *S. pneumoniae* increased susceptibility of human bronchial epithelial cells to HMPV infection *in vitro*, lead the authors to conclude that there is indeed a link between *S. pneumoniae* and HMPV infection that warrants further investigation [334].

Mice have also been used to compare the immune response induced by HMPV and HRSV (table 3). Moreover, several studies into the precise role of specific immune cells in both antiviral immunity and pathogenesis have also been performed in mice specifically through depletion studies (table 4). Whether these models are representative of human HMPV infections remains to be evaluated.

Table 4: Immune cell depletion studies in mice

Reference publication	Cell type of interest	methodology	results
[167]	Alveolar macrophages (AM)	BALB/c mice depleted of AM by i.n. instillation of dichloromethylene bisphosphonate (L-CL2MBP) liposomes	HMPV-infected mice lacking AM exhibited improved disease in terms of body weight loss, lung inflammation, airway obstruction and hyper-responsiveness, compared to AM-competent mice.
[166]	CD4+ and CD8+ lymphocytes	BALB/c mice depleted of CD4+ and/or CD8+ lymphocytes by i.p. instillation of anti-CD4 and/or anti-CD8 antibodies	• $CD4^+$ and $CD8^+$ T cells are important for viral clearance during primary infection • $CD4^+$ more than $CD8^+$ T cells also enhanced clinical disease and lung pathology. • Concurrent depletion of $CD4^+$ and $CD8^+$ T cells completely blocked airway obstruction as well as hyper-responsiveness. • Despite impaired generation of neutralizing anti-HMPV antibodies in the absence of $CD4^+$T cells, mice had undetectable viral replication after HMPV challenge and were protected from clinical disease.
[13]	T cells and natural killer (NK) cells	BALB/c mice depleted of T cells and NK cells by i.p. instillation of anti-CD3ε/ anti-αβTCR or anti-CD49b/Pan-NK cell antibodies	Both T cells and NK cells have a role in limiting early and late HMPV replication
[341]	NK cells	C57BL/6 mice depleted of NK cells by i.p. instillation of anti-NK1.1	HMPV-infected mice had higher numbers of functional lung NK cells than mock-treated mice, comparing NK cell-depleted and control mice did not reveal differences in lung viral titers, histopathology, cytokine levels or T cell numbers or function.

Advantages of inbred mice are: 1) Rapid and easy breeding, 2) Availability of a wide variety of transgenic and gene-deleted mice, 3) The availability of a vast selection of mouse-specific reagents and molecular tools, and 4) The small size and ease of housing and handling makes them cost-effective. Yet the murine model also has its limitations: 1) because mice are not a natural host to HMPV infection, high viral inoculums are needed in order to achieve viral replication in mice, 2) Weight loss is a cheap and reproducible, objective measure used as an index for disease severity. Yet, weight loss is not a symptom of HMPV infection in humans, 3) There are marked differences in the innate and adaptive immune responses between humans and mice, such as differences in the type of cytokines/chemokines (e.g.,

absence of IL-8 but presence of KC in mice), ratio of neutrophils/lymphocytes in blood is higher in humans, pattern-recognition receptors (e.g., TLR signaling) are not the same, and the leukocyte surface expression of several specific clusters of differentiation markers differ and 4) The pulmonary anatomy of mice differs from that of humans. For example, mice have fewer bronchioles, less complex airway branching, and a relatively large caliber of the airway lumen [30].

Other rodents such as hamster, cotton rats and ferrets do not develop symptoms upon experimental HMPV infection even though they do support viral replication silently [205]. The cotton rat model has been extensively used for the study of HRSV infection [49]. This makes it an obvious candidate model for HMPV. Inoculation of young adult cotton rats with HMPV results in significant viral replication in the lower respiratory tract, accompanied by pronounced pulmonary inflammation [135, 348, 354]. HMPV infection causes up-regulation of IFN-γ, RANTES, MIP-1α, MCP-1 and IL-2 mRNA. HMPV infection of cotton rats induces high serum neutralizing antibody titers and protects from subsequent HMPV challenge. For this reason, the model has since been used for the evaluation of HMPV vaccines [71, 225, 361]. Although less well characterized, the hamster model has also frequently been used to evaluate candidate HMPV vaccines [51, 141, 142, 260, 286, 315]. Housing and handling of ferrets is more complex and only limited reagents (such as antibodies and probes) are commercially available, making this a far less suitable model for HMPV.

7.2. Non-human Primates

Cynomolgus macaques were the first non-human primates to be experimentally infected with HMPV [173]. The virus replicated in the respiratory tract of the animals and virus shedding peaked at day 4 pi and decreased to zero by day 10 pi. Symptoms of rhinitis were also observed.

African green monkeys also support viral replication and develop high titers of cross-neutralizing antibodies following experimental infection [205, 300]. Due to this robust humoral immune response, the African green monkey model has since been used to evaluate HMPV vaccines [33, 260, 314].

Rhesus macaques support some viral replication and develop low neutralizing antibody titers, but do not develop symptoms when infected with 4 different HMPV strains [205, 300].

Of all the non-human primate models develop so far, only chimpanzees present a high seroprevalence for HMPV, indicating the possibility of human handler-to-animal and animal-to-animal transmission [300]. When chimpanzees were infected experimentally with HMPV, they demonstrated signs of clinical disease including nasal discharge, thick mucus, cough and decreased appetites. Although the level of HMPV shedding was low, chimpanzees also developed a robust immune response [300].

8. Prophylactic and therapeutic modalities

At present, no HMPV-specific prophylactic or therapeutic modalities are approved. However, a number of strategies have been investigated *in vitro* or in animal models and have yielded promising results.

8.1. Vaccines

Different approaches for the development of vaccines against HMPV have been investigated; for instance, chimeric viruses (containing the genetic information of more than one virus), live-attenuated viruses and subunit vaccines. All of these strategies have advantages and disadvantages and different strategies might be needed for different populations (table 5). Major target populations for HMPV

vaccination are; (1) unprimed infants, as they are most at risk for severe HMPV disease (2) primed children as they may pose a reservoir for the virus, (3) elderly individuals especially in the context of long-term-care facilities and (4) pregnant women in an effort to protect neonates.

Table 5 Advantages and disadvantages of different vaccine-approaches against HMPV [118]

Type of vaccine	advantages	disadvantages	Possible target populations for HMPV vaccination
Live attenuated vaccines	• activation of all components of the immune system • can stimulate an immune response to each of the protective Ags of a virus, • Immunity induced by live virus vaccines is generally more durable and more effective. • Can be administered mucosally	• Can contain contaminations • Can cause direct illness • Can lose attenuation by reversion or by second-site compensatory mutations • Transmission is possible • Can lose infectivity during storage and transport • In the case of vectored vaccine, Abs directed against the vectors, can diminish the immunogenicity	Unprimed populations such as infants might need replicating virus in order to mount a complete immune response to the entire antigenic repertoire.
Inactivated virus	• Immunization with the entire antigenic content of the virus with little or no risk of infection. • Reduced risk of live contaminants	• Typically have to be given parenterally • less effective in stimulating a primary CTL response • Formaldehyde, heat, and oxidation can produce reactive carbonyl groups on protein antigens that promote Th2 differentiation of $CD4^+$ T cells • Risk of vaccine-enhanced disease has been demonstrated for several paramyxoviruses	Might be useful to boost immunity in primed populations such as elderly individuals without the risk of transmission
Virus-like particles	• No viral nucleic acids are present therefore they are non-infectious, chemical inactivation is not required and no transmission-risk • conformational epitopes are presented to the immune system in the same way as native infectious particles	• higher cost and complexity of manufacturing • no ongoing production of Ag following vaccination	Could be safe for all populations, needs further investigation

	• good alternative for viruses that grow poorly in cell culture		
Subunit vaccines	• Production of recombinant viral proteins or synthetic peptides diminishes the risk of contamination by unrecognized pathogens. • free of nucleic acids, so no transmission risk or risk of viral disease • Might reduce antigenic competition and focus the response on the most relevant protective antigenic sites.	• Need for highly pure proteins with appropriate posttranslational modifications • Possible need for adjuvants to increase immunogenicity • Some subunit vaccines have also been shown to induce vaccine enhanced disease	Could be useful to boost immunity in primed populations such as the elderly, primed children and pregnant women
DNA vaccines	• Combine the immunogenicity advantages of live vaccines with the safety advantages of nonliving vaccines. • Use the host cell to transiently produce the viral antigens, assuring native conformation and modification • Genes can be modified to enhance antigen presentation • DNA vaccines are more stable than protein-based vaccines	• So far, DNA immunization shows limited immunogenicity in humans, despite vaccine-induced protection in animal models • May induce immunological tolerance to the host-expressed antigens • Integration into host DNA is a theoretical possibility • Alterations in promoter design, manufacturing process, and delivery might be necessary	Do not appear to be immunogenic enough to be used in naïve populations. priming with DNA, followed with a boost with the respective subunit (protein) vaccine might increase immunogenicity

To date, no commercial vaccines are available to protect against HMPV infections, one live attenuated vaccine is currently in a phase I clinical trial [233]. An overview of vaccines for HMPV under investigation follows in the next sections.

8.1.1. Inactivated vaccines

Caution is warranted when inactivated vaccines are developed. An inactivated measles vaccine initially prevented measles but when vaccinees were infected with naturally circulating measles virus years later, they developed an atypical illness with accentuated systemic symptoms and pneumonia [107].

This atypical disease is believed to be mediated in part by a Th2-biased immune response and immune-complex depositions [262, 263]. The administration of an experimental formalin-inactivated HRSV (FI-HRSV) vaccine induced serum-neutralizing antibodies but did not protect the youngest vaccines in a clinical trial held in the 1960's [159]. On subsequent natural infection with HRSV, 80% of vaccinees developed severe HRSV lower respiratory tract disease and 2 children died. Possibly, the protective antigenic sites on the F protein were altered by fixation, and the altered Ag induced an aberrant T-cell (Th2-biased) response leading to enhanced disease [117].

Similarly, both heat- and formalin-inactivated HMPV induce a dramatic increase in lung pathology associated with enhanced Th2 response in animal models [79, 131, 361]. Therefore, inactivated HMPV vaccines have not been developed further.

8.1.2. Live-attenuated vaccines

In order to alter the genetic composition of a virus, the technique of reverse genetics can be used. In forward genetics, the goal is to discover the genetic foundations of observed phenotypes. Conversely, in reverse genetics a specific gene is modified and then the phenotype is analysed. Thus, less virulent (or attenuated) viruses can be created. As HMPV is a negative sense RNA virus, its genome alone is not sufficient for viral replication. In fact, the virus packages its RNA-dependent RNA polymerase into its virion in order to be infectious. For this reason reverse genetics of (-) sense RNA viruses is more complex than that for (+) sense RNA viruses or DNA viruses. Several different plasmids need to simultaneously be transfected; plasmids containing the genes of the proteins needed to form the RdRP, plasmids containing the antigenome sequence of the virus one wants to produce as well as a plasmid encoding the nucleoprotein needed to encapsidate newly synthesized viral RNA (Figure 10). As is the case for other (-) sense RNA viruses a bacteriophage T7 RNA polymerase is used to transcribe all of the transfected

plasmids; to this end, a baby hamster kidney fibroblast cell-line stably expressing T7 (BHK-21 clone BSR T7/5) is used for recombinant rHMPV rescue [35, 140].

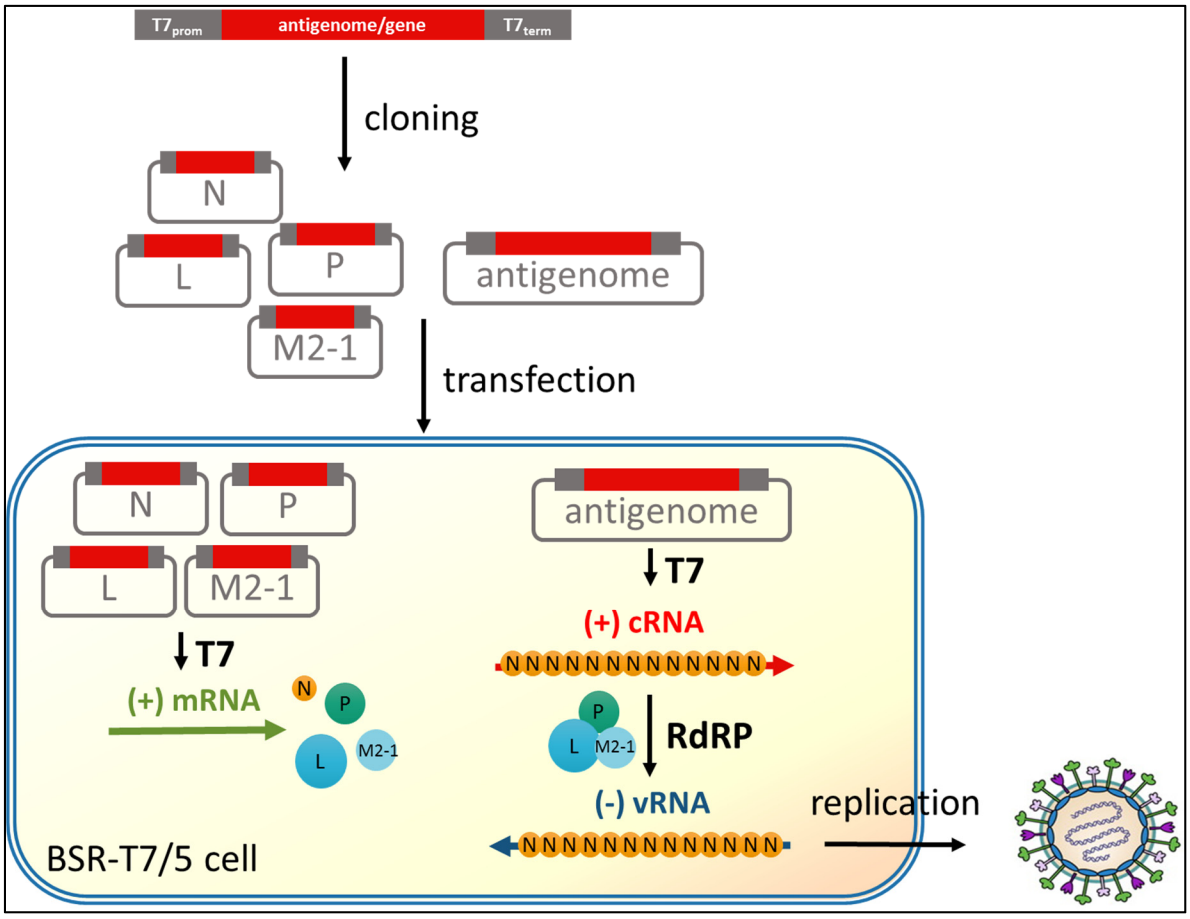

Figure 10 : Reverse genetics for HMPV (adapted from [63]):
The antigenome and genes of the proteins necessary to form the RdRP (N, P, L and M2-1) are cloned separately into plasmids flanked by the T7 promoter and terminator regions. These plasmids are subsequently transfected into cells expressing the T7 RNA polymerase (BSR-T7/5 cells). The T7 polymerase transcribes (+) mRNA from the gene containing plasmids which are translated into the N protein and the proteins that make up the RdRP. The T7 polymerase also produces (+)-sense antigenomes from the antigenome-containing plasmid which is swiftly encapsidated by the N protein. The RdRP then produces the (-)-sense viral genome needed for viral replication starting from the newly synthesized and encapsidated antigenome.

Live-attenuated vaccines, generated by reverse genetics, have been evaluated in small animals and in primates. Deletion mutants, i.e. viruses lacking the M2–2, SH, or G open reading frames were successfully tested in hamsters and African green monkeys, showing high serum neutralizing antibody

titers together with protection against wild-type HMPV challenges [33, 36, 51, 286, 317]. One group created live-attenuated vaccines by removing N-linked glycosylations from the viral F protein [363]. Recombinant HMPV viruses carrying these mutant F proteins could only be detected in the lungs of infected mice by molecular techniques and not by classic titration. Furthermore, immunization of mice provided complete protection from homologous virus infection and substantial cross-protection from heterologous virus infection for at least 56 days after inoculation [196]. Recently, a panel of recombinant HMPVs was created, defective in ribose 2'-O methyltransferase (MTase), an essential part of viral mRNA methylation [369]. These MTase-defective viruses were genetically stable and attenuated but retained high immunogenicity in cotton rats.

A combination of both forward and reverse genetics was used to create temperature sensitive (ts) HMPV viruses. Repeated passage of HMPV at low temperatures in Vero cells results in mutations in the viral genome. Introduction of these mutations into recombinant HMPV resulted in ts viruses. Replication of ts HMPV viruses was attenuated in the upper respiratory tract and undetectable in the lower respiratory tract of hamsters. Immunization of hamsters with these ts HMPV viruses resulted in high titres of HMPV-specific antibodies and protection against challenge infection with a heterologous HMPV strain [141].

Vectored vaccines represent yet another strategy of live attenuated vaccines. Live recombinant human para-influenza virus type 1 (PIV-1) containing the HMPV F protein or a chimeric bovine/human PIV-3 also expressing the HMPV F protein have been evaluated. Both approaches elicited neutralizing antibody production against HMPV and protected animals against subsequent wild-type HMPV and PIV challenges [299, 300, 314, 315]. Recently, immunization of mice with rBCG (*Mycobacterium bovis* bacillus Calmette–Guérin) strains expressing the P protein from HMPV protected against weight loss, airway inflammation and viral replication in the lungs after HMPV infection [246]. The rBCG vaccine also induced

the activation of HMPV-specific T cells producing IFN-γ and IL-2, which could protect from HMPV infection when transferred to recipient mice.

Chimeric recombinant HMPV, in which the P open reading frame was replaced with its counterpart from the closely related AMPV subgroup C (HMPV-Pa), replicated to 25-fold higher titers than WT HMPV in Vero cells, yet titers were 100-fold lower in hamsters and up to 1000-fold lower in African green monkeys [260]. A Phase I study using rHMPV-Pa is currently ongoing; rHMPV-Pa is delivered as nose drops to (1) Adults 18 to 49 years of age, (2) HMPV-seropositive children 12 to 59 months of age, and HMPV-seronegative infants and children 6 to 59 months of age. Participants are monitored for reactogenicity and neutralizing antibody production [233]. This is the only HMPV vaccine in clinical trials, at this time.

8.1.3. Virus-like particles

Viral-like particles (VLPs) are structures built up out of proteins that mimic the organization and conformation of a viral particle but lack the genetic material necessary for replication. In between live-attenuated viruses and VLPs are alphavirus replicon particles (RPs); these are single-cycle, propagation-defective particles, unable to spread beyond the initial infected cells. One group developed alphavirus RPs based on Venezuelan equine encephalitis virus that encode the HMPV F protein and tested their immunogenicity in mice and cotton rats [225]. Intranasal immunization with RPs carrying HMPV F, resulted in virus-neutralizing antibodies in serum and immunoglobulin A antibodies in respiratory mucosa secretions following intranasal HMPV challenge in immunized animals.

Two groups have used two different approaches for developing HMPV-based VLPs. Our group developed retroviral-based VLPs displaying HMPV glycoprotein F (and G). These VLPs induced neutralizing antibody responses in mice and induced protection against both homologous and heterologous HMPV challenge in our study [182]. The second group found that the HMPV matrix (M) and fusion (F) proteins

are sufficient for VLPs formation [67]. Mice immunized with the VLPs mounted an F-specific antibody response and generated $CD8^+$ T cells recognizing an F protein-derived epitope. Furthermore, two doses of VLPs conferred complete protection from HMPV replication in the lungs of immunized mice.

8.1.4. Subunit vaccines and DNA vaccines

Vaccines containing only one or more viral proteins are called subunit vaccines. Since the F protein is the only HMPV surface glycoprotein that induces the production of neutralizing antibodies [299], this is the protein of choice in HMPV subunit vaccines. Subunit vaccines consisting of a soluble F protein or plasmid DNA encoding the HMPV-F protein have been evaluated in hamsters, cotton rats and cynomolgus macaques [71, 142, 144]. However, only animals vaccinated with F protein and an adjuvant (TiterMax Gold [71], Specol or Iscom matrix [142]) were protected against homologous and heterologous infections. Moreover, a study in cynomolgus macaques demonstrated that protection wanes quickly, as animals were no longer protected from infection 8 weeks after the boost [144]. Finally, caution is warranted when developing subunit vaccines against pneumoviruses since cotton rats immunized with 5 μg of purified HRSV F protein exhibited enhanced bronchiolar and alveolar pathology when challenged 3 or 6 months post-vaccination [64, 230]. In chapter 5 of this thesis, a subunit vaccine containing both the HMPV F and M proteins is discussed.

8.1.5. Adjuvants

In any vaccine the antigen (Ag) determines the specificity of the immune response that is elicited; however, the formulation and delivery of this antigen greatly influence the location, duration, quality and magnitude of the immune response. Live attenuated vaccines provide efficient Ag presentation and innate immunity stimulation, reducing the requirement for exogenous adjuvants. Conversely, inactivated virus

vaccines and subunit vaccines have relatively weak immunogenicity, especially in naive hosts, and might therefore benefit from exogenous adjuvants or the use of very specific mechanisms of administration. Adjuvants can improve immunogenicity by optimizing the duration of Ag presentation and/or by enhancing certain innate stimulatory pathways [118]. The most widely used vaccine adjuvant is Alum. The latter is derived from aluminum hydroxide or aluminum phosphate and is formulated as a gel to precipitate protein Ags to create aggregates. It makes a depot from which Ag, electrostatically bound to alum, is slowly released at the site of inoculation. More recent studies have shown that some of the adjuvant activity conferred by alum could be mediated through activation of the inflammasome signaling pathway [304]. Alum is still the standard adjuvant used in licensed vaccines; however, alum is known to elicit a powerful Th2-biased humoral response. The discovery of TLRs and their signaling pathways has created the possibility for the rational design of vaccine adjuvants that could improve immunogenicity and guide the immune response towards a more balanced Th2/Th1 direction.

8.2. Therapeutics

Although several HMPV-specific therapeutic modalities have been investigated, none of them are currently available for use in humans. Some non-specific therapeutics such as ribavirin and immunoglobulins have on occasion been used in severe cases of HMPV infection and will be discussed below. Antibiotics, bronchodilators, oxygen administration, corticosteroids and mechanical ventilation appear to be the most used adjunctive therapies [127, 279, 330, 344, 351, 357] .

8.2.1. Ribavirin

Ribavirin (1-ß-D-ribofuanosyl-1,2,4 triazol-3-carboxamide), a nucleoside analogue of guanosine, has similar antiviral activity (i.e. similar 50 % inhibitory concentration values) against HMPV and HRSV when evaluated *in vitro* [353]. In BALB/c mice treated with ribavirin, corticosterone or both, ribavirin

significantly decreased both HMPV replication in lungs and total pulmonary inflammation, whereas glucocorticoids reduced only alveolar and interstitial inflammation, compared to controls [133]. In a lung transplant recipient, intravenous ribavirin also seemed to show benefits for treating HMPV infection [270].

8.2.2. Immunoglobulins

Immunoglobulin showed good inhibitory activity against HMPV *in vitro* [353]. In 2 hematopoietic stem cell recipients, a combination of oral and aerosolized ribavirin and intravenous immunoglobulins (IVIG) was successful in treating HMPV infection [296]. The same combination was successful in 3 immunocompromised children [43, 163, 295] diagnosed with severe HMPV infection, yet the treatment was unsuccessful in a 10-year-old girl with chronic graft-versus-host disease following allogeneic hematopoietic stem cell transplantation for secondary chronic myeloid leukemia [86]. In a prospective study of HMPV-associated lower respiratory tract infection after allogeneic hematopoietic stem cell transplantation (HSCT), 5 out of 6 patients receiving the combination of IVIG and oral ribavirin survived but red blood cell transfusion support was needed in 4/6 ribavirin-treated patients [95]. Finally, in a retrospective study, no significant difference in the 30-day mortality rate was observed between ribavirin-treated and nonribavirin- treated patients with hematological diseases [252].

8.2.3. Monoclonal antibodies

Palivizumab, (Synagis®, MedImmune, Inc.), a humanized monoclonal antibody (MAb) against the HRSV fusion protein, is not effective at neutralizing HMPV [353]. However, a series of neutralizing MAbs

against the HMPV F protein have been generated [324, 345]. One of these MAbs (MAb 338) had the ability to neutralize strains from the 4 HMPV subgroups *in vitro* and the F epitope recognized by this MAb, called epitope group 4 (AA 238-245,) is similar to the one mapped for Palivizumab, epitope group II on the HRSV F protein [323]. When administered in hamsters 24 h before infection intramuscularly, lung viral titers were significantly decreased. This MAb was also evaluated in a BALB/c mouse model in which prophylactic [130] and to a lesser extent, therapeutic [132] administration of MAb 338 improved acute and late consequences of HMPV. The same study also identified epitope groups 5 and 6 (AA 386-397), which overlap with epitope region IV on the HRSV F protein [323]. Recently, a human monoclonal antibody (MPE8) was described, which cross-neutralizes HRSV and HMPV as well as two animal paramyxoviruses: bovine RSV (BRSV) and pneumonia virus of mice (PVM) *in vitro* as well as in mouse models [65]. MPE8 binds a peptide (YLSALRTGW), conserved in the pre-fusion states of both the HMPV and HRSV F proteins, which is located close to, but distinct from the Palivizumab-binding site.

8.2.4. Glycosaminoglyans and sulfates

NMSO3, a sulfated sialyl lipid and heparin, also a sulfated molecule, significantly inhibit the replication of several HMPV strains *in vitro* [355]. The two compounds are more effective when present before viral inoculation suggesting that they may prevent attachment or penetration of the virus into the cells. NMSO3 also had significant benefits in a mouse model of HMPV infection when administered at the time of infection [303].

One group has evaluated the antiviral effect of different marine algae extract against HMPV and found them to have low cytotoxicity and high virucidal effects *in vitro* [220-222]. Most of these extracts inhibit viral entry into the cell, either by direct interaction with the viral particle or by interaction with cellular receptors.

8.2.5. Fusion inhibitors

The exogenous addition of synthetic HRA or HRB peptides has been shown to inhibit fusion and consequently infection of HMPV both *in vitro* [84, 223] and *in vivo* [84]. The first group used homology modeling of the F protein of HMPV, SV5, HRSV and HPIV3 to predict fusogenic core peptides in both the HRA and HRB of the HMPV F protein. They found that the peptide derived from HRA was a more potent fusion inhibitor (IC_{50} = 46 nM) than the peptide derived from HRB (IC_{50} = 165 nM) in infected LLC-MK2 monolayers [223]. The second group examined 3 peptides derived from HRA and 6 peptides derived from HRB of HMPV and found all 3 HRA peptides and 2 out of 6 HRB peptides to show good viral inhibition *in vitro* [84]. The most potent peptide (IC_{50} = 2.1 nM) was capable of inhibiting HMPV viruses of all 4 subtypes and in a lethal BALB/c mouse model of hMPV infection; simultaneous intranasal administration of the peptide with HMPV was found to inhibit mortality and clinical signs. Moreover, no infectious viruses could be recovered from the lungs of treated mice on day 5 post infection. However, delayed treatment (by 24 h) was not protective.

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8.2.6. SiRNA

Highly efficient small interfering RNAs (siRNAs) inhibits HMPV replication *in vitro* [82], and partially inhibits viral replication in a mouse model [74]. In the first study, 200 potential siRNAs were identified, from which 2 siRNAs (as well as the dicer substrates derived from these siRNAs) proved to be highly efficient against all 4 HMPV subgroups; siRNA45 (targeting the N mRNA) showed an IC_{50} < 0.078 nM and siRNA60 (targeting the P mRNA) showed IC_{50} values of 0.090 - <0.078 nM *in vitro*. In the second study 128 dicer substrate sIRNAs (DsiRNA) were identified and tested *in vitro [74]*. DsiRNA C11 had a sequence similar to siRNA45 and was used to investigate *in vivo* activity. Intranasal administration of DsiRNA C11 30

min prior to HMPV infection, led to a significant (half a log) decrease in viral load but had no significant effect on clinical disease [74].

8.2.7. IFN

In one study, HMPV seemed to be partially resistant to the antiviral activity of type I interferons *in vitro* [284].

8.2.8. Anti-inflammatory agents

Severe cases of HMPV infection are associated with significant inflammatory pathology and cells of the immune system have been found to participate in HMPV pathogenesis (table 4). In mouse models of HRSV infection, inhibition of MIP-1α-, IL-4- as well as RANTES-signaling reduces pulmonary inflammation as does the inhibition of leukotriene synthesis [278]. Resveratrol, a known anti-inflammatory agent, also reduces HRSV-mediated airway inflammation and airway hyper-responsiveness in mice [365]. Moreover, inhibition of MIP-1α-signaling in addition to ribavirin treatment of pneumonia virus of mice (also a member of the *pneumovirinae* sub-family) increased survival from 20% for ribavirin alone to 60% for combined anti-viral and anti-inflammatory therapy. Taken together, these data make a strong case for the investigation of immunomodulatory and anti-inflammatory strategies to prevent or treat severe pneumovirus disease. However, until now, this approach remains uninvestigated for HMPV.

8.2.8.1. Protease-activated receptors

Protease-activated receptors (PAR) are transmembrane, G-protein–coupled cellular receptors (Figure 11). Four PAR have been discovered: PAR1 to PAR4. They are part of the coagulation system but, in recent years, an important role for PARs in immunomodulation has been discovered. Irreversible proteolytic cleavage at a specific site within the N-terminal ectodomain exposes a tethered ligand that

binds to the receptor and induces intracellular signaling [5, 244, 305]. All PARs are activated primarily by thrombin except PAR2 for which the main activating protease is trypsin.

PAR1 specifically, is a ubiquitous receptor present in platelets, endothelial and epithelial cells, smooth muscle cells, leukocytes, neurons, fibroblasts and tumor cell lines [305]. It is implicated in many physiological processes including the cardiovascular, respiratory and central nervous systems as well as in embryogenesis, cancer and inflammation [5]. There is a growing interest in the role of PAR1 during infection (Table 6). In addition, more and more PAR agonists and antagonists are being developed making the study of these receptors possible as well as presenting an attractive approach for therapeutics [267]. The effects of PAR1 during HMPV infection are the topic of discussion in chapter 4 of this thesis.

