Thesis for doctoral degree (Ph.D.)
2008

SARS CORONAVIRUS
THE ROLE OF ACCESSORY PROTEINS AND NITRIC OXIDE IN THE REPLICATION CYCLE

Sara Åkerström

Karolinska Institutet

Thesis for doctoral degree (Ph.D.) 2008
SARS CORONAVIRUS THE ROLE OF ACCESSORY PROTEINS AND NITRIC OXIDE IN THE REPLICATION CYCLE
Sara Åkerström

Karolinska Institutet

From the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet
and the Swedish Institute for Infectious Disease Control, Stockholm, Sweden

SARS CORONAVIRUS

THE ROLE OF ACCESSORY PROTEINS AND NITRIC OXIDE IN THE REPLICATION CYCLE

Sara Åkerström

Karolinska Institutet

Stockholm 2008

Published by Karolinska Institutet.

ISBN 978-91-7409-086-04

Printed by
REPROPRINT AB
Stockholm 2008
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

TO MY MOTHER AND FATHER

ABSTRACT

Severe acute respiratory syndrome coronavirus (SARS-CoV) caused the first pandemic of the 21st century. The etiological agent was identified as a novel coronavirus. Until then, human coronaviruses (HCoVs) had only been known to cause the "common cold". Data indicated that the virus originated from animals. The palm civet (*Paguma larvata*) was identified as the source of transmitting the virus to humans, however not to be the natural host, but most likely acting as an amplifier before the virus was transmitted to humans. SARS-CoV has 14 potential open reading frames (ORFs). Eight of those are specific for SARS and encodes for the accessory proteins. The accessory proteins of SARS-CoV have no known sequence homology to any other accessory proteins found in coronaviruses. The knowledge about the function of most of the accessory proteins are limited. In addition to the four main structural proteins; the spike (S), the envelope (E), the membrane (M) and the nucleocapsid (N) protein, the accessory protein 3a of SARS-CoV has been shown to be a minor structural protein.

SARS-CoV, as the name implies, causes severe lower respiratory syndrome. Person-to-person transmission has been through infectious droplets, and the overall mortality rate is ~10% but can vary with age. Most SARS patients were treated with antiviral drugs and glucocorticoids since no specific treatment has been available. Inhalation of nitric oxide (NO) has been adminstered to only a few patients but with positive effect.

In this thesis we have investigated the importance of accessory proteins 3a/3b and 7a/7b in the replication cycle. Further we compared the neutralizing properties of the endo- and the ectodomian of 3a. We also investigated the antiviral effect of NO on SARS-CoV, and a possible mechanism behind an antiviral effect.

Short interfering RNA (siRNA) was designed to specifically target sgRNA 2, 3 and 7 expressing the S, 3a/3b and 7a/7b protein respectively. The yield of progeny virus was significantly reduced for all three siRNAs. The amount of progeny virus was to some extent lower for siRNA 7, which could be due to the fact that siRNA 7 were able to silence both 7a/7b and 8a/8b protein. Cells expressing the siRNAs specifically silenced the expression of targeted proteins without affecting the infection shown by expression of the N protein. The 3a protein was further investigated, comparing neutralizing properties of antibodies towards the endodomain and the ectodomain of 3a. Antibodies towards both ends were able to detect 3a in lysate from infected Vero E6 cells. However, only antibodies against the ectodomain showed neutralizing properties in Vero E6 cells.

In order to investigate the antiviral affect of NO on SARS-CoV, both an exogenous NO donor, and endogenously produced NO was used. We showed that NO has a clear antiviral effect on SARS-CoV, inhibiting the replication cycle of the virus. To investigate the mechanism behind the antiviral effect of NO, we first confirmed that NO per se was exerting the observed antiviral effect, and not through peroxynitrite interaction. By using a cell-cell fusion assay, we showed that NO inhibits the fusion step by reducing palmitoylation of the SARS-CoV S protein.