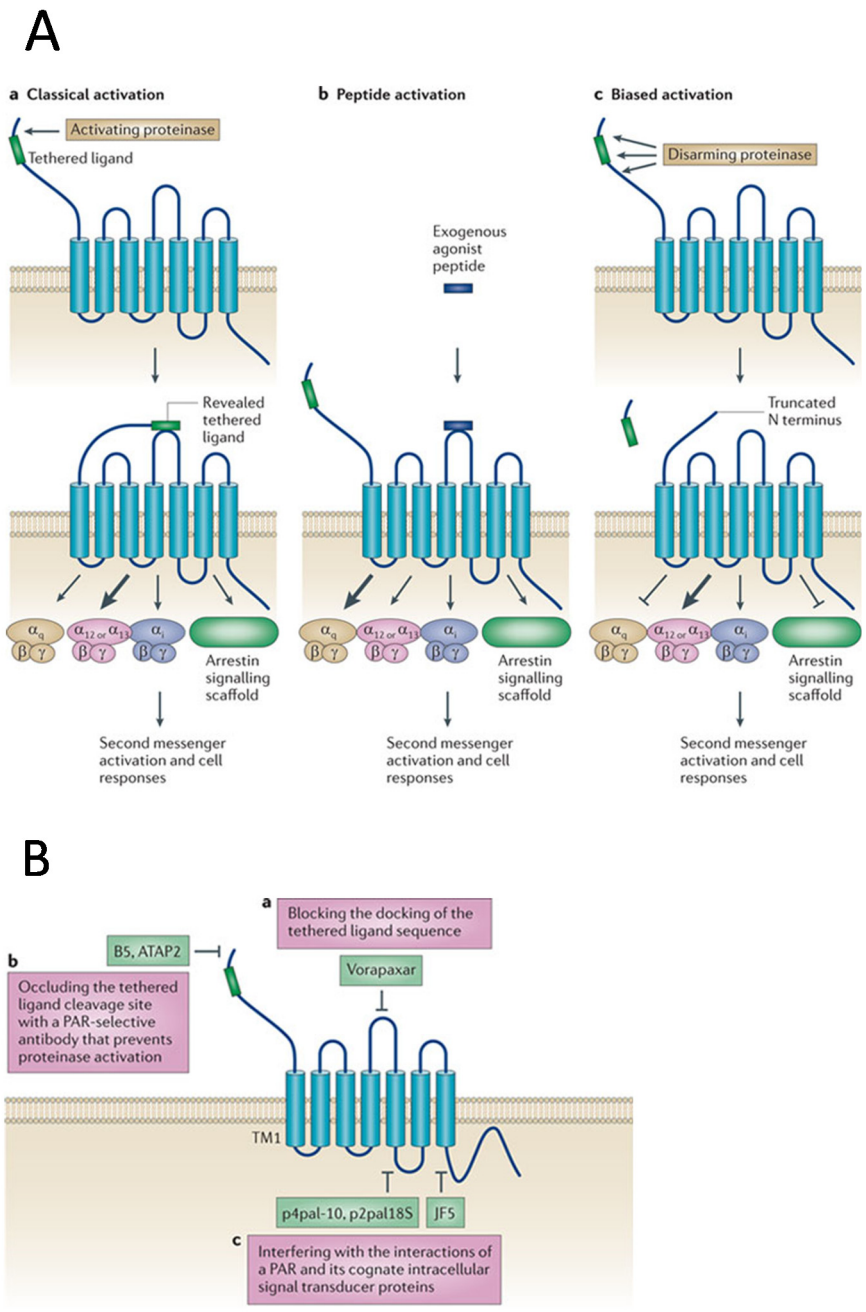

Figure 11 : Mechanisms of PAR activation and inhibition (adapted from [267]):
(**A**) Proteolytic cleavage reveals the tethered ligand to stimulate signalling (a), PARs can be activated by exogenous application of synthetic agonist peptides (b) and disarming of signalling occurs by proteolytic cleavage downstream of the receptor activating site to truncate the tethered ligand and make it unavailable for activating proteinases (c). (**B**) (a) Compounds targeting the second extracellular loop could block tethered ligand docking and receptor activation. (b) Antagonists targeting the proteolytic cleavage of the receptor N terminus can preventing the exposure of the tethered ligand. (c) Cell-permeable compounds targeting the intracellular signaling domains could also act as inhibitors of PAR. Commercially available antagonists are represented in green.

Table 6 : Non-exhaustive overview of studies on the role of PAR1 during infection

Reference publication	Infection	Model used	Results
[50]	HIV	*Ex vivo* (human brain samples)	PAR1 was up-regulated in astrocytes patients with HIV encephalitis. Furthermore, in vitro activation of PAR1 on astrocytes induced neurocytotoxicity.
[370]	Helminthes infection (*Nippostrongylus brasiliensis*)	mouse models	PAR-1 activation induced contraction of murine intestinal smooth muscle that was enhanced during helminthes infection. N. brasiliensis infection alters PAR-1 function and expression
[309, 310]	HSV1 and HSV2	*In vitro*	HSV1 and HSV2 initiate thrombin production to increase the susceptibility of cells to infection through a mechanism involving PAR1-mediated cell modulation
[158]	*Pseudomonas aeruginosa*	*In vitro*	The bacterial exoprotease LepA activates NFκB through human PAR-1, -2 or -4 in transfected COS-cells
[148]	Dengue virus	*In vitro*	Dengue Virus infection up-regulates PAR1 in endothelial vascular cells
[226]	*Candida Albicans*	*In vitro* and mouse models	PAR1 activation results in increased inflammation during fungal infection. *Candida Albicans* modulates the expression of both PAR1 and PAR2 in a TLR-dependent manner.
[264]	Human cytomegalovirus	*In vitro*	Human cytomegalovirus increases the expression of PAR1 and PAR3 on human umbilical vein endothelial cells
[268]	*Leishmania* (parasitic protozoan)	*In vitro*	*Leishmania* promastigotes activate PAR1 on macrophages. Inhibition of PAR1 rendered macrophages resistant to Leishmania infection
[4]	HIV and FIV	*Ex vivo* (dorsal root ganglion neurons)	activation and up-regulation of PAR1 by IL-1β contributed to dorsal root ganglion neuronal damage during lentivirus infections leading to the development of distal sensory polyneuropathy
[108]	*M. Tuberculosis*	*In vitro*	Severe tuberculosis is mediated by MCP1/MMP-1 in a PAR1-dependent manner
[292]	*Streptococcus pneumoniae*	*In vivo*	PAR1-knockout mice showed increased survival, reduced inflammation and reduced bacterial loads compared to WT mice.
[157]	Influenza A	*In vivo*	Administration of a PAR1 antagonist and PAR1 deficiency protected mice from infection with influenza A viruses.
[17]	Influenza A	*In vivo*	PAR1 was found to be protective by enhancing TLR3-dependent IFN-β expression.
[204]	Coxsackie virus B3	*In vivo*	PAR1 knockout mice had increased cardiac injury. PAR1 protects from viral myocarditis in a TLR3-IFNβ-dependent manner
[59]	*Helicobacter pylori*	*In vivo*	PAR1 inhibited both Th1 and Th17 immune responses to H. pylori in the gastric mucosa, mediated the inhibition of proinflammatory cytokine secretion by macrophages. PAR1 also inhibited the ability of vaccinations to combat this infection.

Chapter 2: Research hypothesis and objectives

Ever since the discovery of HMPV in 2001 [326], Dr Boivin's laboratory together with others all over the world have aimed to elucidate how HMPV causes disease and how HMPV disease can be prevented or treated. Unfortunately, many questions still remain unanswered.

In this thesis, three topics will be addressed. First, the contribution of the HMPV fusion protein to HMPV virulence and pathogenesis is examined. Secondly, the effect of PAR1 during HMPV infection in mice is evaluated. Finally, we investigate if the addition of the HMPV M protein to an F protein-based subunit vaccine can improve its immunogenicity and effectiveness.

1. Hypothesis

1.1. Part 1:

HMPV growth in cell culture is difficult and requires regular supplementation with trypsin. CPEs are typically observed after 10 to 14 days of culture and they can vary greatly from strain to strain [128]. Some strains induce focal-rounding of infected cells while others induce syncytium formation (Figure 7).

Few clinical studies have investigated viral determinants for severe HMPV disease, and those that did have focused on HMPV genotype (HMPV sub-lineage) rather than phenotype (syncytium-inducing strain or not) with conflicting results [6, 249, 336].

At a molecular level, transient transfection of HMPV F was found to be sufficient to induce cell-cell fusion, and co-expression of the G protein did not significantly enhance fusion [293]. This initial study into F-mediated cell-cell fusion activity (using transiently transfected cells) suggested that the F protein of HMPV strain CAN97-83 (A2 subtype) requires low pH for fusion [293]. Subsequent mutagenesis studies

determined that Gly_{294} was a key determinant of the low-pH-induced fusion phenotype, but inserting the Gly_{294} residue into pH-independent F proteins did not confer pH sensitivity in all strains [143]. In fact, Gly_{294} is only present in 6% of available F sequences, suggesting that exposure to low pH is not a general requirement for fusion activity. Furthermore, the effect that pH-dependency of the F protein might have on viral replication kinetics and virulence was not investigated.

So far, the effect of a syncytium-inducing phenotype on HMPV replication kinetics and virulence has not been investigated. Therefore the hypotheses for this first part are as follows:

1) The HMPV syncytium-phenotype is fusion-protein dependent

2) The HMPV syncytium-phenotype has an effect on viral replication kinetics *in vitro* and *in vivo*

3) The HMPV syncytium-phenotype has an effect on virulence

1.2. Part 2

Although vaccination (discussed in part 3 of this thesis) might constitute a good preventive measure against HMPV disease in several target populations, severe HMPV disease is often observed in individuals with underlying conditions that might not make them the best candidates for vaccine-dependent prevention of disease. For these populations, a better understanding of the interplay between HMPV pathogenesis and the immune response provoked by the virus, could lead to the developments of compounds that modulate the immune response to better clear the virus without causing excessive and detrimental inflammation. Such immunomodulators temporarily influence the host response to infection rather than impacting on viral replication directly as an antiviral compound would. For this reason, viral resistance to such therapies would be very unlikely.

Protease-activated receptor-1 (PAR1) is an ubiquitous cellular receptor with a wide variety of functions depending not only on the cell type but also on the context in which the receptor is activated [244]. Several human pathogens have been found to up-regulate PAR1 expression often leading to increased infection and inflammation (table 6). Furthermore, the PAR1 antagonist Vorapaxar (Merck) was approved by the FDA on May 8, 2014, for the secondary prevention of atherothrombotic events in stable patients at least 2 weeks after myocardial infarction or those with a history of peripheral artery disease who are also on concomitant standard therapy, including antiplatelets [21]. Thus, there is a great interest to see whether these commercially available agents could also be beneficial in treating certain infections.

We formulated the following hypotheses:

1) PAR1 is implicated in the pathogenesis of severe HMPV disease

2) The prophylactic and/or therapeutic use of PAR1 antagonists is beneficial for HMPV disease

1.3. Part 3

To date, only one live attenuated HMPV vaccine has made it to clinical trials [233]. Although live attenuated vaccines are indeed the most immunogenic, they come with several risks (as discussed in table 5), particularly the risk of reversion and transmission. Subunits vaccines do not pose these risks, yet subunit vaccines based solely on the fusion protein do not confer complete and long-lasting protection [71, 142], making it unlikely that these vaccines would be sufficiently immunogenic in multiply primed populations such as the elderly and pregnant women.

Since free matrix proteins were found to be present in the cytoplasm of HMPV infected cells, a group in Lyon, France hypothesized that these free M proteins could be released from infected cells and might consequently influence the immune response to HMPV infection [20]. Indeed, they observed that recombinant HMPV M protein induces activation of dendritic cells as well as macrophages. Furthermore,

HMPV M-activated DCs stimulated IL-2 and IFN-γ production by allogeneic T lymphocytes [20]. In addition, M protein derived CTL-epitopes were identified and appear conserved across HMPV subtypes [139]. Lastly, in one study, 95 out of 96 tested samples exhibited the presence of anti-M antibodies (IgG) [242]. This study also showed HMPV-specific B cell memory against HMPV M in 40% of individuals tested.

Based on these observations we put forward the following hypothesis:

Addition of the HMPV M protein to an F protein-based vaccine improves immunogenicity and modulates the immune response elicited by such a vaccine.

2. Objectives

2.1. Part 1

To evaluate the involvement of the HMPV fusion protein in syncytium formation and the effects this might have on viral replication and virulence, we intended to use reverse genetics (described in chapter 1 section 9.1.2 Live attenuated vaccines) to create 2 prototype recombinant HMPV viruses, representing either the syncytium-inducing phenotype or the focal-rounding phenotype. We added GFP at the end of the antigenome sequence as a marker for infection.

The next objective was to exchange the F genes of both recombinant viruses in order to examine if the phenotype was F-protein dependent.

Subsequently all 4 recombinant viruses were evaluated for their phenotype, i.e the induction of syncytia vs focal cell rounding, the effect on LLC-MK2 cells state *in vitro* using RTCA (chapter 1 section 5), *in vitro* replicative capacity as well as lung viral titers in a BALB/c mouse model and the effects on virulence.

2.2. Part 2

First, we sought to evaluate the effect of a PAR1 agonist and a PAR1 antagonist on HMPV *in vitro*. We would then evaluate the effect of these compounds in our BALB/ mouse model of HMPV infection using different concentrations and drug regimens.

2.3. Part 3

The objectives of Part 3 were to first express the HMPV F protein using a human embryonic kidney cell expression system to ensure proper post-translational modifications and folding. We would also express the HMPV M protein using a prokaryotic expression system and purify both viral proteins using affinity chromatography.

We would then use the BALB/c mouse model to evaluate immunogenicity and protection using different combinations of HMPV F and M protein, adjuvanted or not with alum.

Chapter 3: Effect of in vitro syncytium formation on the severity of human metapneumovirus disease in a murine model

1. Preface

Chapter 3 contains the paper entitled: "Effect of in vitro syncytium formation on the severity of human metapneumovirus disease in a murine model"written by Laetitia Aerts, Marie-Hélène Cavanagh, Julia Dubois, Julie Carbonneau, Chantal Rhéaume, Sophie Lavigne, Christian Couture, Marie-Ève Hamelin and Guy Boivin , accepted for publication in PLOS ONE on February 5, 2015.

2. Author contributions

Laetitia Aerts:

- designed the experiments
- assisted in plasmid construction
- Transfections and viral rescue
- *In vitro* experiments
- *In vivo* experiments
- Wrote manuscript

Julia Dubois:

- Assisted in *in vivo* experiments

Marie-Hélène Cavanagh:

- Plasmid construction

Julie Carbonneau:

- Assisted in plasmid construction
- sequencing of virus stocks

Chantal Rhéaume :

- assisted in *in vivo* experiments

Sophie Lavigne and Christian Couture

- histopathology

Marie- Ève Hamelin:

- supervised the project
- corrected manuscript

Guy Boivin:

- supervised the project
- corrected manuscript

3. Paper

Title: Effect of *in vitro* syncytium formation on the severity of human metapneumovirus disease in a murine model.

Authors: Laetitia Aerts[1], Marie-Hélène Cavanagh[1], Julia Dubois[1], Julie Carbonneau[1], Chantal Rhéaume[1], Sophie Lavigne [2], Christian Couture[2], Marie-Ève Hamelin[1], Guy Boivin[1]

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Abstract

Human metapneumovirus (HMPV) is an important cause of acute respiratory tract infections (ARTI) in children, elderly individuals and immunocompromised patients. *In vitro*, different HMPV strains can induce variable cytopathic effects ranging from large multinucleated syncytia to focal cell rounding. In this study, we investigated the impact of different *in vitro* phenotypes of two HMPV strains on viral replication and disease severity in a BALB/c mouse model. We first generated two recombinant GFP-expressing HMPV viruses: C-85473, a syncytium-inducing strain (rC-85473) belonging to the A1 subtype and CAN98-75, a focal cell rounding-inducing strain (rCAN98-75) of the B2 subtype. We subsequently exchanged the F genes of both strains to create the chimeric viruses rC-85473_F and rCAN98-75_F. We demonstrated that the F protein was the sole protein responsible for the syncytium phenotype and that viruses carrying a syncytium-inducing F protein replicated to significantly higher titers *in vitro. In vivo*, however, the virulence and replicative capacity of the different HMPV strains did not appear to be solely dependent on the F gene but also on the viral background, with the strains containing the C-85473 background inducing more weight loss as well as increased lung viral titers, pro-inflammatory cytokines and inflammation than strains containing the CAN98-75 background. In conclusion, the F protein is the main determinant of syncytium formation and replication kinetics *in vitro*, although it is not the only factor implicated in HMPV disease severity in mice.

Introduction

Human metapneumovirus (HMPV) is a major cause of acute respiratory tract infections (ARTI) in children, elderly individuals and immunocompromised patients [1]. For instance, HMPV accounts for 10 to 15% of all hospitalizations for ARTI in children [2]. Clinical signs associated with HMPV are similar to those associated with human respiratory syncytial virus (HRSV), ranging from mild respiratory problems to bronchiolitis and pneumonia [3]. Based on phylogenetic analysis of the F and G genes, HMPV strains can be classified into two main lineages (A and B), each containing 2 or 3 sub-lineages (A1, A2a, A2b, B1 and B2) [4,5]. Whether these HMPV lineages are associated with different clinical outcomes remains unclear; some groups found no evidence for differential severity between HMPV lineages [6,7], whereas others suggested more severe clinical disease associated with HMPV-A [8] or HMPV-B [9,10] strains. However, a recent study by our group, suggested that viral load rather than HMPV lineage was an independent risk factor for severe disease [11].

HMPV culture is notoriously difficult. Viruses have to be cultured several weeks before cytopathic effects (CPE) occur and regular addition of exogenous trypsin is required. In addition, CPE differ greatly between strains ranging from typical HRSV-like syncytia to focal cell rounding in tertiary monkey kidney cells such as LLC-MK2 cells (Figure 1a).

Figure 1: Cytopathic effects of HMPV strains and recombinant HMPV viruses: (a) microscopic images of cytopathic effects induced by HMPV infection of LLC-MK2 monolayers. CAN98-75 (B2) induces focal cell-rounding (left) whereas C-85473 (A1) induces multinucleated syncytia (right). Magnification = 10x. (b) Representation of the genomes of the 4 recombinant viruses used in this study; rC-85473 and rCAN98-75 represent the wild-type strains, rC-85473_F represents the chimeric rC-85473 strain in which the F gene has been replaced with that of CAN98-75 and rCAN98-75_F represent the chimeric rCAN98-75 in which the F gene has been replaced with that of C-85473.

HMPV is a member of the *Pneumovirinae* subfamily within the *Paramyxoviridae* family [12]. Paramyxovirus entry into the host cells occurs through fusion of the cell membrane with the viral envelope. This fusion is mediated by viral surface glycoproteins. Membrane fusion of members of the *Pneumovirinae* subfamily (including HRSV and HMPV) is unique among paramyxoviruses, because the fusion (F) glycoprotein alone is sufficient for membrane fusion to occur without the requirement of an additional attachment glycoprotein [13,14]. The paramyxovirus F protein is synthesized as an inactive F_0 precursor protein that requires proteolytic cleavage into 2 disulfide-linked subunits (F_1 and F_2) to be activated and capable of inducing membrane fusion. Proteolytic cleavage reveals a hydrophobic fusion peptide located at the N-terminus of the F_1 subunit, which is inserted into target cell membranes to initiate folding of two heptad repeats within the F_1 subunit, HRA and HRB, into an irreversible six-helix bundle. These conformational changes result in the formation of a fusion pore [15].

Although it is generally recognized that activated F protein-mediated cell-cell fusion is the cause of syncytium formation, the exact reasons why some strains induce large syncytia and others do not remain to be established. In addition, some HMPV strains are dependent on low-pH for membrane fusion *in vitro* [16]. Several studies have tried to elucidate which amino acids could be responsible for this pH-dependency. One group proposed that pH-dependency is dependent on the HMPV lineage [17]. Using 2 subtype A strains and 2 subtype B strains, they suggested that Gly_{294} was responsible for pH-dependency of some subtype A viruses and that subtype A viruses carrying Glu_{294} did not induce syncytium at any pH. On the other hand, both subtype B viruses with Glu at position 294 proved to induce syncytium in a pH-independent fashion. They proposed that a tetrad of variable amino acids at positions 294, 296, 396 and 404 was likely involved in protonation of conserved histidine residues at positions 368 and 435 in pH-dependent strains [18]. Chang et al, on the other hand, used the prototype pH-dependent strain CAN97-

83 (A2) to propose that the tetrad at positions 294, 296, 396 and 438 was involved in destabilizing the histidine at position 435 [19]. However, due to the very limited number of strains analyzed in these studies, it is very difficult to generalize these results. More importantly, the relevance of pH-dependency or –independency on severity of HMPV infection remains unexamined.

Moreover, within our collection of HMPV isolates, we have encountered type A strains that do induce syncytium at neutral pH and type B strains that do not. By comparing the type A1 strain C-85473 (syncytium phenotype) and the type B2 strain CAN98-75 (focal cell rounding phenotype) (Figure 1a), we initially observed increased replication kinetics *in vitro* and increased virulence in BALB/c mice with the former strain. We then generated the recombinant viruses from these strains containing GFP as a reporter gene and we further swapped the F genes in both viruses (Figure 1b). Herein, we report that replacing the F gene of a focal cell rounding-inducing strain with that of a syncytium-forming strain, or vice versa, is sufficient to alter the phenotype of the strain *in vitro*. We also investigated replication kinetics of all 4 recombinant strains in cell culture and in the lungs of infected BALB/c mice. We found that HMPV strains carrying the syncytium-inducing F protein replicated to higher titers *in vitro* than non-syncytium F protein, but that the F protein was not the only contributing factor to HMPV disease severity in animals.

Materials and methods

Cells and HMPV strains

LLC-MK2 cells (ATCC CCL-7) were maintained in minimal essential medium (MEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Wisent). BSR-T7/5 cells (a gift from Dr Ursula Buchholz at the NIAID in Bethesda, MD) were cultured in MEM supplemented with 10% FBS (Wisent), 1% Non-essential amino acids (NEAA) (Life Technologies), 10 mM HEPES (sigma), 1% penicillin / streptomycin (Wisent) and 0.2 mg/ml geneticin (G418, Life Technologies).

The HMPV group A strain C-85473 and group B strain CAN98-75 were grown on LLC-MK2 cells in OptiMEM (Life technologies) supplemented with 0.0002% trypsin (Sigma). Virus stocks were concentrated on Amicon columns (Fisher Scientific) as previously described [20].

Virus quantification

Viral titers were determined by 10-fold serial dilutions of recombinant virus or lung homogenates in 24-well plates containing LLC-MK2 cells as previously reported [21]. Virus titers were reported as 50% tissue culture infectious doses ($TCID_{50}$) per ml. $TCID_{50}$ values were calculated by the Reed and Muench method.

Alternatively, the number of PFU/ml was calculated to determine the MOI for *in vitro* infection experiments (syncytium assay, real-time cell analysis, replication kinetics). Immunostaining of infected cells was performed with MAb 1017, a monoclonal antibody directed against the HMPV F protein (a gift from MedImmune), followed by peroxidase-labeled goat anti-hamster immunoglobulin (Cederlane) and TruBlue peroxidase substrate (KPL/Mandel) as previously described [22].

Construction of antigenome- and supporting protein plasmids

A pSP72 plasmid (Promega) was used to generate the antigenome plasmids as previously reported [23]. Briefly, an NdeI to HpaI fragment was removed from plasmid pSP72 (Promega) and replaced by a T7 terminator, the hepatitis delta virus (HDV) ribozyme and a T7 promoter to yield pSP72-$T7_T$-δ-$T7_P$. cDNA was generated from viral RNA using the Superscript II reverse transcriptase (Life technologies). PCR was carried out using PFU turbo polymerase (Life Technologies). The cDNA encoding the antigenome of C-85473 or CAN98-75 was assembled from 3 or 4 PCR fragments, cloned into temporary pJET plasmids (Thermo Scientific) and sequenced before being cloned into the pSP72 plasmid. The GFP gene was flanked by the N gene start region and the F gene end region of the respective strains and inserted between the N gene and the antigenomic leader sequence using the restriction sites MluI and StuI for CAN98-75 and

MluI and NheI for C-85473. Subsequently, the F genes were interchanged between the 2 stains by site-directed mutagenesis using the Phusion DNA polymerase (New England Biolabs) in the case of CAN98-75_F and using the commercial Gibson Assembly Cloning Kit (New England Biolabs) in the case of C-85473_F (Supplementary Figure S1).

The N, P, L, and M2.1 ORFs of CAN98-75 were amplified by PCR using primers spanning the start and stop codons and flanked by XhoI and NotI restriction sites and were subsequently cloned in the multiple cloning site of the pT_NT vector (Promega) to create supporting protein plasmids.

All plasmids were sequenced using the ABI 3730 DNA analyzer and analyzed using BioEdit, version 7.2.0 prior to further use.

Recombinant virus rescue

BSR-T7 cells were co-transfected with the plasmid containing the antigenome and the 4 supporting protein plasmids using Lipofectamin 2000 (Life Technologies) for 5 h, after which time the medium was replaced by Optimem + 1% NEAA. Transfected cells were incubated at 37°C and 5% CO_2 until GFP expression was observed using fluorescent microscopy. At this point, LLC-MK2 cells were added to the transfected BSR-T7 cells and co-cultured for 2 to 3 days at 37°C and 5% CO_2 with the addition of fresh trypsin (0.0002%) every other day. When infection was observed, cells were harvested, sonicated and centrifuged. The supernatant was then used to infect confluent LLC-MK2 monolayers and virus was cultured until CPE appeared. After 2 passages in LLC-MK2 cells, the recombinant viruses were concentrated using Amicon columns and an aliquot was used to verify the sequence of the F gene (GenBank accession numbers: KM408076.1 and AY145289.1 for C-85473 and CAN98-75, respectively).

Syncytium quantification

Confluent monolayers of LLC-MK2 cells in black 24-well plates with flat and clear bottom (ibidi) were infected with each of the recombinant GFP-expressing HMPV viruses at an MOI of 0.01 in quadruplicate. Trypsine (0.0002%) was added every other day. Syncytium formation was evaluated on a daily basis using a fluorescent microscope. Three photographs were taken of each infected well at 20x magnification. In each field, 40 nuclei were counted and the number of nuclei per cell was calculated.

Real time cell analysis

Real time cell analysis (RTCA) was performed using the xCELLigence System (ACEA). Fifty µl of cell culture medium was added to each well of a 96-well E-Plate (ACEA) to obtain background readings. LLC-MK2 cells were then added at 12 500 cells per well in 100 µl of culture medium. The E-Plates were subsequently incubated for 30 min at room temperature and placed on the RTCA MP station (ACEA) located in an incubator (at 37°C and 5% CO_2). The Cell Index (CI) values were measured automatically every 30 min. When CI reached a plateau (24 h after seeding), cells were washed 2 times with 200 µl of PBS and 6 wells were infected with 150 µl of each of the recombinant HMPV viruses at an MOI of 0.01. Infected E-plates were placed back into the RTCA MP station and the CI values were measured automatically every 30 min for 7 days. Trypsin (0.0002%) was added every other day. Cell indexes were normalized to mock-infected wells and the time until CI was reduced by 50% was calculated for each virus.

In vitro replication kinetics assay

Confluent monolayers of LLC-MK2 cells in 24-well plates were washed twice with PBS and infected with recombinant HMPV viruses at an MOI of 0.01. Trypsin (0.0002%) was added every other day. Three infected wells were harvested every 24 h for 7 days and supernatants were stored at -80°C. End-point titrations were performed on all samples to determine viral titers reported as $TCID_{50}$/ml.

Animal studies

Four week-old BALB/c mice (Charles River Laboratories) were housed in groups of three or four per micro-isolator cage. The mice were infected intranasally with $1x10^6$ $TCID_{50}$ of clinical C-85473 and CAN98-75 HMPV strains or $6x10^5$ $TCID_{50}$ of recombinant rC-85473, rCAN98-75, rC-85473_F and rCAN98-75_F HMPV strains. The animals were monitored on a daily basis for weight loss and the presence of clinical signs such as reduced activity and ruffled fur. Animals were sacrificed when they reached 20% weight loss. For experiments with recombinant HMPV strains, four mice per group were euthanized on days 3 through 6 pi using sodium pentobarbital and lungs were removed for the evaluation of viral titers by cell culture and for the evaluation of cytokine levels using a bead-based multiplex immunoassay. Finally, on day 5 pi, four more mice per group were euthanized and lungs were removed for histopathological analysis. The animal studies were approved by the Animal Protection Committee of the Centre Hospitalier Universitaire de Québec according to the guidelines of the Canadian Council on Animal Care.

In vivo replication kinetics assay

Lungs were removed on days 3 through 6 pi, weighed and homogenized in 1 ml of PBS then centrifuged at 2000 rpm for 10 min. The supernatant was used to determine viral titers reported as $TCID_{50}$/g of lung.

Cytokine analysis

An aliquot of 150 µl of lung homogenates was added to 150 µl of 50 mM KPO_4, pH 6.0 buffer containing 0.2% CHAPS (Sigma) and 0.2% protease inhibitor cocktail (Sigma) and then stored at -20°C. On the day of the experiment, samples were centrifuged at 13,000 × g for 10 min at 4°C and then 50 µl of the supernatant were used for cytokine quantification using a commercial multiplex mouse cytokine bead assay (Bio-Rad) according to the manufacturers' instructions. Experiments were performed in flat bottom 96-well plate and results were analyzed with the Luminex system (QIAGEN).

Histopathological analysis

Lungs were removed on day 5 pi, and fixed with 4% buffered formalin. Fixed lungs were subsequently embedded in paraffin, sectioned in slices of 5 µm, and stained with hematoxylin-eosin. Scoring of histologic parameters was performed by a medical biologist (SL) and an anatomic pathologist (CC), both with experience in pulmonary pathology, independently and blind to experimental data, on digitalized slides scanned at a resolution of 400X magnification (Nanozoomer scanner and viewer, Hamamatsu, Japan). A semi-quantitative scale was used to score bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra alveolar inflammation, capillary vascular congestion and pulmonary edema [21].

Statistical analysis

Repeated-Measure ANOVA's with tukey post-hoc tests were used to analyze all the data except those involving RTCA for which two-tailed Student t-tests were used. All statistical analyses were performed using Prism 6.

Results

***In vitro* and *in vivo* properties of two clinical HMPV strains**

LLC-MK2 monolayers were infected with either C-85473 or CAN98-75 viruses at an MOI of 0.1 to evaluate *in vitro* replication kinetics. The syncytium-inducing C-85473 strain replicated to a significantly higher titer than the focal cell rounding strain CAN98-75 on day 4 pi (8.6 ± 0.8 vs $4.2 \pm 1.3 \times 10^4$ $TCID_{50}$/ml; $p<0.001$) (Figure 2a). After day 4, however, viral titers of strain C-85473 decreased more rapidly than those of the non-syncytium inducing strain.

Figure 2: Replicative capacity of the clinical HMPV isolates C-85473 and CAN98-75 *in vitro* and virulence in BALB/c mice: (a) Replicative capacity of C-85473 and CAN98-75 strans in LLC-MK2 cells at an MOI of 0.1. (b) Kaplan-Meier survival curves of mice infected with $1x10^6$ $TCID_{50}$ of C-85473 or CAN98-75. (c) Weight loss curves of BALB/c mice infected with $1x10^6$ $TCID_{50}$ of C-85473 or CAN98-75 or mock infected. **, $p < 0.01$; ***, $p < 0.001$ comparing C-85473 to CAN98-75 using Repeated Measures Two-way ANOVA.

Intranasal infection of BALB/c mice with 10^6 $TCID_{50}$ of C-85473 led to 37.5% mortality by day 5 post-infection (pi) whereas infection with the same inoculum of CAN98-75 did not induce any mortality (Figure 2b). Mice infected with C-85473 reached their maximum weight loss on day 4 pi (14.4 ± 2.9 %), whereas CAN98-75-infected mice reached their maximum weight loss much later, on day 7 pi (10.3 ± 2.8 %) (Figure 2c). Furthermore, pulmonary viral titers were determined by cell culture on day 5 pi. The C-85473 strain replicated to higher pulmonary viral titers compared to CAN98-75 (18.3 ± 4.6 vs 3.8 ± $1.3x10^3$ $TCID_{50}$/g lung; $p<0.01$).

Generation of recombinant HMPV strains

Based on these observations, we postulated that the F protein, leading to syncytium formation, was also responsible for the increase in HMPV virulence. Thus, we generated recombinant viruses for each strain and swapped the F proteins of both viruses in order to investigate the contribution of individual F proteins in each viral background. Full-length antigenome-plasmids were created for rC-85473, rCAN98-75, rC-85473_F and rCAN98-75 (Supplementary figure S1). Following co-transfection of the antigenome-plasmids and supporting plasmids into BSR T7/5 cells, GFP expression was observed on average 48 h post-transfection. LLC-MK2 cells were then added to amplify viral production and recombinant viruses were rescued within 5 days of transfection.

In vitro phenotype of four recombinant HMPV strains

The *in vitro* phenotype of each HMPV recombinant virus was investigated using fluorescence microscopy. The wild-type (WT) C-85473 strain is known to induce large syncytia and the recombinant strain rC-85473 conserved the same phenotype with 15.9 ± 1.9 nuclei per GFP-expressing cell on day 3 p.i (Figure 3). On the other hand, the recombinant rCAN98-75 induced mostly focal cell rounding, similar to its WT counterpart with 1.2 ± 0.04 nuclei per GFP-expressing cell on day 3 p.i. When the F gene of rC-85473 was replaced with that of rCAN98-75 (rC-85473_F), the phenotype was reverted and less syncytia were observed with 1.2 ± 0.05 nuclei per GFP-expressing cell on day 3 p.i. Finally, rCAN98-75_F induced more syncytia with 23.3 ± 2.1 nuclei per GFP-expressing cell on day 3 p.i. These data confirmed that syncytium formation was primarily dependent on the F gene of the strain C-85473.

Figure 3: Syncytium formation induced by recombinant HMPV strains: (a) LLC-MK2 monolayers in 24 well-plates were infected with rHMPV at an MOI of 0.01 in quadruplicate. On days 2 through 4 pi, pictures were taken using fluorescent microscopy in 3 random fields (20x magnification) per well and the number of nuclei per GFP-expressing cell was calculated. ***, $p < 0.001$ comparing all other strains to rC-85473 and ˚˚˚, $p < 0.001$ comparing all other strains to rCAN98-75 using Repeated Measures Two-way ANOVA. (b) An example of the observed difference in syncytium formation between the 4 recombinant strains on day 3 pi.

Effects of four recombinant HMPV strains on cell state

We also investigated the effect of HMPV infection on the state of LLC-MK2 monolayers using RTCA. This method measures the change in electrical impedance across a cell monolayer in real-time. A parameter called cell index (CI) is used to quantify cell status based on the detected cell-electrode impedance; an elevated CI means that cells have fully adhered to the well and have proliferated, whereas a low CI indicates changes in morphology and viability of the cell monolayer. The changes in CI during an infection

experiment using RTCA is shown in figure 4a and mean time until the normalized CI was reduced by 50% is reported in figure 4b. On average, it took rCAN98-75 28 h longer to reduce the CI by 50% than rC-85473 ((118 ± 9 h compared to 91 ± 8 h). Exchanging the F protein resulted in reverse phenotypes with 50% reduction of CI obtained by 98 ± 4 h and 115 ± 2 h for rCAN98-75_F and rC-85473_F, respectively. These data show that the HMPV strains carrying the syncytium-inducing F protein from C-85473 alter the cell state of infected cells faster than viruses carrying the F protein from CAN98-75.

Figure 4: Real-time cell analysis of recombinant HMPV strains: LLC-MK2 monolayers in 96 well-plates were infected with rHMPV at an MOI of 0.01 (a) Output of one real-time cell analysis (RTCA) experiment; data was normalized using mock-infected wells and normalized cell index is plotted. (b) Mean time until cell index is reduced by 50% from 4 independent experiments is plotted. *, $p < 0.05$ using unpaired, two-tailed Student t-test.

***In vitro* replication kinetics assay**

We next sought to investigate the replicative capacity of each recombinant virus *in vitro*, using an MOI of 0.01 (Figure 5). Over a 7-day period, rC-85473 and rCAN98-75_F (maximum titers of 4.5 ± 0.7 and 3.8 ± 0.8 x10^4 $TCID_{50}$/ml, respectively) replicated to significantly higher titers ($p<0.05$) than rCAN98-75 and rC-85473_F (maximum titers of 1.2 ± 0.5 and 1.3 ± 0.4 x10^4 $TCID_{50}$/ml, respectively). Of note, chimeric viruses (rCAN98-75_F and rC-85473_F) reached their peak titers 24 h later than recombinant WT viruses (rC-85473 and rCAN98-75). This experiment confirms that syncytium-inducing strains replicate to higher viral titers than non-syncytium inducing strains.

Figure 5: *In vitro* replicative capacity of recombinant HMPV strains: LLC-MK2 monolayers in 24 well-plates were infected with 4 rHMPV strains at an MOI of 0.01 in triplicate, one well per condition was harvested every 24 h for 7 days, frozen, sonicated and titrated on LLC-MK2 cells. ***, $p < 0.001$; ** $p <$

0.01 comparing all other strains to rC-85473 and ˚˚, $p < 0.01$; ˚, $p < 0.05$ comparing all other strains to rCAN98-75 using Repeated Measures Two-way ANOVA.

Replication kinetics of HMPV strains in lungs of BALB/c mice

To investigate the replicative capacity of all 4 recombinant viruses *in vivo*, BALB/c mice were infected with $6x10^5$ $TCID_{50}$ of recombinant HMPV strains. Backtitrations of the inoculum confirmed that the same amount of recombinant HMPV was given to all groups (6.3, 6.0, 6.8 and 6.4 x 10^5 $TCID_{50}$/mouse for rC-85473, rCAN98-75, rCAN98-75_F and rC-85473_F, respectively). Such inoculum did not induce mortality in any of the groups. Lungs of infected mice were harvested on day 3 through 6 post-infection to determine viral titers. All 4 recombinant viruses reached their peak of replication on day 4 pi (Figure 6a). rC-85473 replicated to the highest titer (7.2 ± 2.1 $x10^3$ $TCID_{50}$/g lung) whereas rCAN98-75 had the lowest peak (4.6 ± 1.3 $x10^2$ $TCID_{50}$/g lung). Conversely, the chimeric strains rCAN98-75_F and rC-85473_F replicated to similar peak titers (3.2 ± 1.01 and 3.0 ± 0.9 $x10^3$ $TCID_{50}$/g lung, respectively).

Figure 6: lung viral titers and weight loss of BALB/c mice infected with recombinant HMPV strains: BALB/c mice were infected with $6x10^5$ $TCID_{50}$ of rHMPV (as determined by backtitration). (a) On days 3 through 6, four mice per group were euthanized to determine pulmonary viral titers. (b) Six mice per group were monitored for weight loss on a daily basis for 14 days. ***, $p < 0.001$; * $p < 0.05$ comparing all other strains to rC-85473.˚˚˚, $p < 0.001$; ˚, $p < 0.05$ comparing all other strains to rCAN98-75 using Repeated Measures Two-way ANOVA.

Weight loss of HMPV-infected mice

Mice infected with $6x10^5$ $TCID_{50}$ of recombinant HMPV strains or mock infected were monitored for 14 days for clinical signs and weight loss. All infected mice lost between 3 and 7 percent of their initial weight between days 1 and 3, but only viruses with the C-85473 background (rC-85473 and rC85473_F) continued

to lose weight on days 5 - 7, with statistically significant differences observed between rC-85473-viruses and rCAN98-75 viruses on days 6 - 9 (Figure 6b). No significant difference in weight loss was observed between the 2 viruses with CAN98-75 background (rCAN98-75 and rCAN98-75_F). rC-85473-infected mice appeared to recuperate a little bit faster than mice infected with rC-85473_F with a statistically significant difference observed on day 9 pi only. Thus, the severity of the HMPV symptoms correlated better with the viral background than the F protein.

Pulmonary cytokine levels of HMPV-infected mice

On days 3 through 6 pi of the previously described experiment, an aliquot of lung homogenates was used to determine pulmonary cytokine/chemokine levels using a multiplexed bead assay (Figure 7).

Figure 7: Pulmonary cytokine levels of BALB/c mice infected with recombinant HMPV strains: BALB/c mice were infected with $6x10^5$ $TCID_{50}$ of rHMPV (as determined by back-titration). On days 3 through 6, four mice per group were euthanized to determine pro-inflammatory cytokine/chemokine levels in the lungs of infected mice. Mock-infected mice are representad as day 0. ***, $p < 0.001$; **, $p < 0.01$; * $p < 0.05$ comparing all other strains to rC-85473.˚˚˚, $p < 0.001$; ˚˚, $p < 0.01$; ˚, $p < 0.05$ comparing all other strains to rCAN98-75 using Repeated Measures Two-way ANOVA.

IL-2 peaked on day 5 pi for all 4 recombinant viruses and IL-2 levels were similar between groups at each time point. IL-6 also peaked on day 5 pi, but IL-6 levels were significantly higher in viruses with a C-85473 background compared to viruses with a CAN98-75 background on days 5 pi. IL-12 levels remained relatively stable between days 3 and 6 pi, except for rCAN98-75 for which IL-12 levels were reduced by half by day 5 pi. Significantly higher levels of IL-12 were observed with the viruses harboring the C-85473 background compared to the rCAN98-75 virus on all analyzed time-points. However, introducing the F

protein of C-85473 into rCAN98-75 significantly increased IL-12 levels on days 5 and 6 pi. IFN-γ levels reached a plateau on days 5 and 6 pi for both viruses with a C-85473 background as well as for rCAN98-75_F, whereas IFN-γ levels declined from day 3 onward for rCAN98-75. KC levels were highest on day 3 pi, with a second peak on day 5 pi for all viruses except rCAN98-75, for which KC levels increased a day later. KC levels were significantly higher for recombinant viruses with a C-85473 background compared to rCAN98-75. Furthermore, the introduction of the F protein from C-85473 into the CAN98-75 background significantly increased KC levels on days 3, 5 and 6 pi compared to the wild-type rCAN98-75.

MCP-1 levels were consistently significantly higher for viruses with a C-85473 background compared to rCAN98-75. Moreover, on day 5 pi, a significantly higher MCP-1 level was observed in mice infected with rCAN98-75_F compared to rCAN98-75. MIP-1α levels peaked on day 5 pi for all strains except rCAN98-75, which peaked on or before day 3 pi. Furthermore, significantly higher MIP-1α levels were observed on days 5 and 6 pi for rC-85473 and rC-85473_F as well as for rCAN98-75_F, compared to rCAN98-75. In general RANTES levels were lower for strain rCAN98-75 than for the other 3 viruses with statistically significant differences observed at early time-points.

Lung histopathology studies of HMPV-infected mice

On day 5 of the previously described experiment, lungs were harvested from 4 more mice per group to assess pulmonary inflammation. None of the groups showed signs of vascular congestion, pulmonary edema or bronchial inflammation. Moreover, none of the mice infected with rCAN98-75 showed signs of pulmonary inflammation for any of the analyzed parameters. Conversely, mice infected with rC-85473 or rC-85473_F showed mild, moderate or moderate to marked scores, with no significant differences in scores for any of the parameters between these two groups (total inflammation scores of 7.4 ± 0.7 and 7.9 ± 0.5 for rC-85473 and rC-85473_F, respectively) (Figure 8). Importantly, the introduction of the syncytium-inducing F protein into the rCAN98-75 background significantly increased histopathology

scores for peribronchial, perivascular, interstitial and intra-alveolar inflammation (data not shown) as well as total inflammation (total inflammation score of 5.8 ± 0.6). In summary, although the C-85473 background induced significantly more pulmonary inflammation, the introduction of the syncytium-inducing F protein into the rCAN98-75 background significantly increased lung inflammation.

Figure 8: Total pulmonary inflammation scores of BALB/c mice infected with recombinant HMPV strains: BALB/c mice were infected with $6x10^5$ $TCID_{50}$ of rHMPV (as determined by back-titration). On days 5, four mice per group were euthanized to determine histopathology scores of the lungs of infected mice. ***, $p < 0.001$; comparing all other strains to rC-85473.˚˚˚, $p < 0.001$ comparing all other strains to rCAN98-75 using Repeated Measures Two-way ANOVA.

Discussion

In the current study, we examined the effects of the F protein from two different HMPV strains, producing large syncytia in cell culture or not, on *in vitro* and *in vivo* replication kinetics and virulence. We generated recombinant HMPV viruses representing either the syncytium-inducing phenotype (strain rC-85473) or the focal cell rounding phenotype (strain rCAN98-75) and we subsequently exchanged the F genes of both strains. We demonstrated that syncytium phenotype mainly depends on the F protein and that viruses carrying an F protein that induces syncytium-formation replicate to higher titers *in vitro*. However, although the F protein appears to contribute to HMPV virulence, other genetic markers within the HMPV genome seem to impact on disease severity in mice.

HMPV is an important respiratory pathogen that can cause upper and lower RTIs. Virological risk factors for severe HMPV disease, such as HMPV subtype or lineage, have been the object of several investigations, with conflicting results [6,8-10,24]. We first sought to investigate whether the *in vitro* phenotype, namely syncytium formation, might be an indication of efficient HMPV replicative capacity. Previously, the

syncytium-inducing strain NL/1/99 (subtype B1) was found to replicate to higher titers in Vero-118 cells than NL/1/00 (subtype A1), a strain that does not produce syncytium at neutral pH [23,25]. Similarly, we found that the syncytium-inducing strain C-85473 replicated to higher titers in LLC-MK2 cells than the focal cell rounding strain CAN98-75. However, to our knowledge, the impact of *in vitro* phenotype on *in vivo* replication i.e., on lung titers, had not yet been examined. For this purpose, we infected BALB/c mice with either C-85473 or CAN98-75 clinical isolates and found that the former replicated to higher titers in the lungs on day 4 pi than CAN98-75 in 3 independent experiments (data from one representative experiment are shown in figure 2). Furthermore, mortality was only observed in mice infected with strain C-85473.

Similarly to other paramyxoviruses, HMPV enters the host cell by fusion of viral and cellular membranes, a step mediated by surface glycoproteins. On its surface, HMPV carries 3 glycoproteins (F, G and SH) of which the F protein is the most conserved among HMPV strains [12]. As such, the HMPV F protein also shares structural features with other paramyxovirus F proteins; it is a class I viral fusion protein synthesized as inactive precursors (F_0) that must be cleaved into 2 disulfide-linked F_2-F_1 subunits to be fusion-competent [15]. Unlike members of the *Paramyxovirinae* subfamily, but similarly to other members of the *Pneumovirinae* subfamily including HRSV, the HMPV F protein mediates membrane fusion in the absence of a separate viral attachment protein [16,26]. Furthermore, by transfecting cells with recombinant HMPV F proteins, it was demonstrated that the F protein alone is able to induce syncytium formation in cell culture [16,17].

To investigate the role of the F protein on viral replication both *in vitro* and *in vivo*, we used reverse genetics to generate GFP-expressing recombinant viruses with either the C-85473 or the CAN98-75 genome and we further exchanged the F genes of both strains. Inserting the F gene of C-85473 into the

CAN98-75 background restored the syncytium phenotype of strain C-85473, confirming that the F protein is responsible for syncytium formation independently of the genomic background.

Moreover, using RTCA, we demonstrated that the syncytium-inducing rHMPV strains induced changes in cell state 24 h before the focal cell rounding viruses. RTCA is a new electronic cell sensor array, in which the impedance, displayed as CI values, is continuously measured to evaluate cellular integrity [27,28]. This parameter includes cell proliferation, adhesion, viability, morphology and motility. This novel technique has been used to evaluate CPE and their inhibition by neutralizing antibodies directed against flaviviruses [28] and influenza A viruses [27], but also to evaluate antiviral activity [29,30] and cytotoxicity [31]. Here, we demonstrate the usefulness of RTCA in investigating the dynamics of HMPV infection for the first time.

A significant increase in *in vitro* replicative capacity of syncytium-inducing HMPV strains was also confirmed using replication kinetics assays. Laboratory strains were passaged extensively (10 times), which increased their viral titers significantly. On the other hand, we only passaged the recombinant viruses twice to avoid introducing mutations and defective interfering RNAs that could alter the immune response *in vivo* [32], but this led to lower viral stock titers (by about 1 log). Although the change in genomic background did not alter peak viral titers of viruses expressing the same F protein, both chimeric viruses reached their peak of replication a day later than WT rHMPV strains; this could indicate some influence of the genomic background on F protein incorporation and/or expression.

Notably, we investigated whether *in vitro* phenotypes also correlated with *in vivo* replication titers. We observed that peak lung titers occurred on day 4 pi for all recombinant HMPV viruses. Again, rC-85473 was associated with the highest viral titers and introducing the F protein of CAN98-75 into this strain significantly reduced peak viral titers (Figure 6a). Conversely, the rCAN98-75 strain generated the lowest peak viral titers and introducing the F protein of C-85473 significantly increased maximum pulmonary viral titers. However, the overlap in the replication curves of the two chimeric viruses suggests that *in vivo*

properties were not exclusively dependent on the F protein. This was even more evident by looking at weight loss curves where strains were primarily segregated by their genomic background and not by their F protein. Similarly, pro-inflammatory cytokine/chemokine levels and pulmonary inflammation scores were increased in the rC-85473 strains independently of the F protein, although rCAN98-75_F induced higher cytokine levels (particularly MIP-1α, MCP-1, IL-12 and KC) and significantly more pulmonary inflammation than the prototypic rCAN98-75 strain, suggesting that the F protein does have an effect on HMPV pathogenesis as well.

We investigated 8 cytokines/chemokines (IL-2, IL-6, IL-12, IFN-γ, KC, MIP-1α, MCP-1 and RANTES) that had previously been described to be up-regulated in the lungs of HMPV-infected mice [21,33,34]. Although different inoculums and cytokine detection methods were used in previously-published studies, we also observed up-regulation of all cytokines/chemokines in mice infected with C-85473 strains, but only MIP-1α and MCP-1, two chemokines involved in the recruitment and activation of leukocytes, as well as IL-12, a cytokine involved in Th1 differentiation, were significantly up-regulated for rCAN98-75 compared to mock-infected mice (represented as day 0 on the graph).

Unlike HRSV, HMPV does not encode non-structural proteins known to inhibit antiviral immune responses. Therefore, other viral proteins must be involved in immune evasion mechanisms. Among HMPV-expressed proteins, the SH and G glycoproteins, the P phosphoprotein and the M2-2 protein have been found to have immune evasive properties. The presence of the SH protein reduced the expression of TNF-α, IL-6, KC and MCP-1 in mice infected with WT HMPV compared to mice infected with HMPV lacking the SH protein, in an NF-κB-dependent manner [35]. The G protein was found to reduce cytokine/chemokine levels in cell cultures by inhibiting RIG-1 signaling [36,37]. Furthermore, the G protein inhibited TLR4-signaling in dendritic cells [38]. Goutagny et al. observed that a HMPV-B1 strain impaired type I IFN production, specifically by prevented RIG-I-mediated sensing of HMPV viral RNA, in a P protein-

dependent manner [39]. Finally, the M2-2 protein inhibited MAVS-induced IFN-β gene transcription *in vitro* [40]. One or several of these viral proteins may be implicated in the observed differences in pathogenesis between strains with the C-85473 and CAN98-75 genomic background and this will be further investigated.

We did not investigate pH-dependency of our different HMPV strains. In contrast to previous reports [17,18], our syncytium-inducing virus is a subtype A strain and the focal cell rounding virus is a subtype B strain. This suggests that, although low-pH-triggered syncytium-formation may be subtype-dependent, syncytium formation at neutral pH does not appear to be subtype-dependent. Furthermore, the physiological relevance of low pH-dependent syncytium-formation remains to be elucidated. Interestingly, our large syncytium-inducing strain carries EKRN at the proposed tetrad positions of the F protein, which are the same as NL/17/00, a poorly fusogenic strain [18]. Moreover, our focal cell-rounding strain carries EDRP, the same tetrad as NL/1/94, a pH-independent syncytium-inducing strain [18]. This suggests that residues at other positions are likely to influence syncytium formation at neutral pH. Given the importance of the heptad repeats located in F_1 subdomain of the F protein for viral fusion, it would be interesting to examine the effect of the five amino acid changes in HRA and the two amino acid changes in HRB that differentiate our two prototype strains, on syncytium formation at neutral pH.

In conclusion, we have demonstrated the importance of the HMPV F protein for syncytium formation and *in vitro* replication and further showed that the F protein contributes to some extent, but not exclusively, to the virulence potential of different strains in mice. Therefore, it is unlikely that the *in vitro* phenotype of an HMPV strain alone is sufficient to predict the severity of HMPV disease. Other syncytium-inducing and non-syncytium inducing HMPV strains of different subtypes should be studied to confirm our results. Furthermore the effects of other viral genes on HMPV pathogenesis should be investigated using recombinant viruses in different animal models

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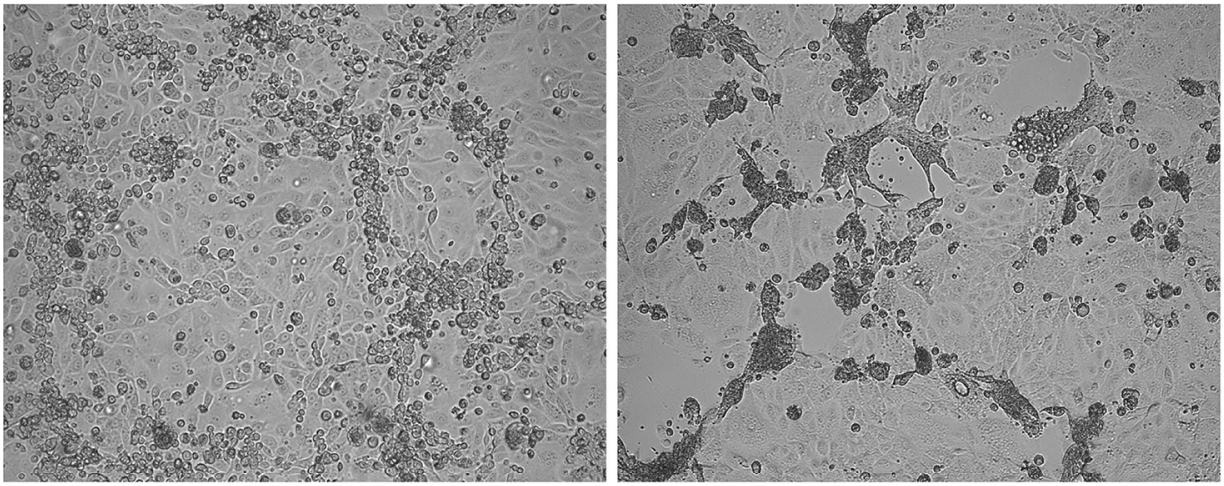
Supplementary Figure legends

S1 Figure: Construction of HMPV antigenome plasmids: Schematic representation of the cloning steps for rCAN98-75 (a) and for rC-85473 (b). Three or four cDNA fragments (A-C for rC-85473 and A-D for rCAN98-75) were cloned into the pSP72-$T7_T$-δ-$T7_P$ vector. The obtained antigenome plasmids were then used to swap the F genes. (c) Schematic representation of the cloning steps used to obtain rCAN98-75_F; a fragment covering M to SH was amplified from the rCAN98-75 antigenomic plasmid and cloned into the temporary pJET vector. Simultaneously, the F gene was amplified from C-85473 cDNA and also cloned into pJET. Both plasmids were digested and the F gene was ligated into the temporary pJET plasmid. From this vector, a fragment covering the region M to M2 was amplified and used as primers for site-directed mutagenesis of the rCAN98-75 antigenomic plasmid. (d) A schematic representation of the cloning steps used to obtain rC-85473_F; a fragment containing the region P to M2 of rC-85473 was cloned into a temporary pJET vector. A fragment of the F gene of rCAN98-75, flanked by the restriction sites EcoRV and PacI, was amplified by PCR and subsequently cloned into the temporary pJET vector containing the rC-85473 fragment. From this vector, fragment 1 was amplified by PCR. Fragment 2 was amplified directly from the plasmid containing the rCAN98-75 antigenomic vector and fragments 3 and 4 were amplified from the rC-85473 antigenomic vector. Finally the pSP72 vector was digested to obtain fragment 5. All 5 fragments were then ligated using the Gibson Assembly cloning kit.