LIST OF PUBLICATIONS

I. Sara Åkerström, Ali Mirazimi, Yee-Joo Tan
Inhibition of SARS-CoV replication cycle by small interference RNAs silencing specific SARS proteins, 7a/7b, 3a/3b and S
Antiviral Research. 2007, Vol. 73, p.219-227. PMID 17112601

II. Sara Åkerström, Yee-Joo Tan, Ali Mirazimi
Amino acid 15-28 in the ectodomain of SARS coronavirus 3a protein induces neutralizing antibodies
FEBS Letters. 2006, Vol.580, p.3799-3803. PMID 16781713

III. Sara Åkerström, Mehrdad Mousavi-Jazi, Jonas Klingström, Mikael Leijon, Åke Lundkvist, Ali Mirazimi
Nitrix Oxide Inhibits the Replication Cycle of Severe Acute Respiratory Syndrome Coronavirus
Journal of Virology. 2005, Vol.79, No.3 p.1966-1969. PMID 15650225

IV. Sara Åkerström, Choong Tat Keng, Yee-Joo Tan, Ali Mirazimi
Nitric oxide inhibits SARS CoV replication directly and decreases the fusion activity of the spike protein
Submitted

CONTENTS

LIST OF ABBREVIATIONS

3CLpro	3C-like main proteinase
aa	amino acids
ACE2	angiotensin-converting enzyme 2
APC	antigen presenting cells
ARDS	acute respiratory distress syndrome
CBP	CREB-binding protein
CoV	coronavirus
CS	core sequence
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
E	envelope
eNOS	endothelial nitric oxide synthase
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
hACE2	human angiotensin converting enzyme 2
HCoV	human coronavirus
HIV	human immunodeficiency virus
IFA	indirect immunofluorescence assay
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon induced protein of 10 kDa
IRF	interferon regulatory factor
kb	kilo base
kDa	kiloDalton
L-NMMA	N^G-monomethyl-D-L-arginine
L-SIGN	liver/lymph node specific ICAM-3 grabbing non-integrin
M	membrane
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MHV	murine hepatitis virus
MIP-1α	macrophage inflammatory protein 1α
N	nucleocapsid
NAP	N-acetylpenicillamine
NendoU	nidovirus uridylate-specific endoribonuclease
NF-κB	nuclear factor kappa B
NK	natural killer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
nsp	nonstructural protein
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PLpro	papain-like accessory proteinase

poly (A)	polyadenylate
pp	polyprotein
PRR	pathogen recognition receptor
RBD	receptor binding domain
RdRp	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RTC	replication/transcription complex
rVV-L-S	recombinant vaccinia virus expressing S protein
S	spike
SARS	severe acute respiratory syndrome
sgRNA	subgenomic RNA
SGT	small glutamine-rich tetratricopeptide repeat
siRNA	small interfering RNA
SNAP	S-nitroso-N-acetylpenicillamine
TGEV	transmissible gastroenteritis coronavirus
TGF	transforming growth factor
T_H	T helper
TNF	tumor necrosis factor
TRS	transcription-regulating sequence
VLP	virus like particle
WHO	World Health Organization

1 INTRODUCTION

Coronaviruses belong to the family *Coronaviridae* and are enveloped viruses with a positive sense ribonucleic acid (RNA) genome ranging in size from a 27.1-31.6 kilo base (kb). Common to all known coronaviruses is the organization of the genomes. The 5'two-thirds of the genome consists of the 1a and 1b open reading frame (ORF) encoding the nonstructural proteins (nsps) and the RNA-dependent RNA polymerase (RdRp). At the 3' end of the genome are genes encoding for the structural proteins; the spike (S), the envelope (E), the membrane (M) and the nucleocapsid (N) arranged in the 5' to 3'order they are written here. Interspersed in between there are accessory genes specific for each coronavirus (CoV), differing in both number and size [1,2].

There are 28 identified members of the coronavirus family belonging to three different groups based on sequence comparison of the genomes [3]. Group 1 consists of coronaviruses found in pig, cat, dog, bat and the human. After the outbreak of the severe acute respiratory syndrome (SARS) coronavirus (CoV) group 2 was subdivided into 2a and 2b with 2b being a new group. The classification of SARS-CoV caused a great controversy over which group it should belong to since phylogenetic characterizations of the genome sequence showed approximately equal distance to all three groups. However, further studies suggested that SARS-CoV is an early split off from the rest of the group 2 coronaviruses and should be classified as a group 2b virus since it is very distinct from the rest of the members in the group 2. Group 2a consists of mouse, cat, rat, pig, horse and human coronaviruses and 2b of one human and a few bat coronaviruses. Group 3 only consists of avian coronaviruses so far.