Figure 1

(a)

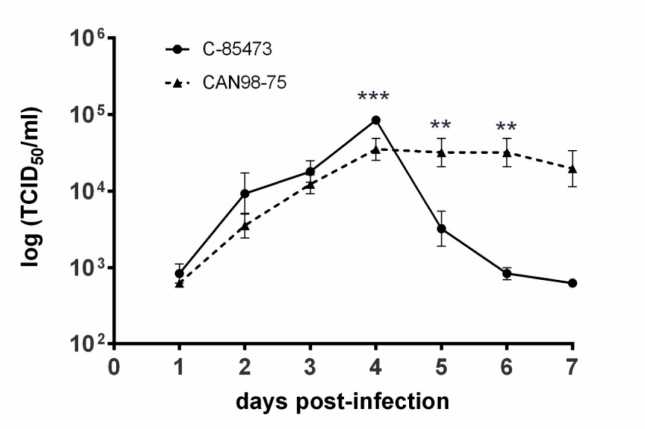


(b)

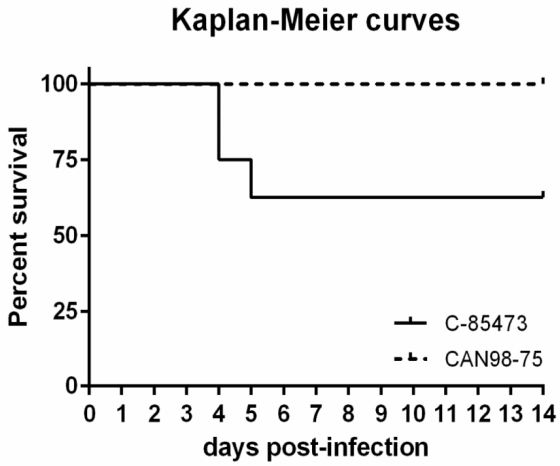

rC-85473
GFP
N
P
M
F
M2
SH
G
L
rCAN98-75
GFP
N
P
M
F
M2
SH
G
L
rC-85473_F
GFP
N
P
M
F
M2
SH
G
L
rCAN98-75_F
GFP
N
P
M
F
M2
SH
G
L

Figure 2:

(a)


C-85473
CAN98-75
log (TCID50/ml)
days post-infection
***
**
**

(b)


Kaplan-Meier curves
Percent survival
days post-infection
C-85473
CAN98-75

(c)

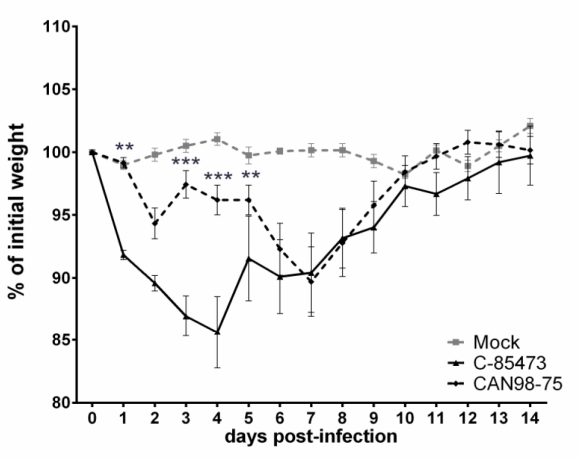

% of initial weight
days post-infection
Mock
C-85473
CAN98-75

Figure 3:

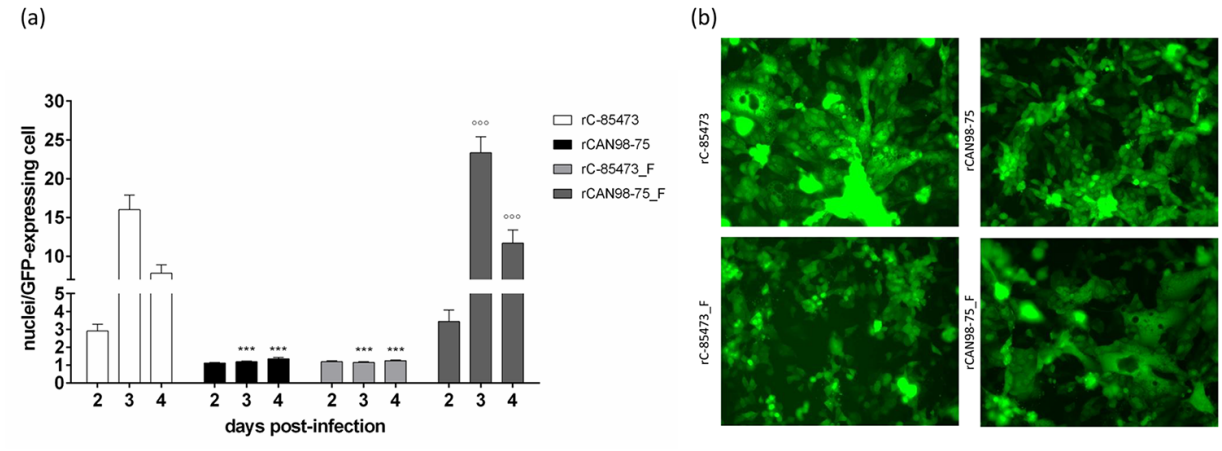


Figure 4:

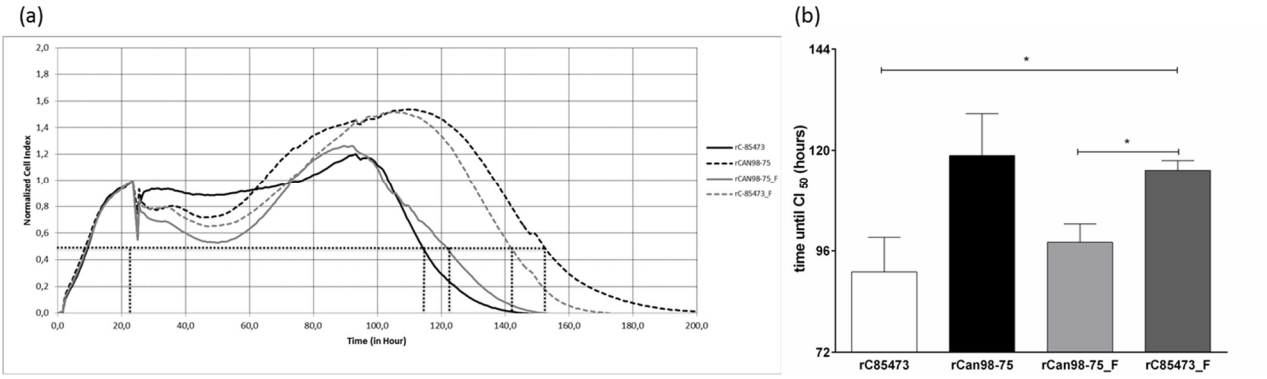
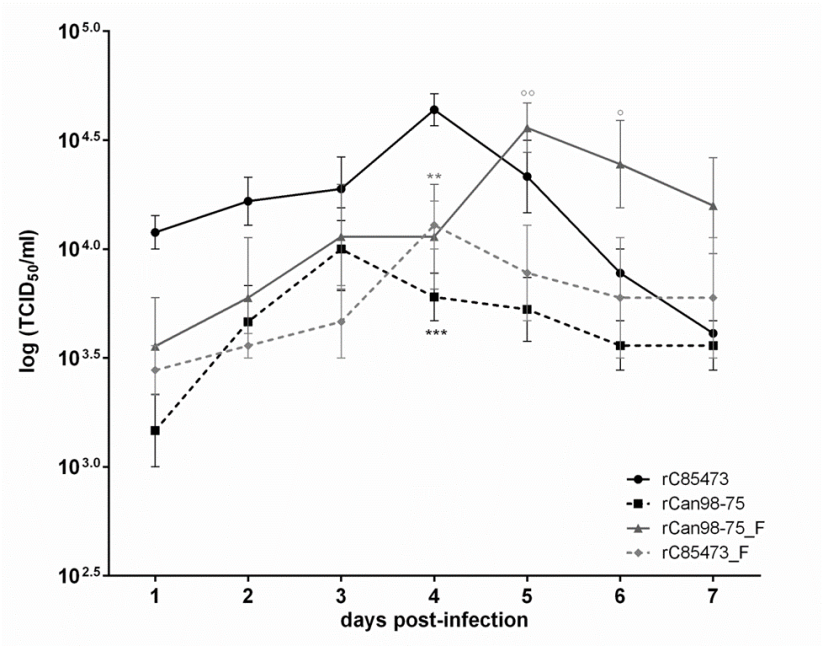


Figure 5:



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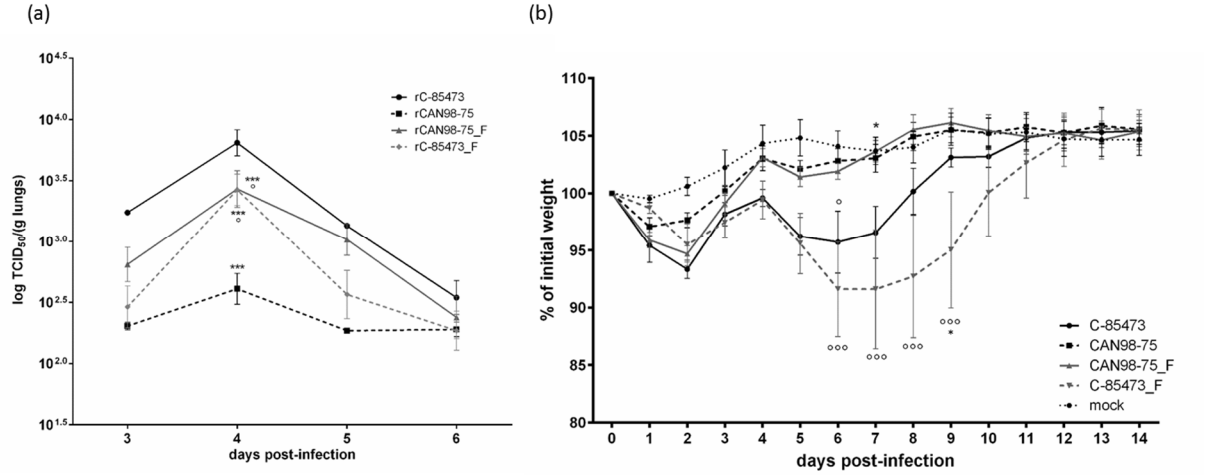

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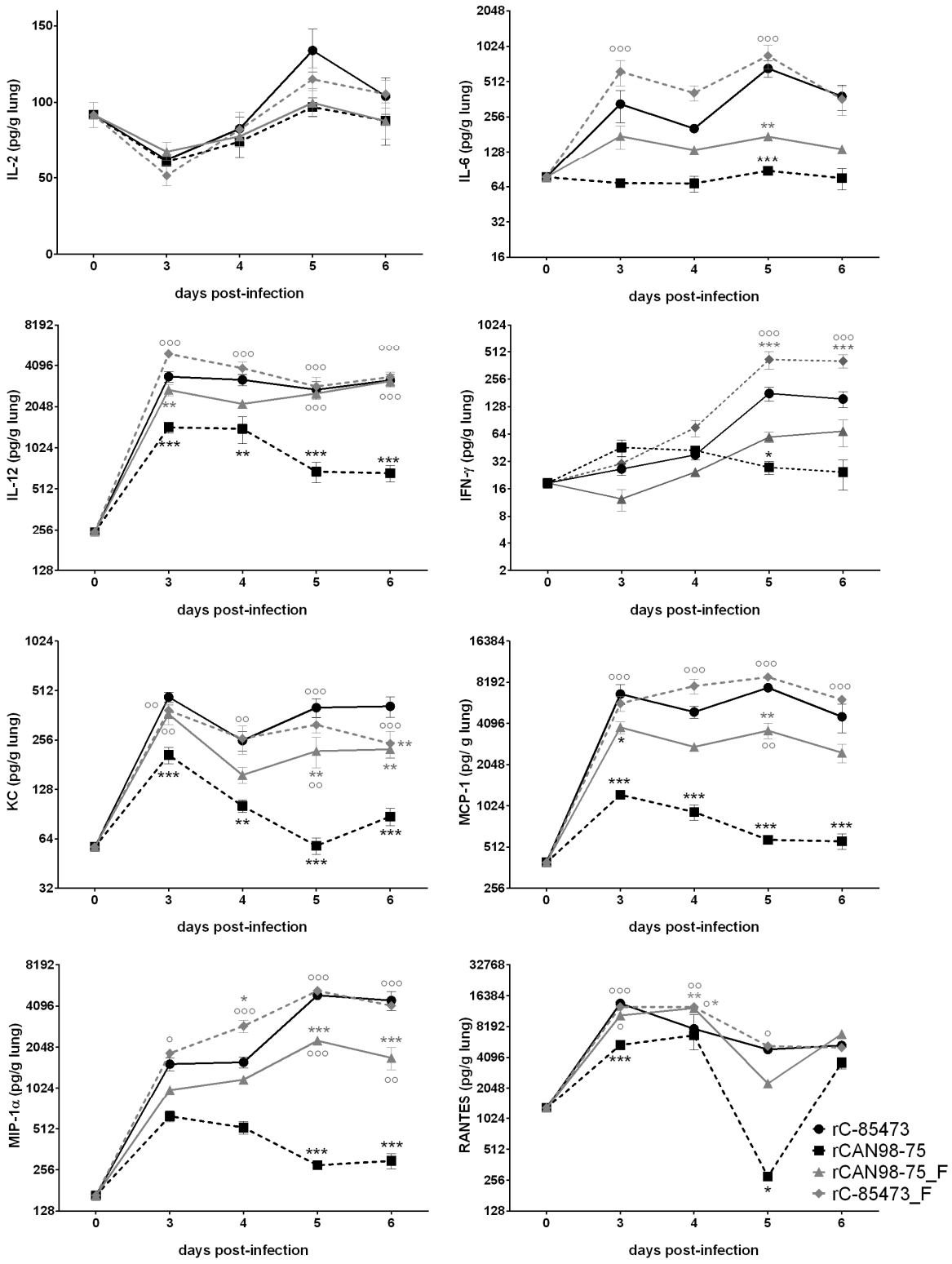
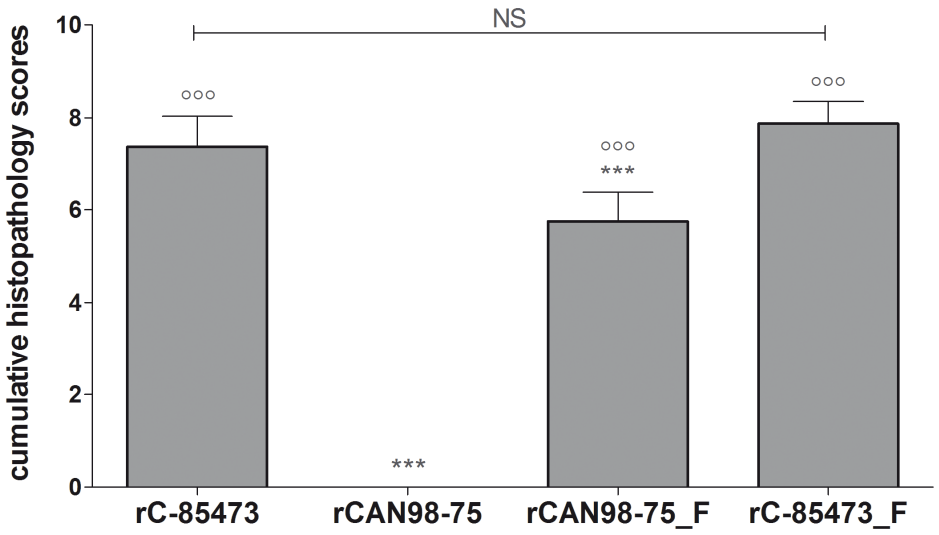
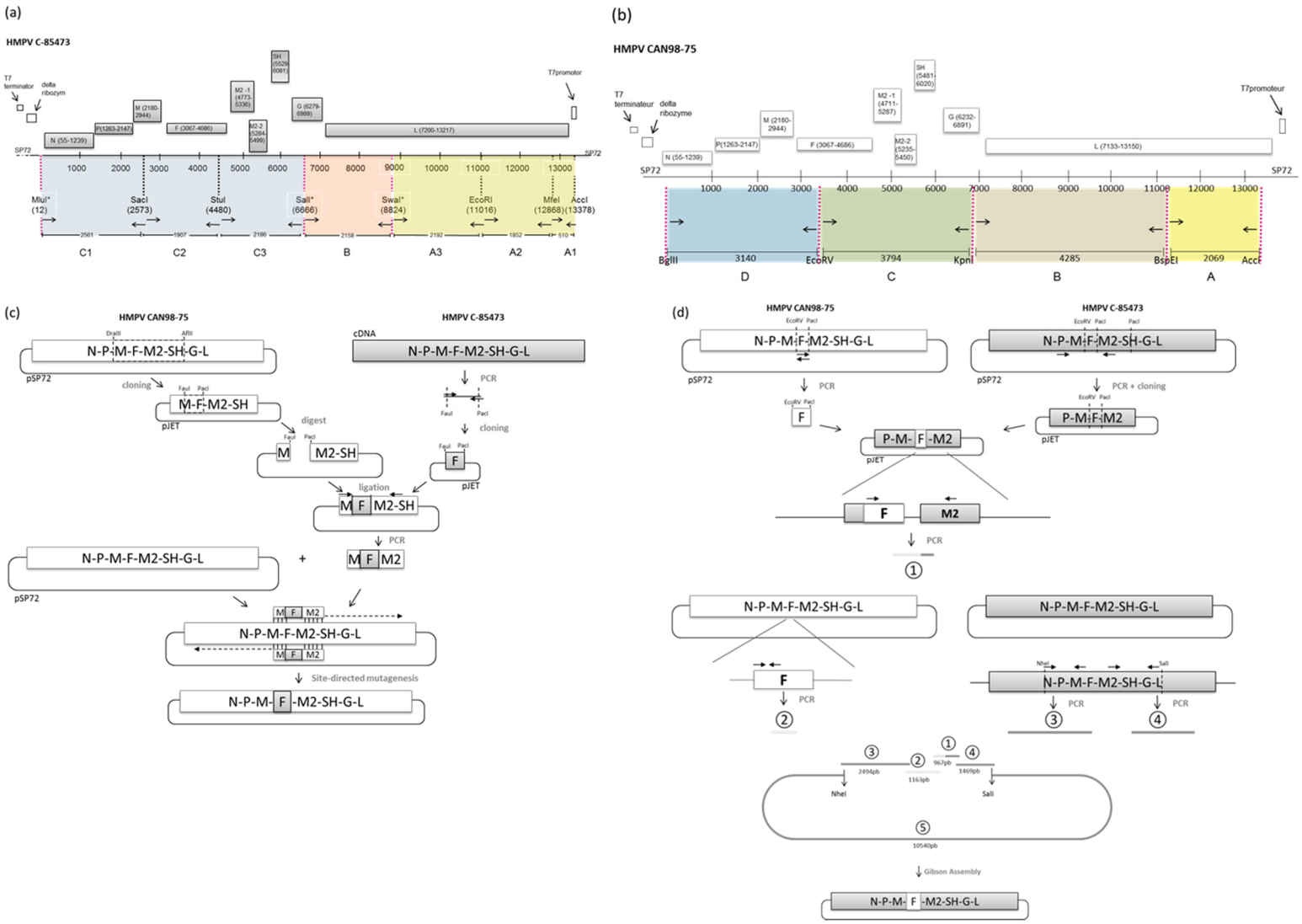

IL-2 (pg/g lung)
IL-6 (pg/g lung)
IL-12 (pg/g lung)
IFN-γ (pg/g lung)
KC (pg/g lung)
MCP-1 (pg/ g lung)
MIP-1α (pg/g lung)
RANTES (pg/g lung)
days post-infection
rC-85473
rCAN98-75
rCAN98-75_F
rC-85473_F

Figure 8 :


NS
cumulative histopathology scores
10
8
6
4
2
0
°°°
°°°
°°°
***
***
rC-85473
rCAN98-75
rCAN98-75_F
rC-85473_F

Supplementary figure 1:



Chapter 4: Modulation of Protease Activated Receptor 1 Influences Human Metapneumovirus Disease Severity in a Mouse Model

1. Preface

Chapter 4 contains the paper entitled: "*Modulation of Protease Activated Receptor 1 Influences Human Metapneumovirus Disease Severity in a Mouse Model*" written by Laetitia Aerts, Marie-Ève Hamelin, Chantal Rhéaume, Sophie Lavigne, Christian Couture, WooJin Kim, Delia Susan-Resiga, Annik Prat, Nabil G. Seidah, Nathalie Vergnolle, Beatrice Riteau and Guy Boivin and published in PLOS ONE on August 28, 2013. DOI: 10.1371/journal.pone.0072529

2. Author contributions

Laetitia Aerts:

- designed the experiments
- *in vitro* experiments
- *In vivo* experiments
- wrote manuscript

Marie- Ève Hamelin :

- supervised the project
- corrected the manuscript

Chantal Rhéaume :

- assisted in *in vivo* experiments

Sophie Lavigne and Christian Couture:

- histopathologial analysis

WooJin Kim, Delia Susan-Resiga, Annik Prat and Nabil G. Seidah:

- furin expression levels
- furin, HMPV-F and PAR1 co-transfection experiment

Nathalie Vergnolle, Beatrice Riteau:

- Investigated specificity of the PAR1 agonist and PAR1 antagonist by evaluating the intestinal permeability of WT and PAR-deficient C57Bl6 mice treated with either compound.
- Supervised the project

Guy Boivin :

- supervised the project
- corrected the manuscript

3. Paper

Title: Modulation of protease activated receptor 1 influences human metapneumovirus disease severity in a mouse model

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Abstract

Human metapneumovirus (hMPV) infection causes acute respiratory tract infections (RTI) which can result in hospitalization of both children and adults. To date, no antiviral or vaccine is available for this common viral infection. Immunomodulators could represent an interesting strategy for the treatment of severe viral infection. Recently, the role of protease-activated receptors (PAR) in inflammation, coagulation and infection processes has been of growing interest. Herein, the effects of a PAR1 agonist and a PAR1 antagonist on hMPV infection were investigated in BALB/c mice. Intranasal administration of the PAR1 agonist resulted in increased weight loss and mortality of infected mice. Conversely, the PAR1 antagonist was beneficial to hMPV infection by decreasing weight loss and clinical signs and by significantly reducing pulmonary inflammation, pro-inflammatory cytokine levels (including IL-6, KC and MCP-1) and recruitment of immune cells to the lungs. In addition, a significant reduction in pulmonary viral titers was also observed in the lungs of PAR1 antagonist-treated mice. Despite no apparent direct effect on virus replication during *in vitro* experiments, an important role for PAR1 in the regulation of furin expression in the lungs was shown for the first time. Further experiments indicated that the hMPV fusion protein can be cleaved by furin thus suggesting that PAR1 could have an effect on viral infectivity in addition to its immunomodulatory properties. Thus, inhibition of PAR1 by selected antagonists could represent an interesting strategy for decreasing the severity of paramyxovirus infections.

Introduction

Human metapneumovirus (hMPV) is a paramyxovirus responsible for acute respiratory tract infections that are especially severe in young children [1,2], elderly people [3,4] and immunocompromized individuals [5,6]. Limited reports in humans [3,7,8] and extensive studies in animals [9-13] have shown that severe hMPV infection is associated with the disruption of the epithelial architecture and the presence of inflammatory infiltrates around and within alveoli. Among the diverse small animal models that have been evaluated, BALB/c mice have emerged as an excellent model for recapitulating hMPV infection, including efficient viral replication in the lungs, concomitant pulmonary inflammation and airway obstruction as well as disease severity that correlates with increasing viral inoculums [10,14-16]. BALB/c mouse studies have also shown that the immune response to hMPV infection can be both disproportionate and inefficient, characterized by a weak innate response and a Th2-biased adaptive immune response, leading to impaired virus clearance and prolonged pulmonary inflammation [10,14-16].

To date, no vaccine or specific antiviral agents are available for the treatment of hMPV infection [17]. Ribavirin, a nucleoside analogue licensed for treatment of severe human respiratory syncytial virus (hRSV) infections, exhibits moderate *in vitro* activity against hMPV and was found to be beneficial in the mouse model [18,19]. Indeed, this antiviral has been used to treat a few severe cases of hMPV pneumonia in immunocompromised individuals [20-22]. The mechanisms of action of ribavirin do not appear to be limited to the disruption of the viral purin metabolism and inhibition of viral RNA polymerase. It has been shown that this drug could also upregulate IL-2, TNF-α, interferon-γ and downregulate Th2 cytokines such as IL-10 *in vitro*, suggesting an immunomodulatory as well as an antiviral effect [23]. However, ribavirin is associated with many side-effects and teratogenicity [24], which limits its widespread use. Thus, the development of new

antivirals and/or immunomodulatory strategies is warranted for the treatment of severe hMPV infections.

Secretory serine proteases have long been known to play an important role in several physiological processes such as hemostasis (primary hemostasis, coagulation and fibrinolysis) and immune responses to infection [25]. Increasing interactions between these two systems are being discovered, including during viral infection[26]. Serine proteases of the coagulation cascade, such as thrombin, are able to activate complement components [27,28] and to regulate cell function through the activation of a family of G-protein coupled receptors called protease-activated receptors (PAR) [29-33]. PARs have a unique mechanism of activation in which irreversible proteolytic cleavage within the extracellular N-terminus of the receptor exposes a new N-terminal sequence that acts as a tethered ligand. The latter interacts with the second extracellular loop of the receptor, hereby activating the receptor [30,34,35]. Four PARs have been characterized until now (PAR1, PAR2, PAR3 and PAR4). They are all activated by thrombin except PAR2 for which the main activating protease is trypsin [30,35].

PAR1 in particular is a ubiquitous receptor present in platelets, endothelial and epithelial cells, neurons, fibroblasts, smooth muscle cells, leukocytes and tumor cell lines [30]. PAR1 is a receptor involved in many physiological processes including the cardiovascular, respiratory and central nervous systems as well as in embryogenesis, cancer and inflammation [35]. In fact, some PAR1 antagonists are currently in clinical trials for their antiplatelet activities [36,37]. The role of PAR1 in the pathogenesis of infectious diseases has recently been investigated for a number of pathogens including feline immunodeficiency virus [38], *Mycobacterium tuberculosis* [39] , dengue virus [40], herpes simplex virus [41,42] and influenza A virus [43,44]. For the latter pathogen, upregulation of

PAR1 was observed in the airways of mice infected with the highly pathogenic influenza A/PR-8/34 (H1N1) virus [43]. Based on these observations, we hypothesized that PAR1 could also play an important role in modulating the immune response during severe hMPV infection. Therefore, we treated hMPV-infected BALB/c mice with either a selective PAR1 agonist (TFLLR-NH_2) or a PAR1 antagonist (SCH79797) and then evaluated disease severity. We observed that PAR1 activation increased weight loss and decreased survival in our hMPV mouse model. In contrast, administration of a PAR1 antagonist decreases disease severity and significantly reduced lung cytokines and inflammation in BALB/c mice. The protective effect of the PAR1 antagonist could also be explained by a decrease in furin-mediated cleavage of the viral fusion (F) protein.

Results

PAR1 agonist (TFLLR-NH_2) and PAR1 antagonist (SCH79797) do not exhibit antiviral activity against hMPV in LLC-MK2 cells. We sought to investigate whether the PAR1 agonist or antagonist had an effect on viral replication *in vitro*. In a first experiment, uninfected LLC-MK2 monolayers were pre-incubated with the compounds and then infected. However, cell monolayers detached at concentrations > 0.8 µM of the PAR1 antagonist (SCH79797), possibly due to the pro-apoptotic effect of this reagent [45] and the toxicity of the vehicle (DMSO). No toxicity was observed using concentrations up to 250 µM of the PAR1 agonist (TFLLR-NH_2). Using concentrations of up to 0.8 µM for the antagonist and 250 µM for the agonist, no antiviral effect was observed. In a second experiment, hMPV was pre-incubated with the compounds (maximum concentration of 0.8 µM) before addition to LLC-MK2 monolayers. No antiviral effect was again observed when the compounds were removed and replaced by fresh medium after the adsorption phase or when the compounds were replenished daily throughout the experiment (not shown).

PAR1 agonist and antagonist have an inverse dose-dependent effect on hMPV infection *in vivo*. In a first *in vivo* experiment, BALB/c mice were infected intranasally with hMPV (4-6x10^5 $TCID_{50}$) or mock infected and simultaneously treated intranasally, for a period of 3 days, with a single daily dose of 50 or 500 µM of PAR1 agonist (TFLLR-NH_2) or PAR1 antagonist (SCH79797). PAR1 agonist- or PAR1 antagonist-treatment of uninfected mice did not induce weight loss, mortality or any signs of toxicity (data not shown). Mortality was only observed in PAR1-agonist treated mice (17% on day 6 post infection (pi) and 50% on day 7 pi for mice treated with 50 µM and 500 µM of PAR1 agonist, respectively). These groups also had a greater weight loss compared to infected, vehicle-treated mice (Fig. 1A). Conversely, weight loss was significantly reduced in a dose-dependent manner in PAR1 antagonist-treated mice compared to the infected, vehicle-treated group (Fig. 1B). No significant difference in pulmonary viral titers was observed between PAR1 agonist-treated mice and vehicle-treated controls. In contrast, viral titers in the lungs of PAR1 antagonist-treated mice were significantly lower than those of vehicle-treated mice by about 1 log (Fig. 1C). Thus, we conclude that PAR1 plays a deleterious role in the pathogenesis of hMPV infections.

Prolonged treatment with the PAR1 antagonist (SCH79797) prevents symptomatic hMPV infection. In the preliminary PAR1 experiment, we observed that a 3-day treatment with the PAR1 antagonist (SCH79797) reduced weight loss in a dose-dependent manner. In order to evaluate if a longer treatment had a more pronounced effect, the compound was administered as a single daily dose of 500 µM for 5 days, starting at the time of infection. PAR1 antagonist-treatment of uninfected mice did not induce weight loss, mortality or any signs of toxicity. PAR1 antagonist-treated and infected mice remained asymptomatic i.e. showed no weight loss, reduced activity or ruffled fur throughout the experiment (Fig. 2A). These findings were confirmed in a second experiment using 18 mice per group (data not shown). Moreover, on day 5 pi, a significant decrease in pulmonary

inflammation was observed in treated mice (Fig. 2B) that correlated with a significant decrease in pro-inflammatory cytokine levels (Fig. 2C). In addition, viral replication in the lungs on day 5 pi, was significantly reduced by about 1 log in PAR1 antagonist-treated mice, compared to controls, confirming the results of the previous 3-day experiment (Fig. 2D). Thus, we conclude that blocking PAR1 protects mice from symptomatic hMPV disease.

Cell recruitment in lungs of mice treated with the PAR1 antagonist. Immune cell populations present in the lungs on day 5 pi were analyzed by flow cytometry in mice treated for 5 days starting at the time of infection with 500 µM of the PAR1 antagonist (Fig. 3). The immune cell populations analyzed included activated dendritic cells ($CD11c^{+}CD11b^{lo}Ly6g^{lo}I\text{-}A/I\text{-}E^{+}$), macrophages ($CD11b^{+}CD11c^{+}F4/80^{hi}$), T lymphocytes ($CD3^{+}CD4^{+}$ or $CD3^{+}CD4^{-}$) and neutrophils ($CD11c^{-}CD11b^{hi}Ly6G^{+}$). Significantly reduced populations of activated dendritic cells, macrophages and $CD4^{-}$ T-lymphocytes were observed in the lungs of PAR1 antagonist-treated mice compared to hMPV-infected untreated mice. Although there appears to be a decrease in neutrophils in infected untreated mice, CD11c-CD11bhiLy6G+ events were extremely low. This population no longer followed a Gaussian distribution and we are therefore reluctant to draw any definite conclusion for this specific population. These results confirm that the PAR1 antagonist protects mice from potentially deleterious lung inflammation induced by hMPV infection.

PAR1 antagonist (SCH79797) treatment decreases furin expression in the lungs of hMPV-infected mice. The proprotein convertases, especially furin, have been shown to process a number of cell surface glycoproteins of infectious viruses both at single and paired basic residues. The minimal cleavage site is **R**XX**R**↓, exhibiting a P1 and P4 Arg residues [46]. In order to tentatively investigate the possible mechanism by which PAR1 compounds can influence viral replication, we analyzed furin

transcripts in the lungs of hMPV-infected animals by RT-PCR. PAR1 compounds were administered for 5 days at a dose of 500 µM, starting at the time of infection, and lungs were harvested on day 5 pi. HMPV infection resulted in a significant increase in furin transcripts (by 38%) in mouse lungs compared to uninfected mice (Fig. 4A). Treatment of infected mice with the PAR1 agonist did not significantly alter furin expression. However, treatment of infected mice with the PAR1 antagonist significantly reduced furin transcript levels (by 18%) compared to hMPV-infected/untreated mice. Using a pIRES vector expressing each proprotein convertase, we previously showed a similar expression of each convertase and their ability to cleave selected substrates [47,48]. Thus, we used these same constructs to show that furin was the only tested proprotein convertase that was able to cleave the hMPV fusion precursor protein into its active form (Fig. 4B) in two cell lines (COS-1 and HEK293). We then investigated the effect of PAR1 on the cleavage of the hMPV fusion precursor protein (Fig. 4C) in this *in vitro* cleavage assay. Treatment of F protein/furin co-transfected HEK293 cells with either the PAR1 agonist or antagonist did not alter F cleavage (43.7 and 41.2% cleavage, respectively, compared to 36.8%). However, co-transfection of cells with cDNA encoding the F protein, furin as well as recombinant human PAR1 (rhPAR1) reduced F cleavage from 36.8% to 12.4%. When these rhPAR1 expressing co-transfected cells were treated with the PAR1 antagonist, F protein cleavage remained low (12.0%), whereas PAR1 agonist treatment restored F protein cleavage to the basal level observed in cells without rhPAR1 expression (38.2%), confirming a role for PAR1 in the cleavage of the hMPV fusion protein.