The first two human coronaviruses (HCoVs), HCoV-229E and HCoV-OC43 were discovered during the 1960s and are members of group 1 and group 2a respectively, and they are responsible for approximately 5-30 % of the "common colds" [4,5]. Table 1 lists representative members of coronaviruses from each group.

After the outbreak of the SARS-CoV pandemic in 2002 the interest in coronaviruses was renewed and two more HCoVs, NL63 (group 1) and HKU1 (group 2), have since been identified. Both of these coronaviruses also results in "common cold" symptoms [6].

Table 1. Representative members of coronaviruses from each group

Group	Virus	Abbreviation	Host
1			
1a	Transmissible gastroenteritits virus	TGEV	Pig
1b	Human coronavirus strain 229E	HCoV-229E	Human
	Human coronavirus strain NL63	HCoV-NL63	Human
2			
2a	Murine hepatitis virus	MHV	Mouse
	Human coronavirus strain OC43	HCoV-OC43	Human
	Human coronavirus strain HKU1	HCoV-HKU1	Human
2b	Severe acute respiratory syndrome coronavirus	SARS-CoV	Human, bat?
	Bat severe respiratory syndrome coronavirus	Bat-SARS-CoV	Bat
3			
	Infectious bronchitis virus	IBV	Chicken

1.1 SEVERE ACUTE RESPIRATORY SYNDROME

SARS was reported to have originated in Guangdong Province in China. Within weeks, SARS had spread to several countries around the world and infected more than 8000 people [7]. By the end of the epidemic in 2004 the World Health Organization (WHO) reported 774 deaths, with a mortality rate of ~10 % (www.who.int/csr/sars/country/table2004_04_21/en/index.html).

The etiological agent causing the disease was rapidly identified as a novel CoV and data indicated that the virus originated from animals [7,8]. Early reports from the Guangdong province of patients with SARS indicated exposure to wild animals in markets trading with restaurants [9]. Animals were sampled at an animal market in Shenzhen early 2003, and in masked palm civets (*Paguma larvata*), raccoon dog (*Nyctereutes procyonoides*) and Chinese ferret badger (*Melogale moschata*) viruses closely related to SARS-CoV were detected [10].

Civets were suggested to be the direct source of infecting humans since the virus sequence between human and civet isolates from the outbreak were very similar [11]. It was however not clear whether the civet was the natural reservoir or just an intermediate host [12]. Experimental infection of civets with two human strains of SARS-CoV resulted in clinical signs of infection, indicating that the civet was not the natural reservoir. In addition, the virus was not widely spread in wild or in farmed civets as could have been expected if the civet was the natural reservoir [13,14]. Studies conducted by different groups suggested that the civet most likely acted as an amplifying host before the virus was introduced to human. The sequence of SARS-like coronaviruses in civets showed that the mutation rate was quite high indicating that it had not adapted to civets but they had consequently acquired the virus from another species [11,14].

In 2005 bat coronaviruses were discovered in different species of horseshoe bats (*Rhinolophus spp*) closely related to SARS-CoV [15,16]. Genome organization was almost identical and the genome sequence showed 88-92 % identity with SARS-CoV [12,17]. This discovery gave an important clue to a possible natural reservoir of SARS-CoV. Subsequent studies have found a diversity of coronaviruses in different bat species and most seem to be species specific, SARS-like coronaviruses have so far only been associated with horseshoe bats and is a strong candidate for being the natural reservoir for SARS-CoV [12,18-20].

1.2 CLINICAL PICTURE

SARS-CoV infects both men and women of all ages. SARS is presented as an acute onset of illness with quick respiratory deterioration, common symptoms are fever, chills, dry cough, headache, malaise and myalgia [21-23]. The major cause of death is severe respiratory failure making the respiratory system the primary target of SARS-CoV [24], but SARS-CoV is also spread to many other organs as well, including kidneys, intestinal tract, sweat glands, liver, and pancreas [24,25]. On admission to hospitals most patients show abnormal chest radiography usually with patchy infiltrates

(Fig. 1a and 1b) [22,23]. Laboratory findings may include low platelet count and low white blood cell count. The lungs of diseased SARS patients show diffuse alveolar damage, large syncytial cells and viral infection of trachea and bronchi epithelial cells [25]. Also detection of infection of T cells, monocytes, B cells, natural killer (NK) cells, pneumocytes and both infected and uninfected macrophages were observed in lungs of patients who died of SARS-CoV infection [25-27].

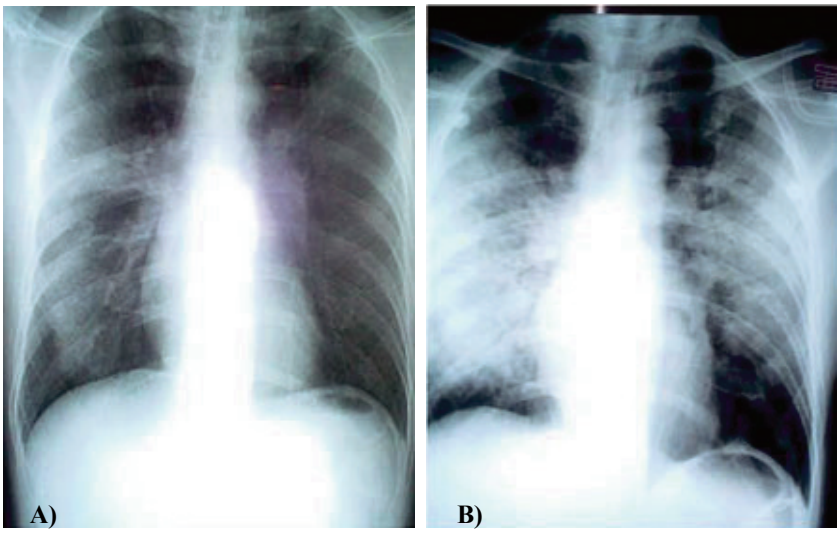

Figure 1. Chest radiograph from patient with SARS showing changes in lung infiltrates. A) Day 8 of symptoms. B) Day 14 of symptoms. Modified from Hsu et al 2003 [28].

1.3 EPIDEMIOLOGY

Early cases of SARS which were more sporadic, were linked to live animal markets as mentioned above [29]. A seroprevalence study among animal traders at three animal markets in Guangzhou revealed that ~9% had IgG antibodies to SARS-CoV without history of any SARS-like symptoms [29,30].

The global spread of the virus started when a professor of medicine from a hospital in Guangzhou, China, arrived and stayed at Hotel M in Hong Kong. Previously upon arrival in Hong Kong he had treated patients with pneumonia at the hospital and acquired the disease. He was taken ill and was brought to a hospital where he later died of respiratory failure. During his stay at the hotel he came in contact with other guests whom he transmitted the virus to. They returned to their home countries and subsequently brought the infection with them [21]. One of the secondary cases from the hotel carried the virus back to Vietnam. At the end of February 2003 a private hospital in Hanoi contacted WHO concerning a patient with an atypical pneumonia. A physician, specialist in infectious diseases at the Hanoi office of the WHO concluded that the hospital was dealing with something unusual. He acted swiftly and the hospital was quickly put under quarantine and additional specialists were flown in [31]. WHO issued a global alert March 12th, 2003

(www.who.int/mediacentre/news/releases/2003/pr22/en/). He died at the end of March 2003 after acquiring SARS while working together with the hospital staff [31].

The mode of spread has been person-to-person transmission (contact with infectious respiratory droplets, fomites etc with mucosa) at homes, public transportation and health care facilities, where a large number of health care workers were affected [7]. Fecal-oral route of transmission has also been suggested since virus was found present in stool [32].

The incubation period is ~2-5 days but could last up to 10 days. Infectious period is ~5 days after onset of disease, and the viral peak has been observed approximately 10 days after symptoms appears (www.who.int./csr/sars/en/whoconsensus.pdf) [32,33].

The overall mortality rate is ~10% however it can range from 0 % to 50% depending on age. People over 65 years of age have a higher fatality rate than younger people and children (www.who.int./csr/sars/en/whoconsensus.pdf).