Discussion

In this study, we showed that PAR1 activation increases weight loss and mortality in a mouse model of hMPV infection. Conversely, an antagonist of PAR1 (SCH79797) prevents clinical signs and reduces lung inflammation and viral titers, when administered at the time of viral infection. The reduction in lung viral titers by the PAR1 antagonist was probably not due to a direct antiviral effect

but was more likely mediated through a reduction in furin expression and/or activity and viral infectivity.

hMPV infection causes a wide spectrum of diseases from mild upper respiratory tract infections (RTI) to severe lower RTI such as bronchiolitis or pneumonia and can result in hospitalization of both children and adults [49]. HMPV is one of the most prominent respiratory pathogens in children, accounting for 5–15% of pediatric hospitalizations for respiratory tract infections [49,50]. A recent study estimated the hospitalization rate for hMPV in older adults to be 9.8 per 10000 residents, a rate similar to those of hRSV and influenza [51]. Hospitalized patients are currently treated symptomatically, since no specific prophylactic or therapeutic modality is available. Although not approved for this indication, ribavirin has been administered as an antiviral agent in a few severe cases of hMPV pneumonia with variable outcomes [20-22]. Several hMPV vaccines have been investigated in animal models, including live-attenuated and subunit vaccines; however, vaccine-induced immunity was often partially protective and/or waned rapidly [52]. Importantly, hMPV-inactivated vaccines have been shown to induce an enhanced disease upon infection reminiscent of hRSV-inactivated vaccines [52-54]. Clearly, alternative approaches for the management of hMPV infections are needed.

We used an agonist (TFLLR-NH_2) and an antagonist (SCH79797) of PAR1 to investigate the role of this receptor during hMPV infection. To further investigate the PAR1 agonist specificity, we examined its well-known effect on intestinal permeability [55] in wild-type and PAR1-knockout mice. To this end, ^{51}Cr-EDTA was infused in the colon of the mice and the effect of the PAR1 agonist on the passage of ^{51}Cr-EDTA to the blood, reflecting intestinal permeability was measured (Supplementary figure 1A). As expected, mice treated with the PAR1 agonist peptide TFLLR

displayed enhanced intestinal permeability, compared to saline control treated mice. In marked contrast, mouse treatment with the RLLFT inactive control peptide had no effect on permeability, arguing against potential nonspecific effects of the agonist. More importantly, the effect of the agonist on increased intestinal permeability was abolished in PAR1-deficient mice. Altogether, these results show that the agonist specifically activates PAR1 in mice. Furthermore, this agonist peptide has been also used to specifically activate PAR1 in mice elsewhere [56-58]. Subsequently, we also investigated whether the PAR1 antagonist was capable of blocking the specific PAR1 signaling in vivo. We found that antagonist treatment prevented increased intestinal permeability associated with administration of the PAR1 agonist peptide to wild-type mice (Supplementary figure 1B). Thus, the effect of the antagonist is likely related to its ability to specifically block PAR1 *in vivo*. Furthermore, the antagonist has also been used to block PAR1 activation in rodent models elsewhere [56,58-60].

In our mouse model, inhibition of PAR1 activation using the antagonist SCH79797 was clearly beneficial by decreasing the severity of hMPV disease. Most strikingly, when treatment was started simultaneously with hMPV-infection and was continued for 5 days, no weight loss or clinical signs were observed (Fig. 2). Evaluation of pulmonary inflammation and viral titers was performed on day 5 pi, the time point at which both parameters peak in untreated hMPV-infected mice [10]. During this acute phase of infection, pulmonary inflammation mostly consists of interstitial, perivascular and alveolar inflammation and all these parameters were significantly reduced by treatment with the PAR1 antagonist. In line with this observation, we also showed a significant reduction in the levels of key cytokines/chemokines that are usually increased in hMPV infection (including IFN-γ, IL-6, KC, MCP-1, MIP-1α and RANTES) [10]. Some of these cytokines namely IL-6, IL-8 (the human equivalent of murine KC) and MCP-1 have been previously shown to be up-regulated by PAR1

activation or down-regulated by PAR1 inhibition in non-infected human respiratory epithelial cells in models of asthma and idiopathic pulmonary fibrosis [61-64]. Furthermore, SCH79797 significantly reduced MCP-1 expression in *M. tuberculosis* H37Rv-infected cells *in vitro* [39]. Finally, Khoufache et al. recently demonstrated that SCH79797 treatment of influenza A-infected mice also significantly reduced IL-6, KC and RANTES levels in broncho-alveolar lavages [44].

We further investigated immune cell populations (activated dendritic cells, macrophages, T lymphocytes and neutrophils) in the lungs of hMPV-infected mice treated with the PAR1 antagonist on day 5 pi using flow cytometry. We did not observe significant differences in pulmonary neutrophils between any of the groups. However, the PAR1 antagonist treatment significantly reduced the recruitment of lung macrophages (Fig. 3). PAR1 is expressed on both human and murine dendritic cells and its activation induces the release of MCP-1, IL-10, and IL-12 from these cells [65]. Moreover, PAR1 signaling on dendritic cells has been shown to sustain a lethal LPS-induced inflammatory response in mice [66]. In our study, we also observed a significant reduction in dendritic cells present in the lungs of PAR1 antagonist-treated mice compared to untreated hMPV-infected mice. Activation of PAR1 on human T cells induces the secretion of IL-6 [67] and may have an effect on T cell receptor signaling [68]. In addition, T cell migration to the liver of PAR1-deficient mice was reduced by 70% in a model of liver fibrosis, suggesting a role for PAR1 in T cell migration [69]. We did not observe a significant difference in $CD4^+$ T lymphocytes between any of the groups, yet significantly less $CD4^-$ T-lymphocytes were found in the lungs of PAR1 antagonist-treated mice. It remains to be confirmed, however, whether these $CD4^-$ lymphocytes represent cytolytic T lymphocytes.

To provide further evidence that blocking PAR1 is protective during hMPV infections, we evaluated the effect of another PAR1 antagonist (SCH530348) on the outcome of hMPV infection in our mouse model. SCH79797 was used as a control (supplementary figure 2). In both treated groups, hMPV-infected mice showed reduced weight loss although the reduction was more important with SCH79797. In fact, as was the case in the first 5-day experiment, the weight of SCH79797-treated mice remained similar to that of uninfected mice and no reduced activity or ruffled fur were observed. Treatment with either antagonist also reduced mortality induced by hMPV infections. Compared to 33% mortality rate for the untreated group, only 17% and 0% mortality rates were observed for the SCH530348- and SCH79797-treated groups, respectively. Thus, these results confirm that blocking PAR1 is protective during hMPV infections.

Immunomodulation, as a therapy, offers several advantages over conventional antiviral strategies. Because the host is the target of such a therapy, immunomodulation avoids the selective pressure on the pathogen that leads to the development of antimicrobial resistance. In addition, because of the non-specific nature of the innate immune system, its modulation could result in broad-spectrum protection against a wide range of microbial pathogens. The use of immunomodulators for viral infections has been most extensively investigated for chronic infections such as HIV and viral hepatits infections and some of these agents like interferon are in clinical use [70]. With the advent of severe respiratory distress syndromes due to A/H5N1 avian influenza and SARS-coronavirus infections and in particular since the 2009 influenza pandemic, the interest for immunomodulators in acute viral infections has been increasing. Several anti-inflammatory therapies for influenza A infections have been evaluated so far, including sphingosine analogues, NF-κB inhibitors, antimicrobial peptides, COX-2 inhibitors and macrolides [71-73]. The latter are antibiotics with known anti-inflammatory and immunomodulatory properties that have shown promising results in

several viral respiratory infections caused by influenza A, rhinovirus and hRSV [74]. Like macrolides, PAR1 antagonists may have a broad spectrum of activity. Indeed, Khoufache et al. recently reported results similar to ours in murine models of influenza A infections including those caused by A/H5N1 and oseltamivir-resistant A(H1N1)pdm09 strains [44]. In addition, unpublished data from our group indicates that PAR1 antagonists can significantly decrease hRSV lung titers and key pulmonary cytokines (IL-6, MCP-1, RANTES) in a similar mouse model highlighting the broad-spectrum potential of these compounds.

We found that PAR1 antagonist-treated and infected mice had reduced pulmonary viral titers on day 5 pi. This observation suggests a direct or indirect effect of PAR1 on viral replication. Such direct antiviral effect was not observed in our *in vitro* cell culture experiments, although testing was limited by cytotoxicity so that we cannot completely rule out a direct antiviral effect. On the other hand, viral entry into the host cell is mediated by the hMPV F surface glycoprotein. This protein is synthesized as an inactive precursor protein that requires proteolytic cleavage to become active [75]. The cleavage motif of the HMPV F protein at **R**SQ**R**↓FV contains a minimal furin cleavage site, i.e., **R**XX**R**↓ present in many of its *in vivo* substrates [76,77]. Even though cleavage of the HMPV F protein requires the addition of trypsin *in vitro*, other enzymes could be involved in activating the F protein *in vivo*. Indeed, we found furin expression levels to be significantly increased in the lungs of hMPV-infected mice compared to mock-infected mice. In addition, using an *in vitro* cleavage assay, we clearly showed for the first time that, of all tested proprotein convertases, only furin was capable of cleaving the hMPV F precursor protein. Moreover, expression of recombinant human PAR1 (rhPAR1) reduced F protein cleavage in this assay; whereas treatment of rhPAR1 expressing cells with the PAR1 agonist restored F protein cleavage, treatment with the PAR1 antagonist did not. Since activation of PAR1 quickly initiates the internalization and degradation of the receptor [78] we

hypothesize that the presence of unactivated PAR1 at the cell surface inhibits furin-mediated F protein cleavage. These *in vitro* observations could, at least partly, explain the slight reduction in viral titers observed in the lungs of hMPV-infected, PAR1 antagonist-treated mice. Considering that endogenous activators of PAR1 are present during *in vivo* infection, the inhibition of PAR1 activation with the PAR1 antagonist and the subsequent inhibition of PAR1 internalization and degradation could lead to reduced F cleavage and could therefore contribute to decreased hMPV infectivity. Taken together, these data suggest that furin levels and/or activity could have an effect on hMPV infectivity. On a similar note, PAR1 antagonists have been shown to decrease the plasmin-mediated cleavage of the influenza hemagglutinin protein with subsequent reduction in mouse lung viral titers [44]. Although PAR1 modulation of furin levels and/or activity and their subsequent effect on viral infectivity could offer a possible explanation for the reduced viral titers observed in the lungs of PAR1 antagonist-treated mice, it needs to be confirmed that PAR1 inhibition can reduce the infectivity of other hMPV strains as well. Of interest, replication of several paramyxoviruses is furin-dependent, and this immunomodulatory approach may thus be beneficial not only for hMPV, but for other paramyxovirus infections as well.

In conclusion, we report that PAR1 inhibition, using a non-peptide PAR1 antagonist, is beneficial in hMPV-infected mice. The protective mechanisms conferred by PAR1 antagonists are mediated by a modulation of the innate and adaptive immune responses, and also possibly through a reduction of viral infectivity. Different PAR1 antagonists appear to confer different protection levels during hMPV infection, warranting the evaluation of other PAR1 inhibitors and, importantly, the assessment of delayed treatment on clinical outcome. Importantly, some PAR1 inhibitors are already in clinical trials for other indications, which could accelerate the development of these compounds in the context of acute treatment of viral infections.

Materials and methods

Virus strains and cells. LLC-MK2 cells (ATCC CCL-7) were maintained in minimal essential medium (MEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Wisent, Quebec, QC). The hMPV group A strain C-85473, a clinical isolate that was passaged nine to ten times on LLC-MK2, was grown on LLC-MK2 cells in OptiMEM (Life technologies, Carlsbad, CA) supplemented with 0.0002% trypsin (Sigma, St. Louis, MO). Virus stocks were concentrated on Amicon™ columns (Fisher Scientific, Waltham, MA) as previously described [15]. The same protocol was used with 16 flasks of uninfected cells for mock-infected mice.

Viral titrations. Viral titers were determined by 10-fold serial dilutions of virus in 24-well plates containing LLC-MK2 cells as previously described [10]. Virus titers were reported as 50% tissue culture infectious doses ($TCID_{50}$) per ml. $TCID_{50}$ values were calculated by the Reed and Muench method.

Compounds. PAR1 agonist (TFLLR-NH_2) (Genscript, Piscataway, NJ) was reconstituted in H_2O at a concentration of 10 mM, aliquoted and stored at -20°C. Immediately before intranasal administration, the compound was diluted to 50 µM or 500 µM in OptiMEM. As a control, H_2O was diluted 1/20 in OptiMEM before administration. PAR1 antagonists (SCH79797 and SCH530348 (Vorapaxar)) (Axon MedChem, Groningen, The Netherlands) were reconstituted at a stock concentration of 22 mM in DMSO and stored at -20°C. Immediately before intranasal administration, these compounds were diluted to 50 µM or 500 µM in OptiMEM. As a control, DMSO was diluted 1/44 in OptiMEM before administration.

Antiviral activity *in vitro*. In a first experiment, confluent LLC-MK2 cells were incubated for 2 h with 2.5-fold dilutions of PAR1 compounds. Subsequently, cells were washed once with PBS, before infection with 200 $TCID_{50}$ of hMPV. After 5 h, the medium containing the virus was removed and replaced by fresh infection medium. Trypsin was added on days 3 and 5 post infection (pi) and titers were determined on day 6 pi. In a second experiment, hMPV was pre-incubated at 37°C with 2.5-fold dilutions of the compounds for 2 h before being transferred onto confluent monolayers of LLC-MK2 cells. Five hours later, the medium was removed and replaced with fresh infection medium containing the same 2.5-fold dilutions of compounds. Dilutions of compounds were added daily and trypsin was added on days 3 and 5 pi. Titers were then determined on day 6 pi.

Furin cleavage experiment. HEK293 and COS-1 cells (ATCC CRL-1650) were co-transfected with cDNA from the F protein of the hMPV strain C-85473, tagged with V5 at the C-terminus and cDNA encoding each of the following proprotein convertases (furin, PC5/6, PACE4, PC7 or SKI-1) with/without cDNA encoding hPAR1 (2:1 ratio of hMPV:PC for the double transfection and 2:1:2 ratio of hMPV:PC:hPAR1 for the triple transfection). Twenty hrs post-transfection, cells were treated with hPAR1 agonist, TFLLR-NH_2 100 µM, or antagonist, SCH79797 0.1 µM for 4 h, and then incubated for 24 h in fresh serum-free media. Protein lysates from each transfectant were separated on SDS-PAGE followed by Western blot analysis with an anti-V5 mAb as reported [47]. Quantitation was performed using Image J software (National Institutes of Health), and normalization was reported to β-actin.

Quantitative RT-PCR assay for furin transcripts. Lungs were removed on day 5 pi and snap frozen in liquid nitrogen. RNA was extracted using Trizol/chloroform and RNA quality was verified on an agarose gel. cDNA was prepared using 250 ng of total RNA. Quantitative PCR was performed on the MX3005 platform (Stratagen, Santa Clara, CA) using QUANTA Sybergreen (VWR, Radnor, PA). cDNA

synthesis and QPCR were performed as previously described [79]. Specific primers sitting on neighboring exons were used for the simultaneous amplification of the normalizing cDNA for ribosomal protein S16 [80].

BALB/c mouse studies. Four to six-week-old BALB/c mice (Charles River Laboratories) were infected intranasally with 5-8×10^5 $TCID_{50}$ of hMPV in 25 µl of OptiMEM. As a control, 4–6-week-old BALB/c mice were mock infected. Mice were treated intranasally with 50 or 500 µM of PAR1 agonist (TFLLR-NH_2) or antagonists (SCH79797 and SCH530348) or with equivalent dilutions of H_2O or DMSO depending on the compounds. Animals were housed in groups of three to five in micro-isolator cages. The animals were evaluated on a daily basis for weight loss and the presence of clinical signs such as reduced activity and ruffled fur. The humane endpoint was determined at 20% weight loss in accordance with the guidelines provided by the Animal Protection Committee of the Centre Hospitalier Universitaire de Québec. On day 5 pi, six mice per group were euthanized using sodium pentobarbital and lungs were removed for the evaluation of viral titers by cell culture, cytokine levels using a bead-based multiplex immunoassay, histopathological changes using hematoxylin-eosin staining, cell recruitment by flow cytometry, and furin expression levels by quantitative RT-PCR.

C57Bl/6 mouse studies: Male C57Bl/6 mice (6 weeks old; Charles River Laboratories, Quebec, Canada) were housed in a controlled environment (22°C, 40% humidity, 12:12 h photoperiods) and had free access to food and water. In a first protocol, after a 12 h fast, under light halothane anaesthesia, a polyethylene catheter was inserted intra-rectally to 3 to 4 cm from the anus. A single administration of TFLLR-NH2 (200 µg/mouse each) or RLLFT-NH2 (200 µg/mouse each) was performed into the distal colon through the catheter, in a volume of 100 µl. Three hours later and

under deep anaesthesia (ketamine 60 mg/kg and xylazine 25 mg/kg), mice received an intracolonic infusion of 75 µl of ^{51}Cr-EDTA at 2x10^6 cpm/h for 3 h. Intestinal permeability was assessed by measuring the passage of ^{51}Cr-EDTA from the colonic lumen to the blood. Blood was collected by cardiac puncture and was then measured for counts using a gamma counter. In a second protocol, after a 12 h fast, mice received an intraperitoneal injection of the PAR1 antagonist SCH79797 (5mg/kg) or its vehicle (carboxymethyl cellulose), then, one h later, under light halothane anaesthesia, a polyethylene catheter was inserted intra-rectally to 3 to 4 cm from the anus. A single administration of TFLLR-NH2 (200 µg/mouse each) or saline was performed into the distal colon through the catheter, in a volume of 100 µl. Three hours later and under deep anaesthesia (ketamine 60 mg/kg and xylazine 25 mg/kg), mice received an intracolonic infusion of 75 µl of ^{51}Cr-EDTA at 2x10^6 cpm/h for 3 h. Intestinal permeability was assessed by measuring the passage of ^{51}Cr-EDTA from the colonic lumen to the blood. Blood was collected by cardiac puncture and was then measured for counts using a gamma counter.

Ethics statement. The present studies in BALB/c mice were approved by the Animal Protection Committee of the Centre Hospitalier Universitaire de Québec according to the guidelines of the Canadian Council on Animal Care (CPAC 10-082-3). Studies with C57Bl/6 mice were conducted under the standards of the Canadian Council on Animal Care and approved by the Animal Care Committee of the University of Calgary.

Pulmonary viral titers. Lungs were removed on day 5 pi and snap frozen in liquid nitrogen. The lungs were subsequently weighed, homogenized in 1 ml of PBS and then centrifuged at 2000 rpm for 10 min. The supernatant was used to determine viral titers reported as $TCID_{50}$.

Pulmonary cytokine levels. An aliquot of 250 µl of lung homogenates was added to 250 µl of 50 mM KPO_4, pH 6.0 buffer containing 0.2% CHAPS (Sigma, St. Louis, MO) and 0.2% protease inhibitor cocktail (Sigma, St. Louis, MO) and then stored at -20°C. On the day of the experiment, samples were centrifuged at 13,800 × g for 10 min at 4°C and then 50 µl of the supernatant were used for cytokine quantification. Levels of interleukin (IL)-4, IL-6, IL-12(p40), IL-12(p70), IFN-γ, KC, MCP-1, MIP-1α, RANTES were determined using 9-plex mouse bead kits (Millipore, Billerica, MA) according to the manufacturers' instructions. Experiments were performed in a 96-well filter plate and results were analyzed with the Luminex system (Qiagen, Germantown, MD).

Histopathology. Lungs were removed on day 5 pi, and fixed with 4% buffered formalin. Fixed lungs were subsequently embedded in paraffin, sectioned in slices of 5 µm, and stained with hematoxylin-eosin. The histopathological scores were determined by two independent researchers who were blinded to experimental data. A semi-quantitative scale was used to score bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra-alveolar inflammation as previously described [10].

Polychromatic flow cytometry. Lungs were removed on day 5 pi, perfused with 1 ml of endotoxin-free PBS (Cederlane, Burlington, ON) and incubated at 37°C in 1 ml (1 mg/ml) Collagenase D (Roche, Nutley, NJ) in Hank's Balanced Salt Solution (HBSS) (Wisent, Quebec,QC). After 45 min, 2 U of Dnase I were added and the lungs were incubated at 4°C for another 15 min. Tissues were disrupted mechanically through a 40-µm filter, and red blood cells were lysed using the ACK buffer (Life technologies, Carlsbad, CA). Cells were incubated with FcBlock (BD Biosciences, Franklin Lakes, NJ) and fluorochrome-conjugated antibodies in endotoxin-free PBS containing 1% FBS and 1% HEPES for 20 min at 4°C. After washing, cells were fixed in 2% formaldehyde in endotoxin-free PBS

containing actinomycin D (20 µg/ml) (Cederlane, Burlington, ON). Surface marker antibodies were specific for murine CD3, CD4, CD11b, CD11c, Ly6G, I-A/I-E (BD Biosciences, Franklin Lakes, NJ) and F4/80 (Biolegend, San Diego, CA). The 7-AAD dye (20 µg/ml) (Biolegend, San Diego, CA) was used to exclude dead cells. Cells were analyzed using a BD FACSCantoA system and gating of positive cells was based on fluorescence minus one (FMO) controls. All analyses were performed using the FCS express V4 software.

Statistical analysis All statistical analyses were performed using the Prism 5 software. Weight loss, pulmonary cytokine/chemokines levels and histopathology scores were analyzed by two-way ANOVA. Lung viral titers and furin expression levels were analyzed using a Student t-test and cell recruitment was analyzed using one-way ANOVA.

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Figure legends

Figure 1: PAR1 agonist or antagonist dose-dependent effect on hMPV infection during a 3-day treatment in mice. Groups of 12 mice were infected intranasally with hMPV (4-6 $x10^5$ $TCID_{50}$) or mock infected and simultaneously treated for 3 days with a single daily dose of 50 or 500 µM of PAR1 agonist (TFLLR-NH_2), PAR1 antagonist (SCH79797) or their respective vehicles. A) and B) Weight loss and mortality were monitored daily for 14 days for mice treated with the PAR1 agonist and antagonist, respectively. The horizontal bar underneath the graphic indicates the timing and duration of treatment. Arrows and numbers indicate the mice that reached the endpoint and were sacrificed (full line: mice treated with 50 µM of PAR1 agonist, dotted line: mice treated with 500 µM of PAR1 agonist). Significant differences in weight loss were observed between mice treated with 50 µM (†) or 500 µM (*) of compound compared to untreated mice based on a two-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, † $p<0.05$, †† $p<0.01$). C) Viral titers were determined by $TCID_{50}$ in lung homogenates at day 5 pi. Significant differences were observed between treated or untreated mice as determined by one-way ANOVA. (* $p<0.05$, ** $p<0.01$) The bar indicates the lower limit of detection.

Figure 2: Effect of PAR1 antagonist on hMPV infection during a 5-day treatment in mice. Groups of 18 mice were infected intranasally with hMPV (6-8 $x10^5$ $TCID_{50}$) or mock infected and simultaneously treated for 5 days with a single daily dose of 500 µM of PAR1 antagonist (SCH79797) or with their respective vehicle. A) Weight loss and mortality were monitored on a daily basis for 14 days. The horizontal bar underneath the graphic indicates the timing and duration of treatment. * indicate a significant difference between PAR1 antagonist (SCH79797) treated mice and untreated controls based on a two-way ANOVA (* $p<0.05$, *** $p<0.001$). B) On day 5 pi, 6 mice per group were euthanized and lungs were removed, fixed, embedded, sectioned and stained for histopathology.

The degree of inflammation was determined using a semi-quantitative scale. (a: bronchial/endo-bronchial inflammation; b: peribronchial inflammation; c: perivascular inflammation; d: interstitial inflammation; e: pleural inflammation; f: intra-alveolar inflammation). Significant differences in pulmonary histopathological scores were observed between treated and untreated mice based on a two-way ANOVA(*** $p<0.001$). C) Pulmonary cytokine/chemokine levels in lung homogenates were determined by Luminex (IFNγ, IL-4, IL-6, IL-12(p40), IL-12(p70), KC, MCP-1, MIP-1α and RANTES). IL-4 and IL-12(p70) data were removed from the graph since no detectable values were obtained for these cytokines in any of the groups. Significant differences in pulmonary pro-inflammatory cytokine/chemokine levels were observed between treated and untreated mice based on a two-way ANOVA (*** $p<0.001$). D) Viral titers were determined in lung homogenates by TCID50. Significant differences in pulmonary viral titers were observed between PAR1 antagonist-treated mice and infected untreated mice based on a Student t-test. (* $p<0.05$)

Figure 3: Cell recruitment in the lungs of hMPV-infected mice treated with the PAR1 antagonist. Groups of 6 mice were infected intranasally with hMPV (7 $x10^5$ $TCID_{50}$) or mock infected and simultaneously treated for 5 days with a single daily dose of 500 µM of PAR1 antagonist (SCH79797). Mice were sacrificed on day 5 pi, lungs were removed, homogenized in HBSS and analyzed by flow cytometry for the presence of (A) dendritic cells expressing the MHC II molecules I-A/I-E ($CD11c^+CD11b^{lo}Ly6G^{lo}$I-A/I-$E^+$), (B) macrophages ($CD11c^+CD11b^+F4/80^{hi}$), (C) T lymphocytes ($CD3^+CD4^+$ or $CD3^+CD4^-$) and (D) neutrophils ($CD11c^-CD11b^{hi}Ly6G^+$). Significant differences in recruited cells were observed between treated and untreated mice based on a one-way ANOVA. (* $p<0.05$, *** $p<0.001$)

Figure 4: Effect of PAR1 agonist or antagonist treatment of hMPV-infected mice on furin expression. A) Groups of 6 mice were infected intranasally with hMPV ($7x10^5$ $TCID_{50}$) or mock

infected and simultaneously treated for 5 days with a single daily dose of 500 µM of PAR1 agonist (TFLLR-NH_2) or PAR1 antagonist (SCH79797). Mice were sacrificed on day 5 pi, lungs were removed and snap frozen. RNA was extracted and furin transcript levels were determined using RT-PCR. Significant differences in furin transcript levels were observed between treated and untreated mice based on a Student T-test (* p=0.05, ** p<0.01) B) COS-1 and HEK293 cells were co-transfected with a plasmid encoding the hMPV F protein containing a V5-tag and a plasmid encoding one of the proprotein convertases. Cell lysates were analyzed by western blot using a V5 mAb. Furin is the only convertase capable of cleaving the full length precursor protein (F0) into its activated form, resulting in the shorter C-terminal subunit (F1). C) Recombinant human PAR1 and furin are co-tranfected with the hMPV F protein containing a V5-tag in HEK293 cells with/without hPAR1 agonist, (100 µM) or antagonist (0.1 µM). Western blot analysis of cell lysates using an anti-V5 mAb is shown.

Supplementary figure 1: Specificity of the PAR1 agonist and PAR1 antagonist in male C57Bl6 mice. A) Effects of intralumenal administration of the PAR1 agonist (TFLLR-NH_2) (200 µg) or the inactive control peptide (RLLFT) (200 µg) , in the colon of wild-type (PAR1+/+) or PAR1-deficient mice (PAR1-/-) on intestinal permeability: passage of a macromolecule (CrEDTA) from the lumen to the blood, observed 3 h after the intracolonic administration of TFLLR or RLLFT. N=8 per group in each group. (** p<0.01) Significantly different from saline or control peptide (RLLFT)-treated group. B) Effects of intralumenal administration of the PAR1 agonist (TFLLR-NH_2) in the colon of mice that were treated with the PAR1 antagonist (SCH79797) or its vehicle, on intestinal permeability: passage of a macromolecule (CrEDTA) from the lumen to the blood , observed 3 h after the intracolonic administration of TFLLR or RLLFT. N=8 per group in each group. (** p<0.01) significantly different from saline-treated group.

Supplementary figure 2: Weight loss in hMPV-infected mice treated with PAR1 antagonist SCH79797 or SCH530348. Groups of 6 mice were infected intranasally with hMPV (7 x10^5 $TCID_{50}$) or mock infected and simultaneously treated for 5 days with a single daily dose of 500 µM of one of two PAR1 antagonists, SCH79797 or SCH530348, then monitored daily for weight loss and mortality during 14 days. The horizontal bar underneath the graphic indicates the timing and duration of treatment. Significant differences in weight loss were observed between SCH79797-treated mice (*) or SCH530348-treated mice (†) and untreated mice based on a two-way ANOVA (** $p<0.01$, *** $p<0.001$, † $p<0.05$). Arrows and numbers indicate the mice that reached the endpoint and were sacrificed (full line: untreated mice, dotted line: SCH530348-treated mice).