The outbreak was brought under control by quarantine measures, patient isolation, travel advisory, and infection control in hospitals (www.who.int/mediacentre/news/releases/2003/pr23/en/) [31,34]. The global outbreak was declared over by the WHO in July 2003 when no more new cases were reported [35]. In late 2003-2004 there were four cases of SARS in China but they were mild and with no spread to contacts. There was one case in Singapore and one in Taiwan in 2003 and two cases in China in 2004 of laboratory-acquired SARS infections with no further spread to contacts [22,36,37].

1.4 GENOME AND STRUCTURE

The genome of SARS-CoV is ~29.7 kb long and is predicted to have 14 functional ORFs (Fig. 2A). ORF1a and 1b which make up about 2/3 of the genome, encode 16 nsps, the last 1/3 of the genome encodes the four structural proteins common to all known coronaviruses and eight accessory proteins specific for SARS [38]. The four common structural proteins are the S, E, M and the N proteins. The virus is 80-120 nm in diameter and the nucleocapsid protein is bound to the positive sense RNA genome [2,39]. A lipid bilayer surrounds the capsid and at least three main structural proteins are anchored in the lipid bilayer, the S, E, and M protein (Fig. 2B) [39]. The 5' of the genome is capped and the 3' has a poly (A) tail, like all other known coronaviruses and untranslated regions flank the 5' and 3' end of the genome. Unlike most other group 2 CoVs, the genomic sequence of SARS-CoV does not contain a gene for hemagglutinin-esterase (HE) protein [38].

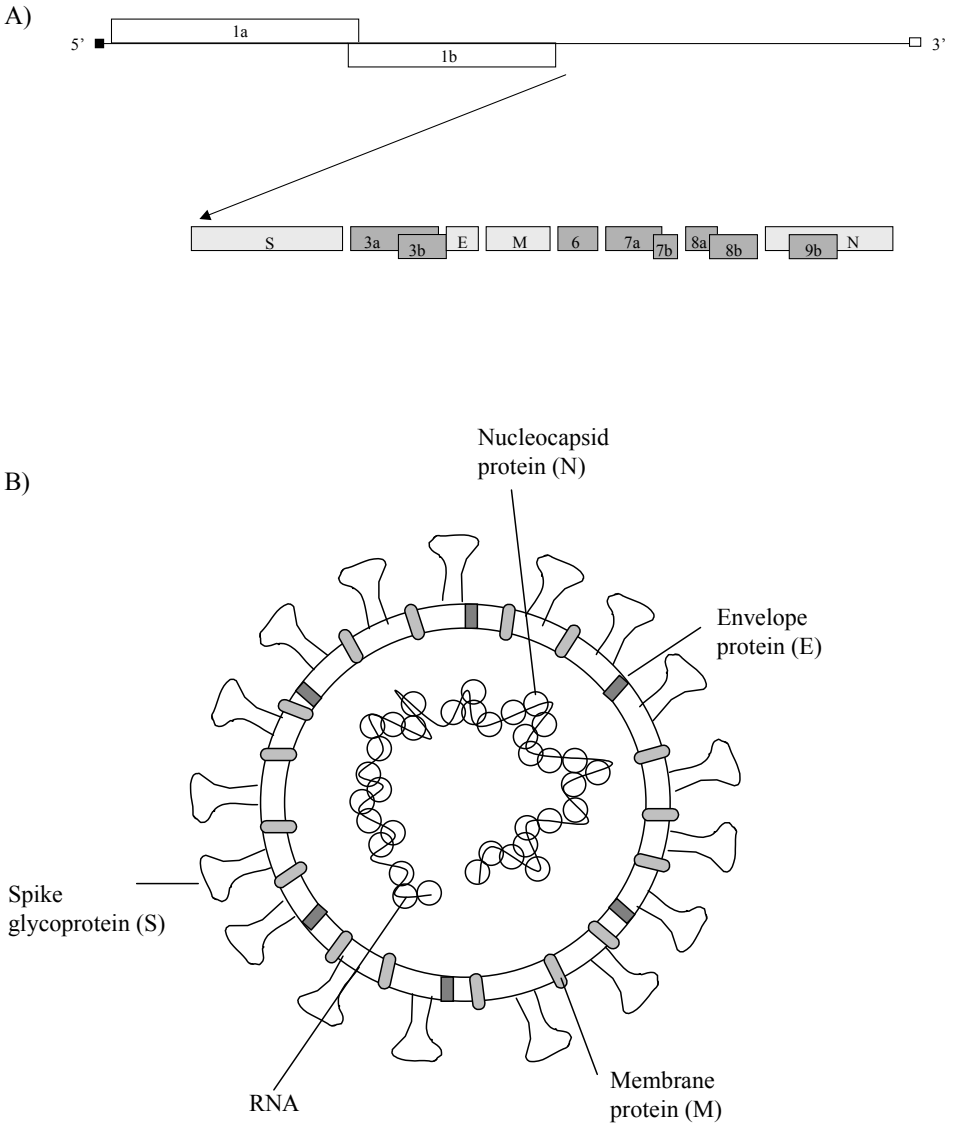

Figure 2. Schematic drawing of the SARS-CoV genome (A) and virion (B).

1.5 STRUCTURAL PROTEINS

The spikes on the envelope are composed of three monomers of viral S glycoproteins and mediate receptor binding and fusion of virus to host cell. The S protein of SARS-CoV is ~1200 amino acids (aa) in length (~180 kiloDalton (kDa)), and can be divided into two subunits, S1 and S2 [40]. The S1 domain at the N-terminal forms the head region containing a receptor binding domain (RBD) which is located close to the carboxy-terminal of S1 and is responsible for binding to the cellular receptor [41]. The main cellular receptor has been identified as the angiotensin-converting enzyme 2 (ACE2) for SARS-CoV [42]. However, liver/lymph node specific ICAM-3 grabbing non-integrin (L-SIGN) found on dendritic cells (DCs) can also function as an alternative receptor for SARS, but it has less receptor activity then ACE2 [43].

The S2 domain makes up the stalk region and is responsible for fusion of the virus envelope and the cell membrane [40,44]. Many coronaviruses are cleaved by furin-like proteases to create a S1 and a S2 unit, however the S glycoprotein of SARS-CoV is not cleaved by furin and do not seem to contain a furin-like cleavage site. The cleavage of S is most likely done by endosomal cathepsin L proteases but can also be cleaved by trypsin [45,46].

In the S2 region are two heptad repeat regions divided by an inter-helical domain suggested to assist in movement of the two heptad repeat regions to form a bundle of six helices seen in the post-fusion state [40,44].The transmembrane domain is followed by a short endodomain, both the transmembrane and endodomain are important for incorporation of S into virons [3,44]

S-palmitoylation is the attachment of 16-carbon fatty acid, palmitate, to cysteine by thioester linkage to cellular as well as viral proteins. The attachment of palmitate to proteins is catalyzed by palmitoyl acyl transferases (PATs). It is a reversible modification; the thioester linkage between the protein and the palmitate can be cleaved by palmitoyl thioesterases allowing palmitoylation and depalmitoylation of proteins. S-palmitoylation is involved in many protein functions such as modulation of protein-protein interactions, trafficking, membrane interaction and signaling [47-49]. There is no requirement for a specific consensus sequence motif for S-palmitoylation except presence of cysteine residues.

The S protein of SARS-CoV transmembrane and endodomain contain 9 cysteine residues. Compared to other coronaviruses 6 of the cysteine residues are quite well conserved [50]. S-palmitoylation of the cysteine residues in the endodomain of SARS-CoV proved to be important for S-mediated cell-cell fusion [50]. Previously the endodomain of murine hepatitis virus (MHV) was shown to be S-palmitoylated [51] and demonstrated to be important for production of infective virions as well as for interaction with the M protein for effective incorporation into virions [52]. The amino acid sequence of the SARS-CoV E protein display three cysteins shortly after the transmembrane domain in the carboxy terminal, and all three cysteins are S-palmitoylated, this modification could be important for protein-protein interaction. S-

palmitoylation of the E protein has previously been shown for MHV and infectious bronchitis virus (IBV) [53-55]. Substitution mutations of the cysteine residues in the carboxy domain of the MHV E protein affected viral production and stability but not entry or transport [56]. The removal of the S-palmitoylation sites in the cytoplasmic domain of human immunodeficiency virus (HIV)-1 envelope glycoprotein 160 (gp160) decreases viral infectivity and form viruses with low levels of incorporated gp160 [57].