Figure 1:

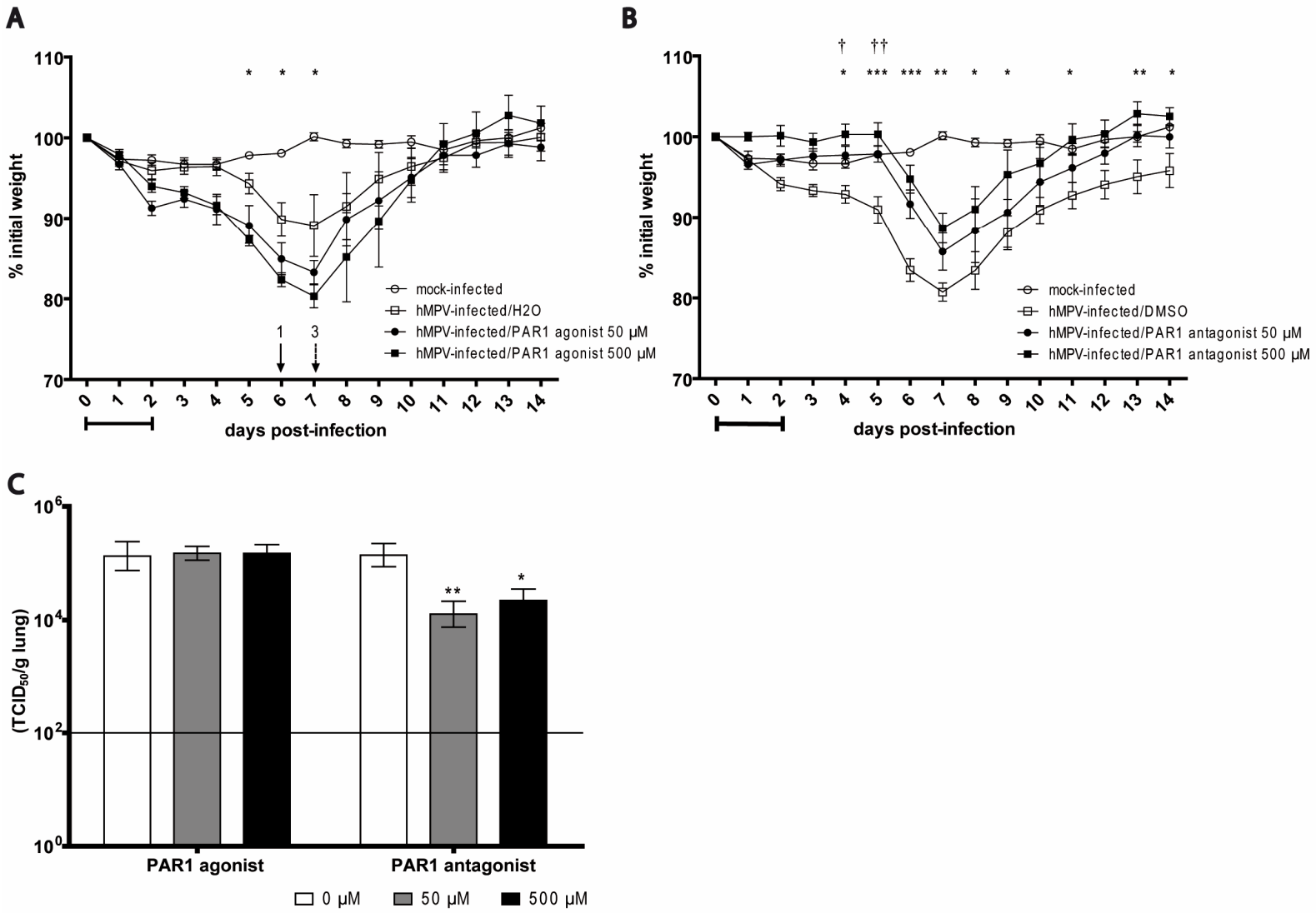

A
% initial weight
days post-infection
mock-infected
hMPV-infected/H2O
hMPV-infected/PAR1 agonist 50 µM
hMPV-infected/PAR1 agonist 500 µM
B
% initial weight
days post-infection
mock-infected
hMPV-infected/DMSO
hMPV-infected/PAR1 antagonist 50 µM
hMPV-infected/PAR1 antagonist 500 µM
C
(TCID50/g lung)
PAR1 agonist
PAR1 antagonist
0 µM
50 µM
500 µM

Figure 2:

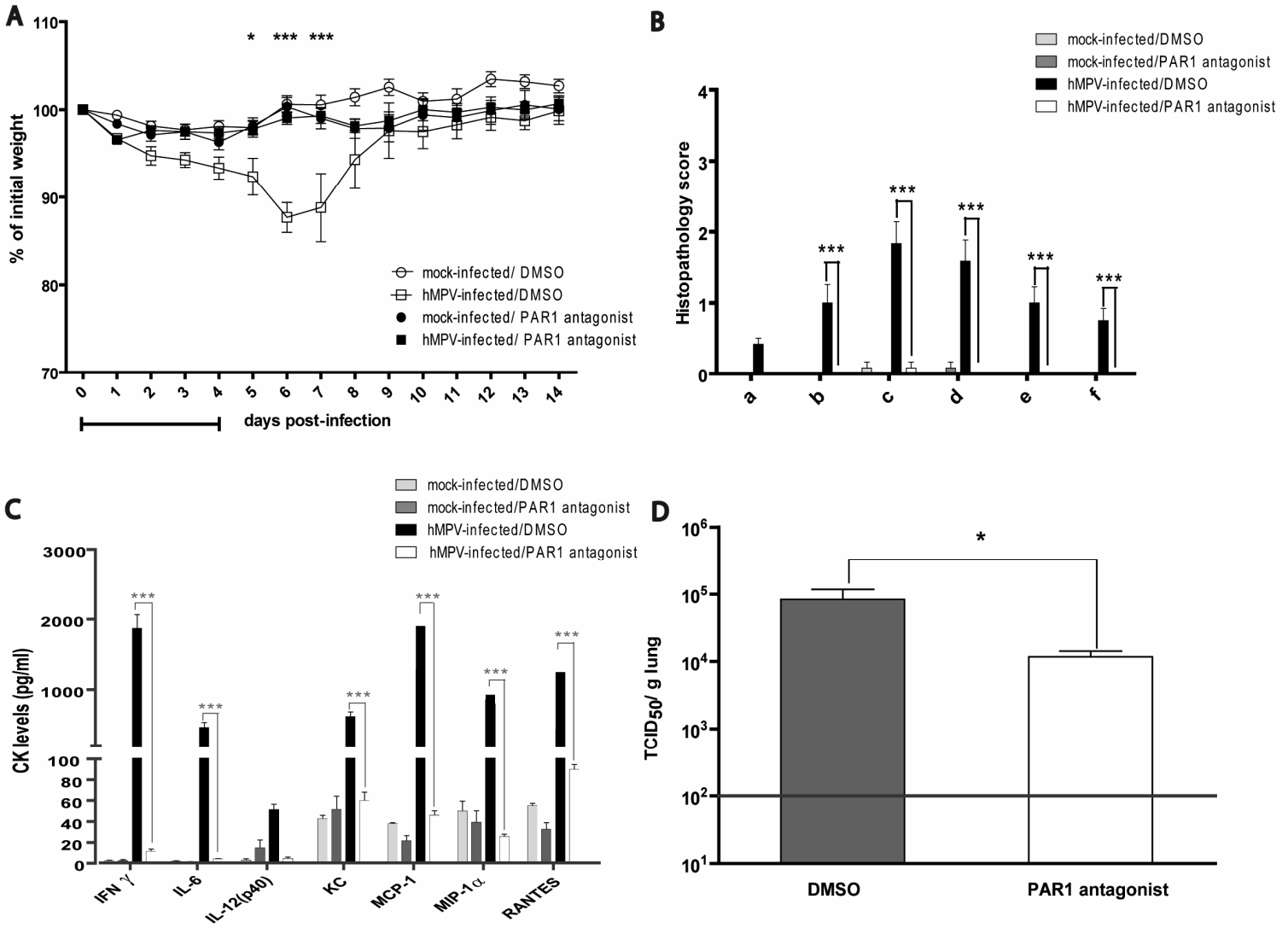

A
% of initial weight
days post-infection
mock-infected/ DMSO
hMPV-infected/DMSO
mock-infected/ PAR1 antagonist
hMPV-infected/ PAR1 antagonist
B
Histopathology score
mock-infected/DMSO
mock-infected/PAR1 antagonist
hMPV-infected/DMSO
hMPV-infected/PAR1 antagonist
C
CK levels (pg/ml)
IFN γ
IL-6
IL-12(p40)
KC
MCP-1
MIP-1α
RANTES
D
TCID50/ g lung
DMSO
PAR1 antagonist

Figure 3:

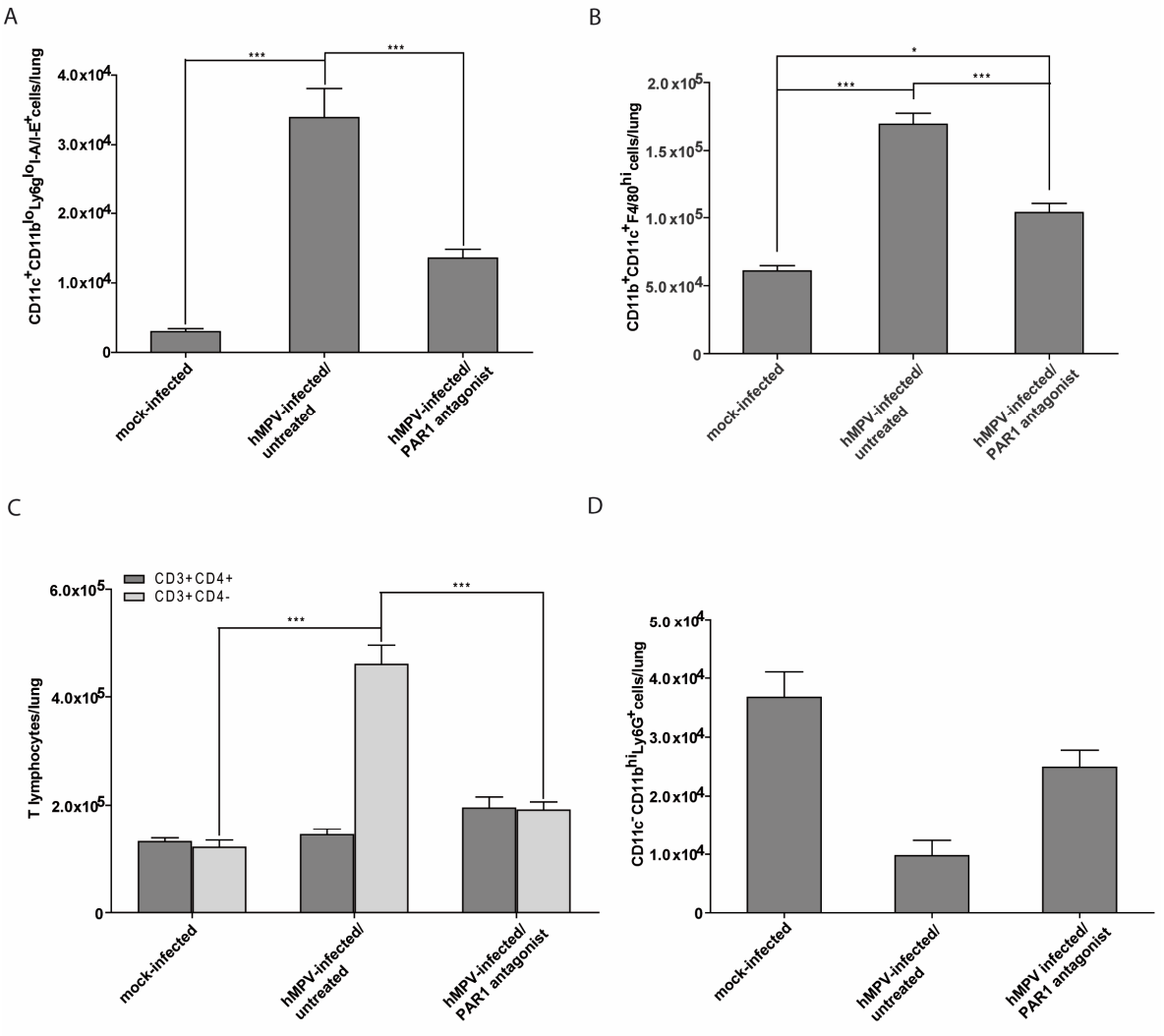
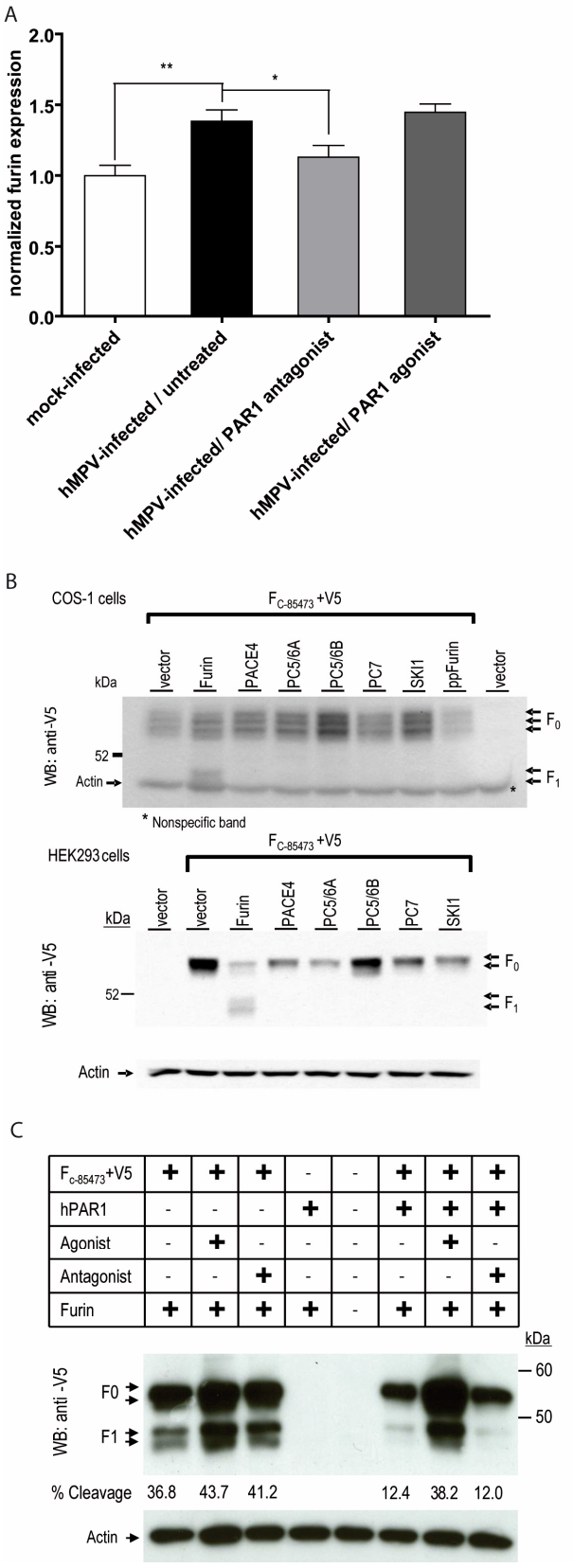

A
B
C
D
CD11c+CD11bloLy6gloI-A/I-E+cells/lung
CD11b+CD11c+F4/80hicells/lung
T lymphocytes/lung
CD11c-CD11bhiLy6G+cells/lung
CD3+CD4+
CD3+CD4-
mock-infected
hMPV-infected/ untreated
hMPV-infected/ PAR1 antagonist
hMPV infected/ PAR1 antagonist
***
*

Figure 4:


A
normalized furin expression
2.0
1.5
1.0
0.5
0.0
**
*
mock-infected
hMPV-infected / untreated
hMPV-infected/ PAR1 antagonist
hMPV-infected/ PAR1 agonist
B
COS-1 cells
FC-85473+V5
kDa
vector
Furin
PACE4
PC5/6A
PC5/6B
PC7
SKI1
ppFurin
vector
WB: anti-V5
52
Actin
F0
F1
* Nonspecific band
HEK293 cells
FC-85473+V5
kDa
vector
vector
Furin
PACE4
PC5/6A
PC5/6B
PC7
SKI1
WB: anti -V5
52
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C
FC-85473+V5 + + + - - + + +
hPAR1 - - - + - + + +
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Antagonist - - + - - - - +
Furin + + + + - + + +
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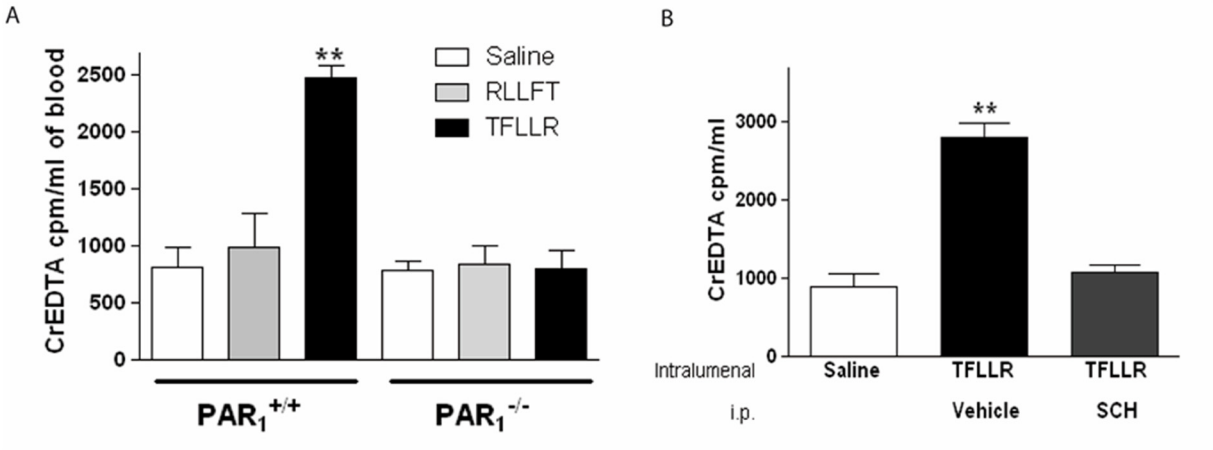
WB: anti -V5
F0
F1
60
50
% Cleavage 36.8 43.7 41.2 12.4 38.2 12.0
Actin

Supplementary figure 1:



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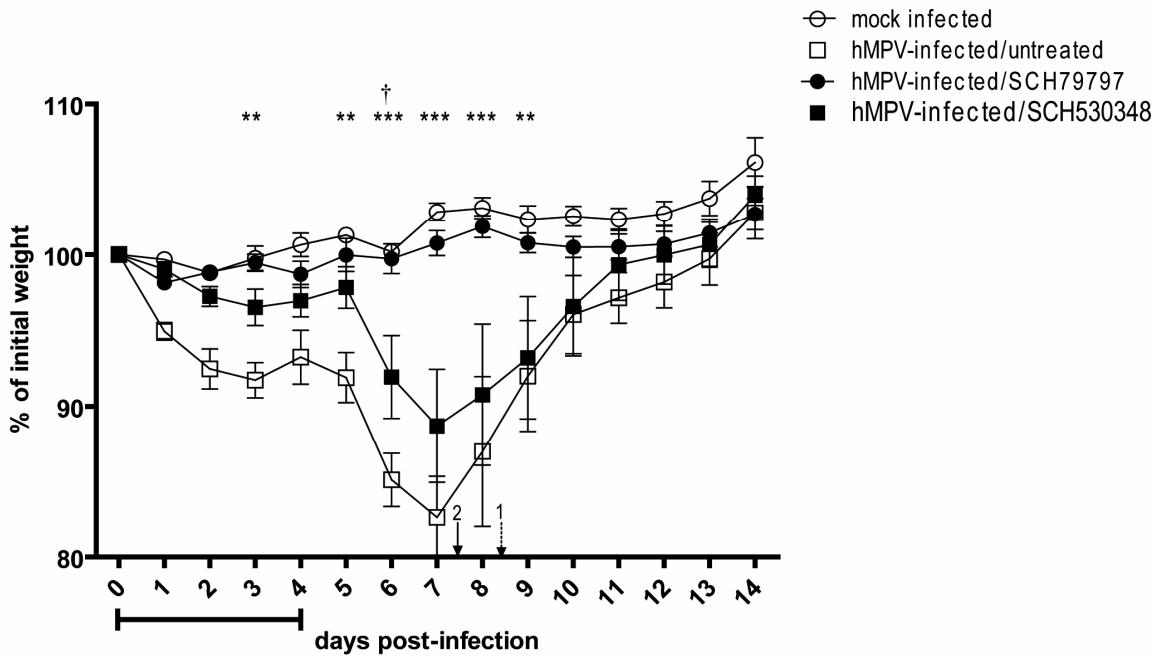

Chapter 5: The adjuvant effect of the human metapneumovirus (HMPV) matrix protein in HMPV subunit vaccines

1. Preface

Chapter 5 contains the paper entitled: "*The adjuvant effect of the human metapneumovirus (HMPV) matrix protein in HMPV subunit vaccines*" written by Laetitia Aerts, Julie Carbonneau, Chantal Rhéaume, Marie-Ève Hamelin, Sophie Lavigne, Christian Couture and Guy Boivin and published in the Journal of General Virology on December 17, 2014.

2. Author contributions

Laetitia Aerts:

- designed the experiments
- plasmid construction, expression and purification of viral proteins
- *In vivo* experiments
- Wrote manuscript

Julie Carbonneau:

- plasmid construction, expression and purification of viral proteins

Chantal Rhéaume :

- assisted in *in vivo* experiments

Marie- Ève Hamelin :

- supervised the project

- corrected the manuscript

Sophie Lavigne and Christian Couture:

- histopathologial analysis

Guy Boivin :

- supervised the project
- corrected the manuscript

3. Paper

Title: The adjuvant effect of the human metapneumovirus (HMPV) matrix protein in HMPV subunit vaccines.

Running title: HMPV subunit vaccines

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Summary

The HMPV fusion protein (F) is the most immunodominant protein, yet subunit vaccines containing only this protein do not confer complete protection. The HMPV matrix protein (M) induces the maturation of antigen presenting cells *in vitro.* The inclusion of the M protein into an F protein subunit vaccine might therefore provide an adjuvant effect. We administered the F protein twice intramuscularly, adjuvanted with alum, the M protein or both, to BALB/c mice at 3-week intervals. Three weeks after the boost, mice were infected with HMPV and monitored for 14 days. At day 5 post challenge, pulmonary viral titers, histopathology and cytokine levels were analyzed. Mice immunized with F+alum and F+M+alum generated significantly more neutralizing antibodies than mice immunized with F only (titers of 47±7 ($p<0.01$) and 147±13 ($p<0.001$) vs 17±2). Unlike F only ($1.6\pm0.5x10^3$ $TCID_{50}$/g lung), pulmonary viral titers in mice immunized with F+M and F+M+alum were undetectable. Mice immunized with F+M presented the most important reduction in pulmonary inflammation and the lowest Th2/Th1 cytokine ratio. In conclusion, addition of the HMPV M protein to an F protein-based vaccine modulates both humoral and cellular immune responses to subsequent infection, thereby increasing the protection conferred by the vaccine.

Keywords. HMPV; vaccines; fusion protein; matrix protein; adjuvants

Introduction

Human metapneumovirus (HMPV) is a leading cause of acute respiratory tract infections in children, the elderly and immunocompromised individuals, for which no vaccine is currently available (Feuillet *et al.*, 2012). It is anticipated that these different target populations will require different immunization strategies. One recombinant live-attenuated HMPV vaccine (rHMPV-Pa) is currently in clinical trial (National Institute of Allergy and Infectious Diseases, 2000-(cited 2014 Aug 18)). Notably, inactivated vaccines against paramyxoviruses have been shown to cause vaccine-enhanced disease upon infection in both humans and animals (Anderson, 2013; Fulginiti *et al.*, 1967). This type of vaccine-enhanced disease has also been observed in rodents when inactivated HMPV was used as an immunogen (Hamelin *et al.*, 2007; Yim *et al.*, 2007). On the other hand, HMPV subunit vaccines are unlikely to induce enhanced disease, especially in seropositive populations. Furthermore, they do not pose the transmission and reversion risk associated with live-attenuated vaccines.

Several groups have demonstrated that the viral fusion protein (F) is the most immunodominant HMPV protein (Biacchesi *et al.*, 2004; Mok *et al.*, 2008; Skiadopoulos *et al.*, 2006). Although prophylactic passive antibody transfer has been shown to be protective (Ulbrandt *et al.*, 2006; Williams *et al.*, 2007), the F protein alone is not sufficient to confer complete and long-lasting protection (Cseke *et al.*, 2007; Herfst *et al.*, 2007). The use of commercially-available adjuvants can improve the protection of F-based subunit vaccines (Cseke *et al.*, 2007; Herfst *et al.*, 2007). However, several studies have recently revealed that other viral proteins are implicated in immunity and pathogenesis of HMPV infections (Kolli *et al.*, 2012; Le Nouen *et al.*, 2014; Ren *et al.*, 2014). Of interest, one group showed that the HMPV matrix (M) protein induces the activation of antigen-presenting cells (APC) *in vitro*, resulting in the maturation of these APC and the secretion of a broad range of inflammatory cytokines. Furthermore, M-activated dendritic cells were shown to stimulate

IL-2 and IFN-γ production by allogeneic T lymphocytes (Bagnaud-Baule *et al.*, 2011). The inclusion of other viral proteins, such as the M protein, into HMPV subunit vaccine formulations, might therefore provide a more balanced and robust immune response.

In the current study, we expressed the HMPV F ectodomain (rHMPV-F) into a mammalian expression system to preserve its native conformation and glycosylation, as well as the full-length rHMPV-M protein into a prokaryotic expression system. We then immunized BALB/c mice twice intramuscularly with the rHMPV-F protein, adjuvanted with alum, the most widely used commercially available adjuvant, the rHMPV-M protein or both, at 3-week intervals. Protection from HMPV infection was evaluated 3 weeks after the boost when mice were challenged with HMPV. The addition of the rHMPV-M protein to the rHMPV-F-based subunit vaccine reduced viral titers below the limit of detection. Importantly, the rHMPV-M protein also modulated the immune response towards a more balanced Th2/Th1 cellular response.

Results

Successful expression of recombinant HMPV F and M proteins.

Analyses of the F protein ectodomain and the full-length M protein by SDS-PAGE followed by Coomassie blue staining or Western blotting revealed that the purified proteins were >90% pure, with a predicted molecular weight of 55 kDa and 32 kDa, respectively (Figure 1a-b). A small second band was observed at around 48 kDa for the HMPV-F protein. Incubation of the protein with increasing concentrations of trypsin increased the secondary band, indicating that this band represents the cleaved F1 form of the protein. In addition, Western blot analysis of the F protein ectodomain under native conditions revealed a band at 165 kDa, demonstrating that the protein is present in its trimeric form (Figure 1c).

Non adjuvanted and adjuvanted rHMPV-F vaccines are immunogenic in BALB/c mice.

Other teams have developed and examined HMPV-F subunit vaccines in rodent models. Herfst et al. immunized Syrian gold hamsters with a HMPV-F subunit vaccine adjuvanted with alum, Specol or Iscom matrix and Cseke et al. immunized cotton rats with a HMPV-F subunit vaccine adjuvanted with TiterMax Gold (Cseke *et al.*, 2007; Herfst *et al.*, 2007). Because different animal models and immunization strategies were used by these groups, we decided to perform a preliminary small-scale experiment, in which BALB/c mice were immunized twice at a 3-week interval with 10 or 30 µg of rHMPV-F or 30 µg of rHMPV-F adjuvanted with alum. Following viral challenge with a lethal dose of virus ($1.4x10^6$ $TCID_{50}$), control mice immunized with supernatant of non-transfected 293F cells, with or without alum, showed more than 80% mortality. Fifty percent mortality was observed in mice immunized with 10 µg of rHMPV-F, whereas mice immunized with 30 µg of rHMPV-F, with or without alum, only showed 17% mortality (data not shown). Based on these observations, 30 µg of rHMPV-F was chosen as the dose for subsequent intramuscular immunizations.

BALB/c mice immunized twice intramuscularly at a 3-week interval with 30 µg of rHMPV-F adjuvanted with alum produced significantly more neutralizing antibodies than mice immunized with 30 µg of rHMPV-F alone (47 ± 6.7 vs 17 ± 1.7, respectively; $p<0.05$) (Figure 2a). Mice immunized with rHMPV-M only did not produce neutralizing antibodies and the addition of rHMPV-M to rHMPV-F did not significantly increase the production of neutralizing antibodies (13 ± 1.7 and 27 ± 3.3 vs 17 ± 1.7 for rHMPV-F + 30 µg rHMPV-M and rHMPV-F + 60 µg rHMPV-M vs rHMPV-F).However, BALB/c mice immunized twice intramuscularly at a 3-week interval with 30 µg of rHMPV-F adjuvanted with alum and 30 µg rHMPV-M produced significantly more neutralizing antibodies than mice immunized with 30 µg of rHMPV-F only (147 ± 13.3 vs 17 ± 1.7, respectively; $p<0.001$) or mice immunized with rHMPV-F+alum (147 ± 13.3 vs 47 ± 6.7, respectively; $p<0.01$).

Immunization of BALB/c mice with adjuvanted rHMPV-F vaccines decreases pulmonary viral titers.

Viral replication in lungs of unadjuvanted and alum-adjuvanted rHMPV-F-immunized mice was significantly reduced ($p<0.01$) compared to mock immunized mice (14.6 ± 3.6 vs 1.6 ± 0.5 and 2.6 ± 1.3 $x10^3$ $TCID_{50}$/g lung for mock-immunized vs rHMPV-F-immunized with and without alum, respectively) and between the 2 immunized groups ($p<0.05$) (Figure 2b). Viral replication in the lungs of rHMPV-M-immunized mice did not differ significantly from mock immunized mice (37.9 ± 13.2 and 14.6 ± 3.6 $x10^3$ $TCID_{50}$/g lung for rHMPV-M-immunized and mock-immunized mice, respectively). However, the addition of rHMPV-M to rHMPV-F did significantly reduce lung viral titers in a dose-dependent manner (1.3 ± 0.4 and 0.35 ± 0.17 vs 2.6 ± 1.3 $x10^3$ $TCID_{50}$/g lung for rHMPV-F + 30 μg rHMPV-M and rHMPV-F + 60 μg rHMPV-M vs rHMPV-F alone; $p<0.05$ and $p<0.001$, respectively). In addition, the viral replication in lungs of mice immunized with the combination of rHMPV-F, rHMPV-M and alum was also reduced below the limit of detection (0.26 ± 0.15 $x10^3$ $TCID_{50}$/g lung; $p<0.001$) compared to rHMPV-F only and rHMPV-F+alum.

Immunization with adjuvanted rHMPV-F vaccines reduces HMPV disease severity in challenged mice.