The E protein is an integral membrane protein which together with the M protein is important for viral assembly and budding [53]. The protein is localized to the ER [58]. The E protein has been shown to alter membrane permeability in cells involving the transmembrane domain and form ion channels, which may be important for viral infection [53,59]. The M_2 protein of influenza A form proton ion channels important for viral uncoating and release [60]. It was demonstrated by reverse genetic techniques that E is not essential for the replication of SARS-CoV in various cell-lines [61]. However, the recombinant SARS-CoV lacking the E gene (rSARS-CoV-DeltaE) grew to lower titers than the recombinant wild-type virus, indicating that the E protein has an effect on virus growth. In addition, the rSARS-CoV-DeltaE virus is found to be attenuated in both hamsters and transgenic mice expressing the SARS-CoV receptor, human angiotensin-converting enzyme 2 (hACE2) [61,62], indicating that E is a virulence factor.

The M protein is ~23 kDa (221 aa), and the most abundant protein, and localized to the ER and Golgi compartments [63]. As mentioned above, M is important for assembly and budding of virions. The protein is N-glycosylated with three transmembrane domains, to the exterior of the virion is the amino terminal ectodomain, and to the interior of the virion is a large carboxy terminal domain [64]. It has been shown that 12 amino acids in the carboxy terminal is the minimal sequence for interaction between the M and the N protein [65]. The activity of the nuclear factor kappa B (NF-κB) has been shown to be suppressed by the M protein, most likely through the interaction with IκB kinase (IKK)β. The interaction was shown to reduce the translocation of NF-κB to the nucleus in Vero E6 cells [66]. Over expression of M has been reported to induce apoptosis in HEK 293T cells. The M protein seemed to down regulate phoshorylation of cellular Akt involved in the cell survival pathway. Consequently, the reduction of the cell survival signal result in induction of apoptosis [67].

The N protein is ~ 46 kDa (422 aa) and binds and encapsulates the genome forming a helical nucleocapsid [3]. The interaction of N with the carboxy terminal of the M protein has been shown to be necessary for formation of virus like particles (VLPs) [65,68]. Packaging of the viral RNA is an essential process in assembly, an RNA binding domain has been identified at the amino terminal region of the N protein important for packaging of the genome [69,70]. Many proteins both cellular and viral are cleaved by caspases [71], the N protein induces the intrinsic apoptotic pathway by activation of caspase 9 and de-phoshorylation of Bad. The N of SARS in infected and transfected cell lines was cleaved by caspase 6 as a result of the activation of caspase 9 and Bad, but the cleaveage was dependant on which cell line that was used [72]. The N protein of transmissible gastroenteritis coronavirus (TGEV) has previously been shown to be cleaved by caspase 6 and 7 [73].

1.6 ACCESSORY PROTEINS

The genome of SARS-CoV contains 14 potential ORFs, eight of those are specific for SARS and encodes for the accessory proteins [74]. Two of the accessory proteins, the 3a and 7a have been investigated to a greater extent and will be discussed separately but the knowledge about the function of most of the accessory proteins are still limited. The ORFs 3, 7, 8 and 9 are bicistronic and translate two proteins from each ORF, ORF 6 is monocistronic and only one protein is translated. All accessory proteins are not essential for SARS-CoV replication in cell culture or small animal models [75]. However, it is still not known if these proteins contribute to viral replication and/or pathogenesis in the natural host. It is also interesting to note that some of these proteins have been shown to be incorporated into the virion [76-79].

ORF 6 encodes a ~7.5 kDa protein (~63 aa) and has been detected in the lung and intestine of infected SARS patients as well as in infected Vero E6 cells. Antibodies have been detected in sera from convalescent patients [80,81]. In cell cultures the localization of ORF 6 has been observed in the ER but also in the cytoplasm in what looks like vesicles. The protein has been reported to increase virus growth in cells, enhance virulence, and speed up replication of a mouse coronavirus [82-85]. An interaction of ORF6 with nsp8 has been observed in SARS-CoV infected cells and is suggested to associate with the replication/transcription complex (RTC) [85].