Upon viral challenge, the mean maximum weight loss of mice immunized with rHMPV-F with and without alum, was significantly reduced compared to mock-immunized mice (20% weight loss), but did not significantly differ between the two groups (10.4 % ± 1.8 vs 10.4 % ± 0.4, rHMPV-F with and without alum, respectively) (Figure 2c). However, the mean maximum weight loss of mice immunized with rHMPV-M only was significantly reduced compared to rHMPV-F-immunized mice (6.5 % ± 1.0 vs 10.4 % ± 0.4; $p<0.01$). Addition of rHMPV-M to rHMPV-F also significantly reduced the mean maximum weight loss (7.2 % ± 1.0 and 6.9 % ± 1.2 for rHMPV-F + 30 μg rHMPV-M and

rHMPV-F + 60 µg rHMPV-M, respectively; $p<0.05$). Furthermore, the mean maximum weight loss of mice immunized with the combination of rHMPV-F, rHMPV-M and alum was significantly reduced compared to rHMPV-F-immunized mice (8.4 % ± 0.4 vs 10.4 % ± 0.4 for rHMPV-F+ rHMPV-M+alum vs rHMPV-F only; $p<0.01$). Figure 3 shows complete weight loss curves of the different groups over 14 days post challenge. Notably, mortality (87.5%) was only observed in mock-immunized HMPV-challenged mice.

Although no statistically significant differences in histopathology scores were observed between any of the groups, pulmonary inflammation did appear to correlate with weight loss. We observed a trend towards higher pulmonary inflammation in mock-immunized mice (mean histopathology score of 11.8 ± 0.5) and pulmonary inflammation remained similar when alum was added to rHMPV-F (11.0 ± 0.5 vs 10.3 ± 0.4 for rHMPV-F with and without alum) (Figure 2d). A trend towards lower pulmonary inflammation was observed in rHMPV-M -immunized mice (mean histopathology score of 7.1 ± 0.2) and when rHMPV-M was added to rHMPV-F (8.8 ± 0.5 and 7.4 ± 1.9 vs 10.3 ± 0.4 for rHMPV-F + 30 µg rHMPV-M and rHMPV-F + 60 µg rHMPV-M vs rHMPV-F alone). Finally, a trend towards lower pulmonary inflammation was also observed for rHMPV-F+rHMPV-M+alum-immunized mice (mean histopathology score of 7.1 ± 1.6) compared to both rHMPV-F and rHMPV-F+alum (10.3 ± 0.4 and 11.0 ± 0.5, respectively).

The rHMPV-M protein modulates Th2/Th1 ratios

IL-4 levels were significantly increased when alum was added to rHMPV-F (1598.5 ± 158.1 vs 1051.2 ± 80.6 pg/g lungs for rHMPV-F with and without alum, respectively; $p<0.05$) (Figure 4a). Conversely, rHMPV-M induced significantly reduced IL-4 levels compared to rHMPV-F only (389.7 ± 41.0 pg/g lungs for rHMPV-M; $p<0.001$). Also, the addition of rHMPV-M to the rHMPV-F+alum vaccine

significantly reduced IL-4 levels in challenged mice (720.3 ± 147.8 vs 1598.5 ± 158.1 pg/g for rHMPV-F+rHMPV-M+alum and rHMPV-F+alum, respectively; $p<0.01$)

Mock immunized mice showed higher levels of IFNγ than rHMPV-F immunized mice (137.0 ± 9.0 pg/g lungs vs 99.7 ± 11.0 pg/g, respectively; $p<0.05$) (Figure 4b). IFNγ levels were also significantly increased when alum was added to rHMPV-F (132.1 ± 2.6 vs 99.7 ± 11.0 pg/g lungs for rHMPV-F with and without alum, respectively; $p<0.05$) and when rHMPV-M (60 μg) and rHMPV-F were combined (141.4 ± 10.2 vs 99.7 ± 11.0 pg/g lungs, respectively; $p<0.05$). Finally, IFNγ levels were reduced in the rHMPV-F+rHMPV-M+alum group compared to the rHMPV-F+alum group (68.8 ± 7.0 vs 132.1 ± 2.6 pg/g lungs; $p<0.001$).

The IL-4/IFNγ ratio was significantly higher in rHMPV-F-immunized mice than in mock immunized mice (5.3 ± 1.0 vs 12.9 ± 1.2 and 11.6 ± 0.8 for mock-immunized vs rHMPV-F with and without alum, respectively; $p<0.01$) (Figure 4c). However, the Il-4/IFNγ ratio was significantly reduced in rHMPV-M-immunized mice compared to rHMPV-F-immunized mice (3.9 ± 0.4 vs 11.6 ± 0.8; $p<0.001$). The addition of the greater dose of rHMPV-M (60 μg) to rHMPV-F showed a trend towards reduced IL-4/IFNγ ratio compared to unadjuvanted F, although the difference was not significant (8.5 ± 1.5 vs 11.6 ± 0.8 for rHMPV-F + 60 μg rHMPV-M vs rHMPV-F alone). There was also a trend towards reduced IL-4/IFNγ ratios for rHMPV-F+rHMPV-M+alum-immunized mice compared to rHMPV-F+alum (10.3 ± 1.3 vs 12.9 ± 1.2, for rHMPV-F+rHMPV-M+alum and rHMPV-F+alum, respectively).

Discussion

Since the detrimental results obtained with formalin-inactivated RSV and measles vaccines (Anderson, 2013; Fulginiti *et al.*, 1967), enhanced disease continues to be an important concern for all types of non-replicating paramyxovirus vaccines. This type of vaccine-enhanced disease might, at least in part, be due to poor induction of neutralizing-antibodies and an imbalanced Th2/Th1 response (Anderson, 2013; Schickli *et al.*, 2009). Moreover, vaccine-enhanced disease accompanied by a Th2 cellular bias has been observed in animals following formalin-inactivated HMPV immunization (de Swart *et al.*, 2007; Hamelin *et al.*, 2007; Yim *et al.*, 2007). However, in a seropositive population, a subunit vaccine could be sufficient to boost existing immunity without inducing enhanced disease. In addition, adjuvants can be used to guide and modulate the immune response to a more balanced response and an increased production of neutralizing antibodies. Bagnaud-Baule et al. demonstrated that the HMPV M protein is capable of activating antigen presenting cells *in vitro* as well as increasing IFNγ production by autologous T cells when co-cultured with HMPV-M-activated dendritic cells (Bagnaud-Baule *et al.*, 2011). These observations led us to hypothesize that the HMPV M protein could have an immunomodulatory effect *in vivo* and that the addition of the HMPV M protein to a HMPV F protein-based subunit vaccine could increase the protection against viral infection in BALB/c mice, even though the HMPV M protein is not an immunodominant protein and does not induce the production of neutralizing antibodies on its own.

As described in other previous studies (Cseke *et al.*, 2007; Herfst *et al.*, 2007), immunization with rHMPV-F induced the production of neutralizing antibodies in our study. Although Herfst et al. did not observe an increase in neutralizing titers in animals immunized with rHMPV-F adjuvanted with alum, we observed almost a 3-fold increase in neutralizing antibodies when rHMPV-F was adjuvanted with alum. This difference might be due to the higher concentration of rHMPV-F (30 vs 10 µg), different source of Alhydrogel or the animal model used (hamsters vs mice).

Cox et al. recently investigated the immunogenicity of virus-like particles (VLP) composed of full-length rHMPV-M and rHMPV-F proteins in C57BL/6 mice (Cox *et al.*, 2014). When the rHMPV-M VLPs were not supplemented with rHMPV-F and not adjuvanted, no neutralizing antibodies were induced in twice-immunized mice. We were also unable to demonstrate the induction of neutralizing antibodies in mice immunized with rHMPV-M only. Immunization with VLPs containing both F and M did induce the production of neutralizing antibodies in the Cox study, but the greatest increase in neutralization titers was observed when these VLPs were adjuvanted with commercially-available adjuvants. Similarly, we observed that the addition of rHMPV-M to the rHMPV-F subunit vaccine did not significantly increase neutralization titers, but when this mixture was adjuvanted with alum another 3-fold increase in neutralizing antibodies was observed.

Interestingly, despite the lack of neutralizing antibody production in mice immunized with rHMPV-M, we observed a significant reduction in weight loss after viral challenge in all groups of mice receiving rHMPV-M. None of the above mentioned studies reported changes in weight loss in HMPV-challenged immunized mice, but our observations indicate an important role for the HMPV-M protein in the immune response following HMPV infection. Similarly to the results reported by Cox et al. with HMPV VLPs, no difference in pulmonary viral titers was found in mice immunized with rHMPV-M only, suggesting that the reduction in disease severity was not due to a reduction in viral replication. However, Cox et al. did observe a reduction in pulmonary viral titers when the rHMPV-M VLPs were adjuvanted. We did not investigate the effect of adjuvanting the rHMPV-M protein, but this might be of interest in future studies. Nevertheless, when we combined rHMPV-M at high dose with rHMPV-F with or without alum, pulmonary viral titers were reduced below the limit of detection. These results are again in concordance with the observations that VLPs composed of rHMPV-M and rHMPV-F adjuvanted or not reduced lung viral titers below the limit of detection.

In our study, as in previous reports (Cseke *et al.*, 2007), there was a discordance between persisting pulmonary inflammation but significantly reduced viral titers on day 5 pi. However, all groups receiving rHMPV-M showed a trend towards reduced cumulative histopathology scores compared to mock immunized- or rHMPV-F and rHMPV-F+alum -immunized mice. Importantly, our results do not include a time-course of pulmonary inflammation, so any difference in kinetics of resolution of pulmonary inflammation could not be evaluated. Furthermore, IL-4 levels were significantly increased in rHMPV-F-immunized mice compared to mock-immunized mice, highlighting once more the Th2-bias induced by HMPV immunization compared to HMPV infection (Hamelin *et al.*, 2005). Similarly to the results obtained by Cseke et al., a significant increase in IL-4/IFN-γ ratio was observed in mice immunized with rHMPV-F compared to mock-immunized mice and this ratio further increased when rHMPV-F was adjuvanted with alum. Conversely, rHMPV-M immunization yielded very low IL-4/IFN-γ ratios and the addition of rHMPV-M to rHMPV-F reduced the IL-4/IFN-γ ratio although the difference was not statistically significant. Finally, the addition of alum to rHMPV-F increased both IL-4 and IFN-γ levels, whereas the addition of rHMPV-M to rHMPV-F+alum reduced both cytokines.

The mechanism by which rHMPV-M modulates the immune response following immunization requires further investigation. It is likely that rHMPV-M influences the cellular immune response, as suggested by the Th2/Th1 cytokine ratios. Although Cox et al. only observed increased recruitment of lymphocytes to the lungs when rHMPV-M VLPs were adjuvanted (Cox *et al.*, 2014), the effect of the M protein on T cell effector function has not yet been investigated and will be the object of future studies.

Despite the fact that rHMPV-M does not induce neutralizing antibodies on its own, we have demonstrated that the addition of rHMPV-M to a rHMPV-F-based subunit vaccine not only

modulates the immune response following viral challenge, but also acts as an adjuvant increasing neutralizing antibodies induced by rHMPV-F and improving the protection following challenge. Additional studies are warranted to complete our evaluation, such as the investigation of cross-protection against other HMPV clades and the long-term benefits of these promising vaccines.

Materials and methods

Virus strains and cells

LLC-MK2 cells (ATCC CCL-7) were maintained in minimal essential medium (MEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Wisent). The HMPV group A strain C-85473, a clinical isolate that was passaged ten times, was grown on LLC-MK2 cells in infection medium consisting of OptiMEM (Life technologies) supplemented with 0.0002% trypsin (Sigma). Virus stocks were concentrated on Amicon™ columns (Fisher Scientific) as previously described (Hamelin *et al.*, 2006).

Viral titrations

Viral titers were determined by 10-fold serial dilutions of virus or lung homogenates in 24-well plates containing LLC-MK2 cells as previously reported (Hamelin *et al.*, 2005). Virus titers were reported as 50% tissue culture infectious doses ($TCID_{50}$) per ml. $TCID_{50}$ values were calculated by the Reed and Muench method.

Microneutralization assay

HMPV neutralizing-antibody titers were determined by an end-point dilution assay as described previously (Hamelin *et al.*, 2007). Briefly, two-fold dilutions of heat-inactivated sera pools were mixed with an equal volume of infection medium containing 500 $TCID_{50}$ of HMPV strain C-85473 and

incubated at 37°C for 2 h. The mixture was then transferred onto confluent monolayers of LLC-MK2 cells in 24-well plates. Following an incubation of 5 h, the antibody/virus mixture was removed, fresh infection medium was added and plates were incubated at 37°C for 4 days. Neutralization titers were defined as the highest dilution of antibody at which culture wells were negative for infection.

Construction and production of the F protein ectodomain

RNA was extracted from HMPV group A1 strain C-85473 using the QIAamp Viral RNA Mini Kit (QIAGEN). Reverse transcription was performed on the extracted material, using random primers (Amersham) and the Superscript II RT Kit (Life Technologies). cDNA was used as template to generate a PCR product encoding the F protein ectodomain (rHMPV-F; GenBank No. KM408076.1) containing a C-terminal polyhistidine (His_6) tag, using the following primers: 5`- AACCAAC TCG AGC ACC ATG TCT TGG AAA GTG GTG ATC-3' and 5`-TTAATT TTA *GTG ATG GTG ATG GTG ATG* GCC AGT GTT TCC TTT CTC TGC-3'. The C-85473 PCR product was cloned into a commercial vector using TOPO TA cloning (pcDNA5; Life technologies), and transformed into One Shot TOP10 chemically competent *E. coli* cells (Life technologies). Plasmids were purified with the HiSpeed Plasmid Maxi kit (QIAGEN). Transient expression of the rHMPV-F was performed by transfecting 293-F cells with pcDNA5- rHMPV-F (1.25 mg) as recommended by the manufacturer (Freestyle 293 Expression System, Life technologies). Transfected cells were collected on day 4 post-transfection, clarified by centrifugation at 300 x g for 10 min and the F protein was purified from the supernatant using nickel-nitrilotriacetic acid (Ni-NTA) (QIAGEN) affinity chromatography under native conditions. The purified protein was dialyzed against phosphate-buffered saline (PBS; Sigma) and concentrated using Amicon™ columns (Fisher Scientific). The concentration of the rHMPV-F protein was

determined by a Bradford protein assay (Bio-Rad). Purified rHMPV-F was further analysed using SDS-PAGE and western blot by using MAb 1017 (a gift from MedImmune).

Construction and production of the M protein

The cDNA from HMPV C-85473 was used as a template to generate the PCR product encoding the M protein (GenBank No. KM408077.1) using the following primers: 5'-ACAA CTC GAG ATG GAG TCC TAC CTA GTA GAC ACT-3' and 5'-CAG GGA TCC TTA TCT GGA CTT CAA CAC AT-3'. The PCR product was digested with restriction endonucleases *XhoI* and *BamH*I and ligated into the pet19b vector (EMD Millipore), containing an N-terminal polyhistidine (His_6) tag. Plasmids were subsequently transformed into One Shot BL21 Star™ (DE3) chemically competent *E. coli* (Life Technologies). Protein expression was induced using isopropyl-β-d-thiogalactoside (IPTG, Sigma) for 4 h at 37°C. rHMPV-M was purified using Ni-NTA affinity chromatography under denaturing conditions and dialyzed against endotoxin-free PBS (Sigma). The absence of endotoxin contamination was confirmed using the Limulus amebocyte assay (Cambrex) and protein concentrations were determined by a Bradford protein assay (Bio-Rad). Purified rHMPV-M was further analysed using SDS-PAGE and western blot by using MAb 4831 (ViroStat).

BALB/c mouse studies.

Four to six-week-old BALB/c mice (Charles River Laboratories) were housed in groups of three to five in micro-isolator cages. The mice (20 per group) were immunized twice intramuscularly at 3-week intervals, with 60 μg of rHMPV-M alone, 30 μg of rHMPV-F alone, 30 μg of rHMPV-F adjuvanted with Alhydrogel (alum) (1:1, Cederlane), 30 μg of rHMPV-F adjuvanted with 30 or 60 μg of rHMPV-M or 30 μg of rHMPV-F adjuvanted with Alhydrogel and 30 μg of rHMPV-M. As a control group, mice were immunized with purified and concentrated supernatant of untransfected 293F

cells. Three weeks post-boost, the mice were infected intranasally with 1×10^6 $TCID_{50}$ of HMPV strain C-85473 in 25 µl of OptiMEM. The day before immunization or infection, blood samples were taken to evaluate humoral responses. Eight animals were monitored on a daily basis for 14 days for weight loss and the presence of clinical signs such as reduced activity and ruffled fur. The endpoint was determined at 20% weight loss. On day 5 post infection (pi), six mice per group were euthanized using sodium pentobarbital and lungs were removed for the evaluation of viral titers by cell culture, cytokine levels using a bead-based multiplex immunoassay and six other mice were evaluated for histopathological changes using hematoxylin-eosin staining. The animal studies were approved by the Animal Protection Committee of the Centre Hospitalier Universitaire de Québec according to the guidelines of the Canadian Council on Animal Care.

Pulmonary viral titers

Lungs were removed on day 5 pi and snap frozen in liquid nitrogen. The lungs were subsequently weighed, homogenized in 1 ml of PBS and then centrifuged at 2000 rpm for 10 min. The supernatant was used to determine viral titers reported as $TCID_{50}$/g of lung.

Pulmonary cytokine levels

An aliquot of 250 µl of lung homogenates was added to 250 µl of 50 mM KPO_4, pH 6.0 buffer containing 0.2% CHAPS (Sigma, St. Louis, MO) and 0.2% protease inhibitor cocktail (Sigma, St. Louis, MO) and then stored at -20°C. On the day of the experiment, samples were centrifuged at 13,000 × g for 10 min at 4°C and then 50 µl of the supernatant were used for cytokine quantification using a commercial multiplex Mouse Cytokine Th1/Th2 Assay (Bio-Rad) according to the manufacturers' instructions. Experiments were performed in flat bottom 96-well plate and results were analyzed with the Luminex system (QIAGEN).

Histopathology

Lungs were removed on day 5 pi, and fixed with 4% buffered formalin. Fixed lungs were subsequently embedded in paraffin, sectioned in slices of 5 µm, and stained with hematoxylin-eosin. The histopathological scores were determined by two independent researchers who were blinded to experimental data. A semi-quantitative scale was used to score bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra-alveolar inflammation as previously described (Hamelin *et al.*, 2005).

Statistical analysis

Statistical analyses were performed using the Prism 5 software. All groups were compared to the unadjuvanted rHMPV-F group using a Student t-test. An additional student t-test compared alum adjuvanted rHMPV-F with rHMPV-F adjuvanted with both alum and rHMPV-M.

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Figure legends

Figure 1: Expression of rHMPV-F and rHMPV-M proteins. a) SDS-PAGE followed by Coommassie Blue staining and b) western blot under denaturing conditions. c) Confirmation of the trimeric confirmation of HMPV-FΔTm by western blot under native conditions.

Figure 2: a) Reciprocal neutralization titers of immunized mice. Serum was collected from 6 mice per group on day -42 and day 0 of the immunization protocol. Sera from 2 mice were pooled, resulting in 3 biological replicates per group. Neutralizing antibody titers were determined using a microneutralization assay. The dotted line indicates the limit of detection of the microneutralization assay; data below the limit of detection were extrapolated. (*): $p<0.05$; (**): $p<0.01$; (***): $p<0.001$ compared to rHMPV-F only and (♦♦): $p<0.01$ compares rHMPV-F+alum to rHMPV-F+rHMPV-M+alum as determined by Student t-test. b) Mean pulmonary viral titers of immunized mice (n=6) infected with $1.0x10^6$ $TCID_{50}$ of HMPV strain c-85473 on day 5 post-challenge. The dotted line indicates the limit of detection, titers below the limit of detection were extrapolated. (*): $p<0.05$; (**): $p<0.01$; (***): $p<0.001$ compared to rHMPV-F only and (♦♦♦): $p<0.001$ compares rHMPV-F+alum to rHMPV-F+rHMPV-M+alum as determined by Student t-test. c) Maximum % weight loss of immunized mice (n=8) infected with $1,0x10^6$ $TCID_{50}$ of HMPV strain c-85473. (*): $p<0.05$; (**): $p<0.01$; (***): $p<0.001$ compared to rHMPV-F only as determined by Student t-test. d) Cumulative histopathological scores of immunized mice (n=6) challenged with $1.0x10^6$ $TCID_{50}$ of HMPV strain c-85473, on day 5 post-challenge. There was no significant change in any group compared to rHMPV-F only.

Figure 3: Weight loss curves of immunized mice (n=8) infected with $1.0x10^6$ $TCID_{50}$ of HMPV strain c-85473 and monitored for 14 days. By day 7 pi, 7 out of 8 mice in the mock-immunized group had reached the endpoint of 20% weight loss, hence the curve was stopped on day 7 for this group.

Figure 4: a) IL-4 levels, b) IFNγ and c) IL-4/IFNγ-ratios of immunized mice (n=6) infected with $1.0x10^6$ $TCID_{50}$ of HMPV strain c-85473 on day 5 post-challenge. (*): $p<0.05$; (**): $p<0.01$; (***): $p<0.001$ compared to rHMPV-F only and (♦♦): $p<0.001$; (♦♦♦): $p<0.001$ compares rHMPV-F+alum to rHMPV-F+rHMPV-M+alum as determined by Student t-test.

Figure 1:

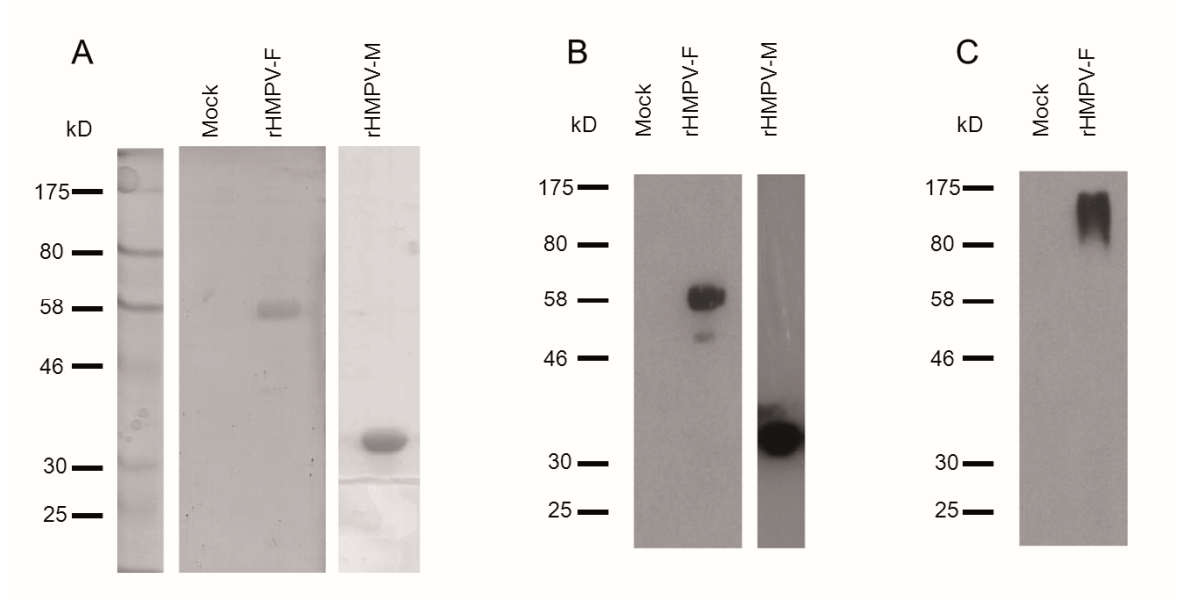


Figure 2:

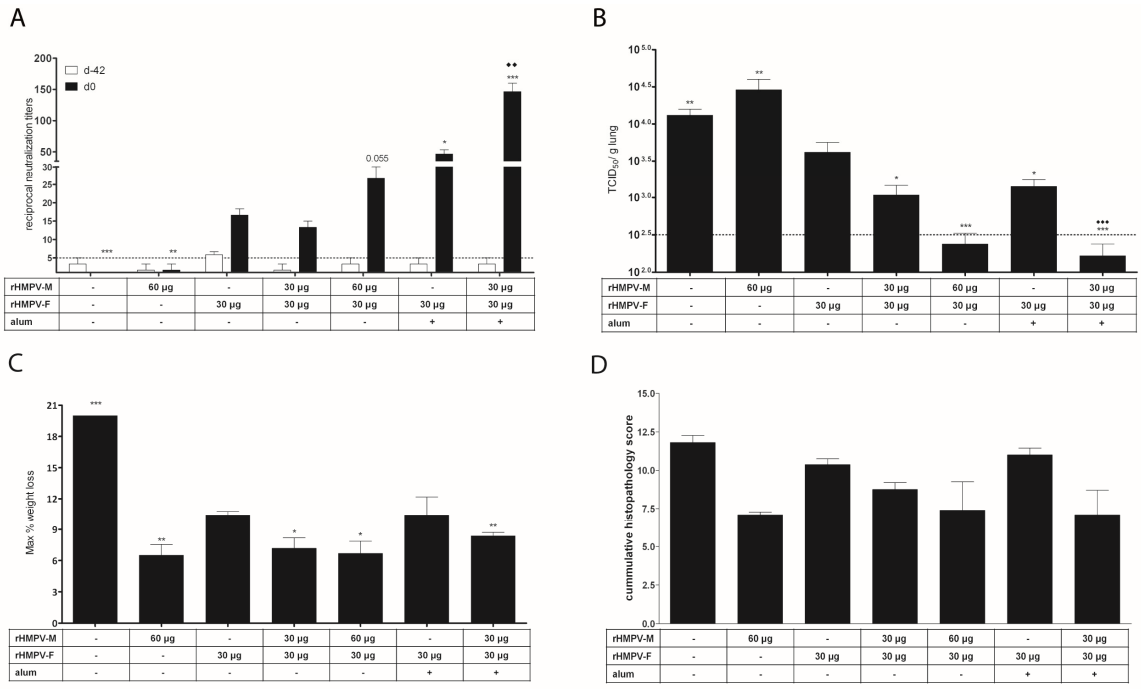


Figure 3:

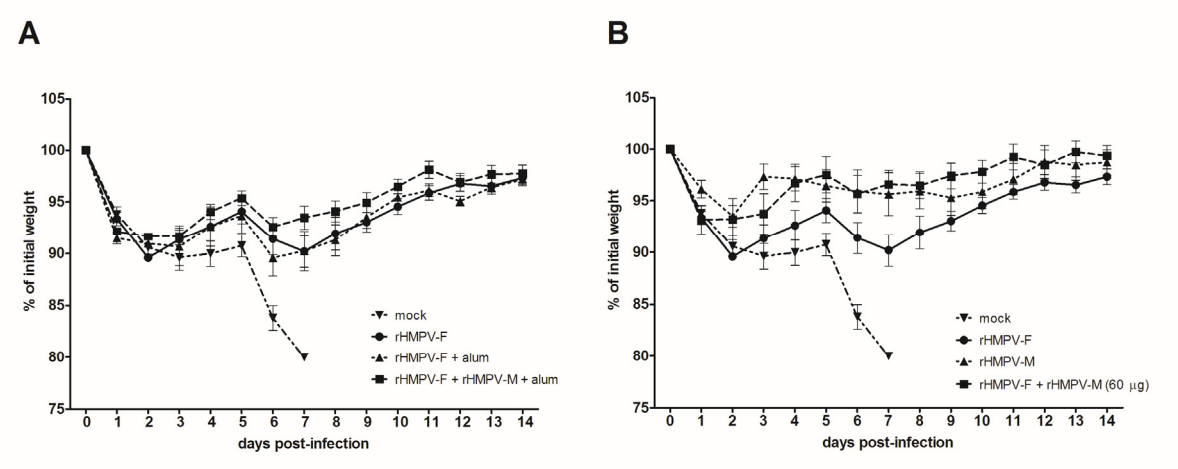
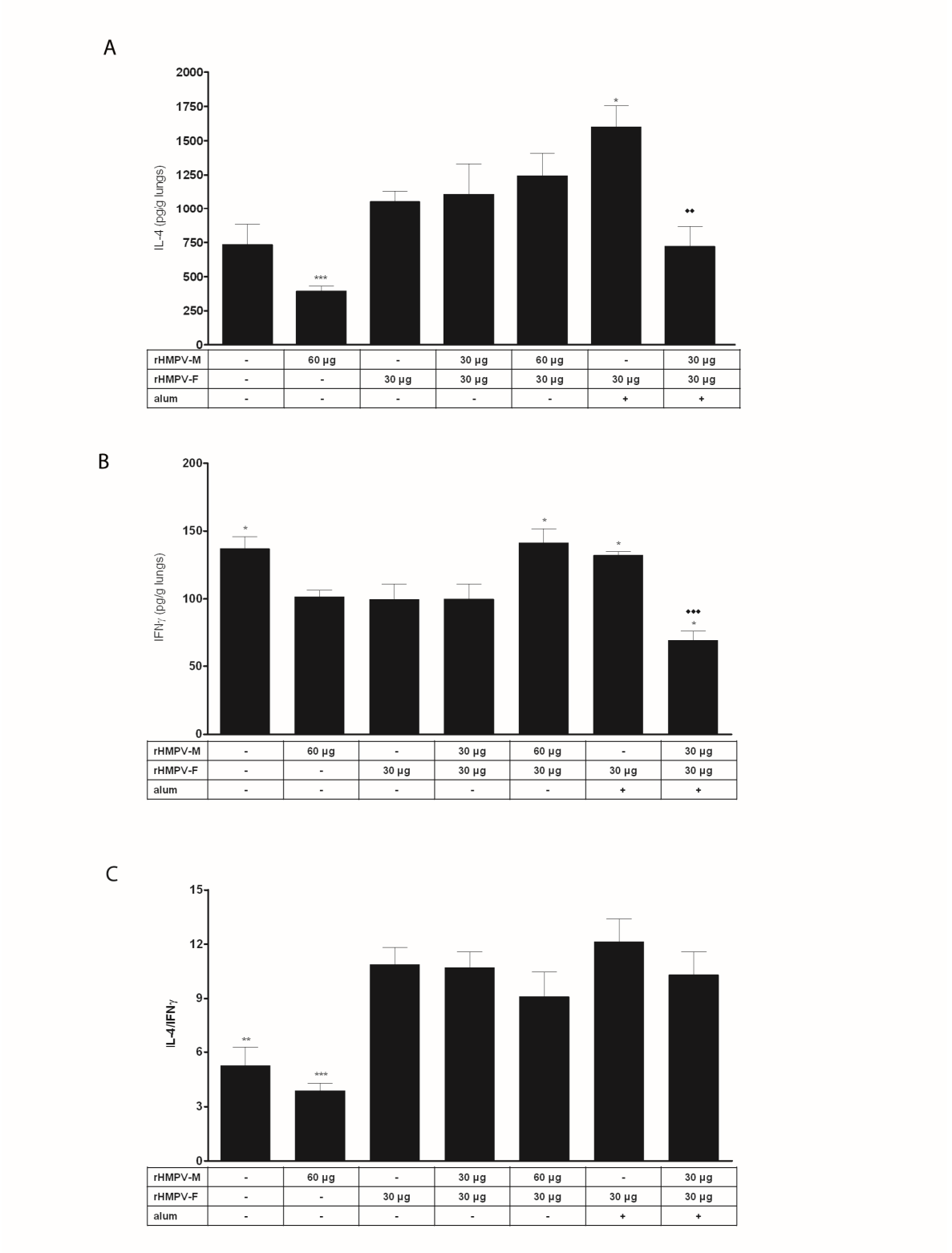

A
B
% of initial weight
days post-infection
mock
rHMPV-F
rHMPV-F + alum
rHMPV-F + rHMPV-M + alum
rHMPV-M
rHMPV-F + rHMPV-M (60 μg)

Figure 4:


A
IL-4 (pg/g lungs)
2000
1750
1500
1250
1000
750
500
250
0
***
*
◆◆
rHMPV-M - 60 µg - 30 µg 60 µg - 30 µg
rHMPV-F - - 30 µg 30 µg 30 µg 30 µg 30 µg
alum - - - - - + +
B
IFNγ (pg/g lungs)
200
150
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rHMPV-M - 60 µg - 30 µg 60 µg - 30 µg
rHMPV-F - - 30 µg 30 µg 30 µg 30 µg 30 µg
alum - - - - - + +
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IL-4/IFNγ
15
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Chapter 6: Discussion

HMPV is an important cause of acute respiratory tract infections. This is particularly the case in infants, whose immune system is not fully developed, elderly patients, whose immune system is decreased due to immunosenescence and immunocompromised patients, such as HIV-patients, solid-organ transplant recipients and hematopoietic stem cell transplant recipients. In fact, HMPV can cause very serious illness in immunosuppressed individuals. This seems somewhat self-contradictory since severe HMPV disease is associated with excessive immune responses and inflammation. Indeed, partial reconstitution of the immune system in hematopoietic stem cell recipients has led to severe and even fatal HMPV infections [38, 86, 98, 113, 146]. A better understanding of the pathogenesis of severe HMPV disease is thus needed.

Animal models are essential tools for the investigation of the pathogenesis of an infectious disease as well as for the pre-clinical investigation of vaccines. Unfortunately, the only natural animal model of HMPV disease appears to be non-human primates, more precisely chimpanzees [300]. Many surrogate animal models have been evaluated (chapter 1 section 8: animal models) and BALB/c mice were found to experience clear HMPV disease, with high viral titers in the lungs that coincide with clinical symptoms and increased pulmonary inflammation [13, 135]. Because of their short generation times and small size, breeding and housing of mice is fairly easy and inexpensive. Moreover, because they have been widely used in research for many years, a large array of immunological tools has been established to study them.

However, using a murine model to evaluate pathogenesis and develop vaccines is associated with some limitations. Some of the general limitations of a murine model were discussed in chapter 1 and will not be addressed again here. Specific considerations for our mouse model of HMPV are:

1) High viral titers of HMPV are needed to obtain viral replication in mice. However, a recent study experimentally infecting healthy adults with 10^6 PFU (also a very high dose) of HMPV only found evidence of infection in 43% of the inoculated population. Therefore, the high dose required for HMPV infection in mice, might in fact be representative of the dose required in humans as well.

2) *In vitro* HMPV passaging and virus concentration are necessary to attain sufficiently high viral titers so that mice can be inoculated with a small volume. Passage in cell culture increases the risk of mutations and might increase the quantity of defective interfering particles which, in turn, might influence the immune response to infection [329].

   Furthermore, in our protocols, clarified supernatant of infected LLC-MK2 cells is concentrated using spin filtration. Soluble cell-components are not removed using this technique, therefore control mice were always mock-infected with concentrated supernatants of uninfected LLC-MK2 cells. An alternative would be to use a sucrose gradient to purify viral particles.

3) There is no standard laboratory strain for HMPV, as is the case for HRSV. Different laboratories use different HMPV strains, different cell lines to culture these strains, different ways of virus quantification and different inoculums, which makes it difficult to compare HMPV studies. BALB/c mice have not been found permissive for all HMPV strains, yet when we infected both BALB/c mice and C57BL/6 with the same inoculum of our laboratory strain C-85473, BALB/c mice were found to be the most permissive.

1. Part 1

We demonstrated that the different phenotypes of HMPV strains are associated with different replication kinetics *in vitro*. By creating recombinant HMPV strains representative of each phenotype and subsequently exchanging the F proteins of these strains, we further established that it is the F protein alone that determines the phenotype. *In vivo*, the F protein also appeared to influence replicative capacity and virulence, but the genomic background was also of great importance.

The HMPV strains routinely used in Dr Boivin's laboratory are C-85473, an A1 subtype virus inducing syncytia and CAN98-75, a B2 subtype virus inducing focal cell rounding. Based on the genomic sequences there is 81.5% nucleic acid homology between the 2 strains (the percentage identity for each viral gene/protein is shown in table 7). Despite this high degree of conservation between the 2 strains, the *in vitro* phenotypes are very different (see Figure 7).

Table 7 : Percentage identity between HMPV strains C-85473 and CAN98-75

	nucleotide identity	Amino acid identity
N	85.9	96.1
P	81.3	85.3
M	85.4	97.2
F	84.5	94.6
M2	83.9	84.6
SH	55.7	42.6
G	53.3	32.6
L	84.1	93.8

We first set out to investigate if the phenotype is associated with the replicative capacity of the virus. Indeed, we observed that the strain that induces large syncytia (C-85473) also replicated to

higher titers *in vitro* and *in vivo*. Moreover, C-85473 induced greater weight loss and mortality in mice.

Studies aimed at elucidating the residues responsible for syncytium formation got side-tracked because the first strain used to investigate HMPV-induced syncytium formation was atypical in the fact that it required low pH to induce syncytium formation [293]. Nevertheless, by transfecting cells with the HMPV F protein, this group and others demonstrated that the F protein alone is sufficient for the formation of syncytia [57, 143, 293]. However, recently, a similar technique of transfecting cells with viral proteins demonstrated that the presence of the HMPV SH protein significantly inhibited F protein-induced syncytium formation, without altering its expression or functionality [211]. This study clearly demonstrated the importance of examining protein function in the context of other viral proteins. Thus, we set out to investigate the influence of genomic background on F-protein induced syncytium formation.

We generated recombinant HMPV viruses representative of the syncytium and non-syncytium phenotype (rC-85473 and rCAN98-75, respectively) and we exchanged the F proteins of both strains (rC-85473_F and rCAN98-75_F). We demonstrated that the exchange of the F protein alone completely restored the phenotype to those of the wild-type strains, indicating that other viral proteins are not involved in syncytium formation. Furthermore, results of RTCA experiments examining the dynamics of HMPV infection of LLC-MK2 cells, also supported these findings. In concordance with this, the strains expressing the F protein of C-85473 replicated to similar maximum titers in cell culture, and these titers were significantly higher than those reached by CAN98-75 F-expressing strains.

In mice, rC-85473 also reached the highest pulmonary viral titers and rCAN98-75 replicated to the lowest titers. Introducing the syncytium-forming F protein into rCAN98-75 significantly

increased pulmonary viral titers and replacing the F protein of rC-85473 with the non-syncytium inducing F protein significantly reduced viral replication. However, full restoration of viral replication was not observed for chimeric viruses, suggesting that other viral proteins contribute to the replicative capacity of HMPV strains *in vivo*.

Moreover, we observed that the genomic background had a great influence on disease severity. Significantly more weight loss was observed in mice infected with recombinant HMPV strains with a C-85473 genomic background compared to rCAN98-75 viruses. Similarly higher levels of pro-inflammatory cytokines were measured in the lungs of mice infected with HMPV strains with the C-85473 genomic background, although the introduction of the syncytium-inducing F protein into the CAN98-75 background significantly increased pulmonary levels of the chemokines MCP-1 and MIP-1α as well as IL-12.

2. Part 2

In the second part of this thesis, we demonstrated the deleterious effect of PAR1 on HMPV pathogenesis in BALB/c mice. PAR1 is a ubiquitous cellular receptor recognized to link coagulation and inflammation, and a receptor that has gained increasing attention for its role in diverse infections (see table 6). We used a commercially-available PAR1 agonist and antagonist to examine the effect of PAR1 on HMPV infection.

We first sought to evaluate if PAR1 had an effect on cellular permissiveness to HMPV infection or an immediate antiviral effect on HMPV replication, *in vitro*. Unfortunately, the PAR1 antagonist was cytotoxic *in vitro* at concentrations above 0.8 µM, this was not the case for the agonist at concentrations up to 250 µM. This concentration (0.8 µM) was used in different conditions; (1) pre-

incubation of the cells with the compounds, to verify if an antiviral cell-state is induced, (2) pre-incubation of the virus with the compounds, to verify if the compounds directly interact with the virus, (3) presence of the compounds only during adsorption phase, to verify the effect on viral entry, or (4) continued presence of the compounds throughout the experiment. No direct or indirect antiviral effect was observed in any of these conditions. On the other hand, when we intranasally administered either the PAR1 agonist or PAR1 antagonist at concentrations up to 500 µM once daily for 5 days to mock-infected mice, no signs of toxicity were observed. Of note, because of its role in coagulation, a PAR1 antagonist (Vorapaxar) has recently received FDA approval for the secondary prevention of atherothrombosis [21]. The use of PAR1 antagonists as antiplatelet compounds is controversial, since increased bleeding was observed after prolonged use in specific populations. Still, it is important to note that in the case of an acute viral infection, such as HMPV, prolonged use would not be required, thereby significantly reducing the risk of such adverse effects.

In the BALB/c mouse model, PAR1 activation, using a PAR1 agonist contributed to disease severity, characterized by increased weight loss and reduced survival, whereas inhibition of PAR1 activation significantly reduced clinical signs. Most strikingly, daily administration of the PAR1 antagonist for a period of 5 days starting on the day of infection, rendered mice asymptomatic without any signs of pulmonary inflammation on day 5 pi and significantly reduced pulmonary cytokine levels and immune cell recruitment compared to infected, vehicle-treated mice.

Interestingly, we systematically observed a 1 log reduction in pulmonary viral titers in mice treated with the PAR1 antagonist. This reduction was not sufficient to explain the decrease in HMPV disease severity but seemed worth exploring. The HMPV F protein is the main protein involved in viral entry and contains a minimal furin cleavage-site (R-X-X-R↓) (see chapter 1 section 3.3.1 Attachment and fusion). Because HMPV replication requires trypsin supplementation, it was

assumed that furin did not take part in F cleavage. However, when we examined furin expression in murine lungs, we found increased furin expression in HMPV-infected and untreated mice and in PAR1-activated mice, whereas infected PAR1 antagonist-treated mice expressed basal levels of furin. We next demonstrated that furin is indeed capable of cleaving the HMPV F protein using transiently transfected cells. When PAR1 was co-transfected, we observed that the presence of uncleaved PAR1 significantly reduced furin mediated F-cleavage *in vitro*. The mechanism by which PAR1 could influence pulmonary furin levels as well as furin function remains to be elucidated.

For the inhibition of PAR1 activation to have a useful therapeutic application, the PAR1 antagonist would have to be administered at the time symptoms appear and not at the time of infection. Therefore, PAR1 antagonist treatment was delayed with 24 h or 48 h (not published). Surprisingly, delayed treatment was less effective in reducing disease severity and differences in reduced weight loss between antagonist-treated and mock-treated HMPV-infected mice were no longer statistically significant. Increased duration of PAR1 antagonist treatment and/or increased doses of the antagonist have not yet been evaluated in a post-infection setting.

3. Part 3

Currently, there is no vaccine available for HMPV. Moreover, different populations might need different types of vaccines. In a primed population, a subunit vaccine is a safe way to boost immunity with no risk of transmission; however, subunit vaccines are less immunogenic than live attenuated vaccines and require the addition of adjuvants.

It is widely accepted that the HMPV F protein is the most immunodominant protein and the only of the surface glycoproteins that induces the production of HMPV-neutralizing antibodies. Yet, other HMPV proteins have been shown to influence the immune response to infection (chapter 1

section 6.2.1 innate immune response). Here, we demonstrate that the addition of another viral protein (the M protein) to an F-based vaccine influences and even increases the immunogenicity of the vaccine.

We started this project by cloning the ectodomain of the HMPV F protein into a commercial expression vector. We chose to express only the soluble ectodomain, which contains the antibody neutralizing epitopes [323, 342], because the preservation of the transmembrane and cytosolic domains would have made the purification of the protein very complex and purified protein yield would have been low. Although this could have given rise to an unnatural conformation of the expressed protein, preceding studies in hamsters and cotton rats had established that the ectodomain alone is sufficient to induce high titers of virus neutralizing antibodies [71, 142]. Additionally, we used a human embryonic kidney cells and not a prokaryotic or non-human mammalian expression system in order to ensure proper post-translational modifications and we purified the protein using affinity chromatography under native conditions.

The HMPV M protein does not require post-translational modifications (chapter 1 section 13.2.2.1 Matrix protein). Although we did attempt to use the same human cell line as the F protein to express HMPV M, the yield was very low. We thus expressed the matrix protein in *E.Coli* cells and purified the protein from cell-lysate under highly denaturing conditions. In fact, the M protein was stored in PBS containing 2M urea because the protein renaturates and precipitates at lower urea concentration. The protein in PBS/urea was only diluted just before administration.

Previous reports showed that the F protein alone was immunogenic but insufficient to fully protect animals from infection. However, when adjuvants such as TiterMax Gold, Iscom matrix or Specol were added to HMPV F, post-challenge lung viral titers were undetectable [71, 142]. Consequently, we chose Alhydrogel (alum), the most widely used adjuvant in human vaccines, as an

adjuvant control. Alum is known for inducing a strong humoral response and a Th2-biased cellular response [191]. Indeed addition of alum increased neutralizing antibody titers 3-fold and increased Th2/Th1 cytokine ratios.

We observed that mice immunized with the HMPV M protein only did not develop neutralizing antibodies and viral replication in the lungs was not reduced. Conversely, these mice showed less clinical signs and less pulmonary inflammation than mice receiving HMPV F with or without alum, indicating an immunomodulatory effect. It would be very interesting to investigate specific T-cell populations induced in HMPV M immunized-mice, in a follow-up study.

When HMPV F and HMPV M were co-administered, mice showed reduced weight loss as well as reduced viral replication despite neutralizing antibody titers similar to F only mice, indicating again an increase in cellular rather than humoral responses. Interestingly, when HMPV F and HMPV M were adjuvanted with alum, a six-fold increase in neutralizing antibodies was observed and viral replication was no longer detectable. Perhaps the depot-effect of alum increases Ag-uptake and presentation by antigen presenting cells or perhaps the M protein improves $CD4^+$ T-cell stimulation of B-cells, increasing quantity and/or quality of antibody production. In any case, examination of the vaccine-induced cellular response seems warranted based on our findings.

Chapter 7: Conclusions and perspectives

1. Part 1

The HMPV F protein was demonstrated to be the main determinant for syncytium formation. Moreover, HMPV strains expressing a syncytium inducing F protein replicated to higher titers *in vitro*. The F protein also influenced, to a lesser extent, viral replication in mice; however, the genomic background in which the F protein was expressed was of great influence on disease severity.

1) Studies investigating the pH-dependency of some HMPV strains to induce syncytia suggest a combination of amino acids in the vicinity of conserved histidine residues within the F1 region of the F protein to be of importance for syncytium formation [57, 210]. However, a study in which the HMPV F protein was replaced by the hyperfusogenic AMPV-C F protein found the F2 subunit to be the determining fragment for the observed increased fusogenicity. It would thus be of great interest to examine which regions of the F protein of C-85473 is responsible for syncytium formation. Table 8 compares the protein sequences of both strains used in this study. Individual swapping of specific regions within the F protein of CAN98-75 by those of C-85473 could elucidate the fragment responsible for syncytium formation at neutral pH. For instance; only the F2 region, only the fusion domain, only the HRA or HRB region or both, or the entire F1 subunit. In total 29 amino acid changes differ between C-85473 and CAN98-75 (see table 8). Given the importance of the HR regions for the functionality of the F protein, The 5 amino acid changes in the HRA and the 2 amino acid changes in the HRB are the most likely candidates and will be investigated first.

Table 8 : Amino acid changes within the F protein of C-85473 and CAN98-75

Amino acid position	C-85473	CAN98-75	Location within the F protein
6	V	M	Signal peptide (1-22)
9	F	I	
61	A	T	F_2 (23-102)
82	R	K	
122	V	I	Fusion peptide (103-125)
135	T	N	HRA (131-172)
139	N	G	
143	K	T	
166	E	K	
167	D	E	
175	R	S	F_1 (173-453)
233	N	Y	
286	V	I	
296	K	I	
312	C	K	
323	E	K	
348	K	R	
404	N	P	
449	V	I	
479	R	K	HRB (454-514)
482	S	N	
503	S	L	TM domain (490-514)
507	L	S	CS domain (515-539)
510	V	I	
511	F	I	
518	K	R	
528	S	N	
533	N	G	
539	N	S	

2) We demonstrated that HMPV strains expressing the same F protein reached similar peak replication titers *in vitro*, but chimeric viruses reached their peak a day later. This could indicate a difference in F protein expression levels between wild-type and chimeric strains. A difference

in expression levels could also partially account for the fact that *in vivo* replication capacities were not fully restored in chimeric HMPV viruses. Quantification of glycoprotein spikes on virions has been performed for other viruses using electron microscopy [227], mass spectrometry [283] and/or immune-capture [236, 261]. However, independent of the technique used to quantify surface glycoproteins, the virions should be highly purified using sucrose gradients and ultracentrifugation to ensure no free glycoproteins or sub-viral particles remain present prior to analysis of the virions.

3) HMPV disease severity did not solely depend on the F protein. Although introduction of the syncytium-inducing F gene increased some pro-inflammatory cytokine/chemokine levels, complete restoration was not observed. Furthermore, the HMPV strains with the C-85473 background induced similar symptoms and similarly high levels of pro-inflammatory cytokines/chemokines, despite their slightly different replicative capacities. This suggests that other viral proteins are involved in the increased virulence of these strains. Viral proteins that have been found to inhibit antiviral responses have been described in chapter 6.2 of the Introduction. Among these proteins are the two other surface glycoproteins G and SH as well as proteins involved in the polymerase complex; P and M2-2. It would be interesting to swap these genes between our two strains and investigate their effect on replicative capacity and virulence. Of course other viral proteins may also be involved, yet those that have already been described to be involved in immune evasion might be a good starting point.

4) Finally, it would be interesting to investigate if a syncytium-inducing HMPV protein is more immunogenic than a non-syncytium-inducing F protein. By collecting serum samples from mice

infected with the 4 recombinant viruses, neutralizing antibody titers against both C-85473 and CAN98-75 could be determined. If one F protein is found to be more immunogenic than the other, this could have an impact on future vaccine design.

2. Part 2

We found that PAR1 significantly contributes to HMPV pathogenesis and that the treatment of HMPV-infected mice with PAR1 antagonists significantly reduces HMPV-induced pathogenesis, as observed by lower weight loss and reduced pulmonary inflammation.

1) We used a non-peptide PAR1 antagonist (SCH79797) to inhibit PAR1 activation. Using a different (less potent) non-peptide PAR1 antagonist (SCH530348), weight loss was only slightly reduced compared to mock-treated mice. However, this compound uses the same mechanism to inhibit PAR1 activation, i.e. inhibiting the interaction between the receptor and its tethered ligand. It would be interesting to test antagonists with a different mechanism of inhibition such as pepducins. These cell-penetrating peptides inhibit PAR1 intracellular signaling instead of PAR1 activation (see also Figure 11). Pepducins are composed of a lipid moiety, such as palmitate, attached to a peptide that corresponds to an amino acid segment from one of the cytoplasmic loops or the C-terminal tail of the target PAR [241]. Depending on the peptide sequence, pepducins can act as agonists, antagonists, or modulators of PAR activity. To date, two PAR1-specific antagonist pepducins (P1pal-12 and P1pal-7) have been used to block PAR1 signaling in mice [61, 190]. We would also have liked to infect PAR1 knock-out mice to confirm our results with the PAR1 antagonist. Unfortunately, there were difficulties mating the PAR1

heterozygote mice, the frequency of KO was only 10-12% and many KO mice died during embryonic development. The line was lost before experiments could begin.

2) The PAR1 antagonist treatment regimen influences its protectiveness. Pathogenesis was less severe in infected mice treated with the antagonist for 3 consecutive days, starting at the time of infection, but when the duration of the treatment was increased (to 5 consecutive days in total) HMPV disease was asymptomatic. This suggests an important role for PAR1 at the peak of viral replication. However, delaying treatment 24 h or 48 h post infection resulted in less striking effects (not published). This suggests that PAR1 is also involved in the innate immune response in the very first hours after infection. PARs have been proposed to modulate TLR signaling [16]. In fact, using two different models of viral infection (Coxsackievirus B3 and Influenza A, both single stranded RNA viruses), PAR1 activation was shown to increase IFN-β and IP-10 levels in a TLR3-dependent manner [17]. Although only a few studies have examined the role of TLR3 during HMPV infection [168], TLR4 has recently been found to contribute to HMPV pathogenesis [333]. Thus, it might be interesting to examine the effect of PAR1 on IFN-β and IP-10 levels during HMPV infection and investigate crosstalk between PAR1 and TLR3 or TLR4 in HMPV-infected mice using antagonists or knock-out mice.

3) The close relatedness of HMPV and HRSV makes it likely that PAR1 also plays a role in HRSV pathogenesis. We have thus developed the BALB/c mouse model for HRSV infection using the laboratory strain HRSV A2. We are currently investigating the role of PAR1 in HRSV infection, but so far no beneficial effect of the PAR1 antagonist is observed in that model (unpublished). Since no reduction in viral titers has been observed during HRSV infection in PAR1-antagonist-

treated mice and furin is known to cleave and activate the HRSV fusion protein [171], it would be interesting to also investigate the effect of PAR1 on furin mediated cleavage of the HRSV F protein.

4) Since HMPV is only released from infected epithelial cells on the apical side (into the lumen), direct spread from one cell to another is limited. DCs have been shown to be permissive to HMPV infection [120], and they might carry the virus from an infected epithelial cell to an uninfected cell, thereby increasing infectivity. Using flow cytometry, we demonstrated reduced recruitment of immune cells, including DCs, to the lungs of HMPV-infected PAR1 antagonist-treated mice. It is therefore possible that the reduction in HMPV pulmonary viral titers is partly due to the reduction in DCs in addition to reduced F cleavage. Furthermore, recent depletion studies in mice showed that alveolar macrophages (AM) contribute to HMPV pathogenesis and infection whereas they are protective during HRSV infection [167]. AM depletion was associated with significantly reduced HMPV titers in the lungs, suggesting that HMPV required AMs for replication in the lung. Conversely, AM depletion increased HRSV pulmonary viral titers, indicating a protective role of AM during HRSV infection. We demonstrated reduced levels of Ma in the lungs of HMPV-infected PAR1 antagonist-treated mice, however we did not examine the population of AM specifically. Since both DCs [358] and AM [277] have been shown to express PAR1, it would be of great interest to investigate the role of DCs and AMs during PAR1 antagonist treatment of both HMPV- and HRSV-infected mice.

5) Antivirals such as ribavirin are effective at reducing viral replication, yet the effect on clinical outcome remains unclear [252]. One possible reason for this discrepancy is the fact that

antivirals are only given in severe cases, when the infection has already induced excessive inflammation and clinical outcome is no longer dependent on viral replication alone. One group used pneumonia virus of mice to demonstrate that the combination of ribavirin and immunomodulatory agents significantly decreases morbidity and mortality compared to either treatment alone [44, 45]. Our group found that the combination of ribavirin and corticosterone showed modest improvements in HMPV-infected mice compared to ribavirin alone [133]. Taken together, it would be interesting to see if the combination of ribavirin and the PAR1 antagonist could result in a more effective HMPV treatment.

3. Part 3

We have demonstrated that a subunit vaccine containing both HMPV F and M proteins is immunogenic and protective in mice. Such a vaccine would be safe to increase immunity in a seropositive population, but might not be immunogenetic enough to be used in a seronegative population. However, further investigation is warranted.

1) As discussed earlier, the evaluation of the cellular immune response could shed light on the mechanism by which HMPV M increases immunogenicity. A fairly straightforward way to evaluate lymphocyte infiltration into the lungs of challenged mice would be the immunohistochemical quantification of CD3-positive cells in the lungs of vaccinated, challenged mice. Intracellular staining (ICS) experiments of spleen cells obtained from vaccinated mice, using the F or M protein or whole virus as stimuli, could subsequently be developed to evaluate lymphocyte effector function. In addition, 3 F protein and 2 M protein CTL predictopes recognized by BALB/c (H-2d) mouse T cells have been evaluated [219] and could serve as stimulating peptides to evaluate CTL effector function in particular, using ICS or an IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT).

2) We have established the dose of HMPV F and M needed to reduce viral replication in the lower respiratory tract; yet, it would also be pertinent to assess if viral replication still occurs in the upper respiratory tract. If the virus is still capable of replicating in the upper respiratory tract, the individual might be protected from disease, but would still be able to transmit the virus to others.

3) Since different HMPV strains can co-circulate, a vaccine would have to be protective against both homologous and heterologous HMPV strains [34, 335]. Challenging vaccinated mice with heterologous virus is thus also needed. Moreover, HMPV immunity wanes over time [144, 327], it would be necessary to investigate the duration of immunity induced by such a vaccine. Quite possibly immunisation would need to be repeated at a regular interval.

4) The use of adjuvants other than alum, for instance TLR ligands, might improve immunogenicity even more and allow for a reduction in the amount of Ag needed. To date, not many studies looking at the roles of specific TLRs during HMPV infection have been published; one study found distinct patterns of upregulation of TLR mRNAs in the lungs of BALB/c mice infected with HMPV or HRSV [87], with levels for TLR3, TLR7 and TLR8 mRNA peaking earlier in HMPV infection than in HRSV infection and TLR3 expression being generally more elevated during HRSV infection. Additionally, TLR4 has recently been found to contribute to HMPV pathogenesis [333] whereas TLR7, but not TLR9, was found to participate in the antiviral immune response to HMPV [116]. The role of TLR agonists as adjuvants in HMPV vaccines specifically, has not been studied extensively. However, studies found TLR agonists i.e., TLR2 (protollin or peptidoglycan), TLR3 (polyinosinic:polycytidylic acid), TLR4 (monophosporyl lipid A) and particularly TLR9 (oligodeoxynucleotide containing an unmethylated CpG motif)

agonists, to improve Th2/Th1 ratios induced by RSV vaccines [136]. In addition, such adjuvants might even permit mucosal (intranasal) administration of the vaccine, which could potentially increase localized immunity. Based on the limited studies into TLR involvement in the immune response to HMPV infection, TLR3 and -7 agonists would seem likely candidates to examine. However, the promising result with TLR9 agonists in vaccine formulations developed against other infectious diseases [285], suggest that TLR9 should also be taken into consideration.

5) Finally, other viral proteins could be investigated as part of a subunit vaccine. For instance, studies predicting CTL-epitopes found several conserved epitopes in the N protein [139] and the N protein has been found to self-assemble into multimeric capside-like particles [259], and this could possibly increase antigen-uptake in comparison with only oligomeric proteins. A trivalent vaccine (F, M and N) might be worth exploring. On the other, hand a recent study showed that mice immunized with rBCG (*Mycobacterium bovis* bacillus Calmette–Guérin) strains expressing only the P protein from HMPV were protected [246]. Therefore the addition of the P protein to a multivalent subunit vaccine might also be worth consideration.

4. General conclusion

HMPV is a recently discovered but important respiratory pathogen. Despite the many advances made in recent years in elucidating the pathogenesis of this infection, much remains to be investigated. The objectives of this thesis were to investigate the contributions of the HMPV F protein and the cellular receptor PAR1 to HMPV virulence and pathogenesis, and to investigate the effect of the HMPV M protein in an HMPV F subunit vaccine in a BALB/c mouse model.

The HMPV F protein was found to be of great importance in viral replication *in vitro*, even though it contributed only to a lesser extent to HMPV virulence. PAR1, on the other hand, was found to contribute significantly to HMPV pathogenesis and the inhibition of PAR1 activation rendered HMPV-infected mice asymptomatic. Furthermore, the addition of the HMPV M protein to F protein-based subunit vaccines increased immunogenicity and protected mice from subsequent pulmonary HMPV infection.

These findings shed light on specific aspects of HMPV pathogenesis and immune responses, yet they also open the door to new lines of investigation which will need to be examined in order to reach a good understanding of HMPV infection and in order to develop targeted prophylactic and therapeutic modalities against HMPV.

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Annex

During my stay at Dr Boivin's laboratory I've had the opportunity to writing an update to the Human Metapneumovirus chapter in the electronic textbook Antimicrobe, which is currently in press. (http://www.antimicrobe.org)

Human Metapneumovirus

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And to co-author two peer-reviewed papers;

Virus-like particle vaccine induces cross-protection against human metapneumovirus infections in mice.

Lévy C, Aerts L, Hamelin MÈ, Granier C, Szécsi J, Lavillette D, Boivin G, Cosset FL.

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