ORF 8a and 8b protein are ~5.3 kDa (~39 aa) and ~9.6 kDa (~84 aa) respectively. There are an additional 29 nt in the ORF8 sequences isolated from animals resulting in fusion to a single protein (8ab) from the 8a and 8b protein which most of the human isolates lack [10]. From the early phase of the outbreak some human isolates of SARS-CoV contained the additional 29 nt whereas in the middle and late phase the 29 nt were deleted [10,16]. The presence of the additional 29 nt do not seem to have any implications for the virus, and civets are equally susceptible to SARS-CoV with or without the 29 nt [13,75]. The 8b has been shown to down-regulate the structural protein E in virus-infected cells [86].

ORF 9b is an alternative ORF within the N gene, ~11 kDa (~98 aa). Antibodies against this protein has been detected in sera from convalescent patients [80]. The structure of 9b has revealed the protein to be a lipid binding protein [87]. Sequence analysis has suggested the 9b protein to contain a motif acting as nuclear export signal [88].

1.6.1 3a and 3b

ORF 3 is located between the S and the E genes. SARS-CoV 3a is a minor structural O-glycosylated protein and the largest accessory protein with a length of 274 a.a [77,89]. The protein is expressed in infected cells, localized to the Golgi apparatus and can be transported to the cell surface where it also can undergo internalization [90]. The expression of 3a has been detected in a specimen from a SARS infected patient's lung. Also, antibodies towards the protein have been detected in sera from convalescent patients [80,81]. It has also been shown that antibodies towards the ectodomain of 3a are able to bind and kill 3a expressing cells with help from the complement system. [91]. The 3a protein has three transmembrane domains with an N-terminal ectodoamin

and a C-terminal endodoamin. The C-terminal contains two sorting motifs, a YxxΦ which is suggested to be involved in directing protein to different intracellular compartments, and the diacidic motif important for transport from ER [92]. The protein interacts with S, E, M and 7a, though demonstrations of co-expression of 3a with the structural proteins S, E, M and N in the incorporation into VLPs show that 3a is not required for formation of VLPs or virus assembly [70,78,93]. 3a can induce cell cycle arrest [94] and apoptosis via a caspase 8 dependant pathway in Vero E6 cells [95]. The expression of 3a in HEK 293T cells have been shown to activate the NF-κB promoter [96,97]. NF-κB and MAP kinases can regulate interleukin (IL)-8 which have been detected at elevated levels in plasma from SARS patients. The activity of the IL-8 promoter was enhanced by the expression of 3a in HEK 293T cells [96]. Reverse genetic studies show that the deletion of 3a resulted in about 1 log reduction of virus yield, suggesting that 3a can modulate viral replication [75].

The 3b protein is 154 aa long and has been reported to be localized mainly to the nucleus [98]. Antibodies against the protein have been detected in sera from a SARS patient [99]. The 3b protein has also been shown to induce apoptosis and cell cycle arrest at G0/G1 phase [94]. 3b has been shown to inhibit induction of IFN-β in 293T cells [98].

1.6.2 7a and 7b

ORF 7 encodes for two proteins, 7a and 7b.The 7a protein is 122 aa long, and one study suggest the 7a protein to be structural protein since the protein is incorporated into virus particles [79]. Antibodies towards 7a has been detected in convalescent sera [80], and the protein is expressed in infected cells and localized to the intermediate compartments [100]. The N-terminal contains a signal peptide and in the C-terminal an ER retrieval motif has been located, important for transport of proteins to the ER and recycling of 7a between ER and Golgi [100]. As mentioned above, the 7a protein can interact with 3a as well as with E and M [101] and with the S protein [79], but these interactions does not seem to be important for 7a incorporation into VLPs [79]. Over-expression of 7a can, via the caspase-dependent pathway, induce apoptosis in different cell lines derived from different organs. The induction of apoptosis was blocked when Bcl-X_L, an anti-apoptotic protein and a member of the Bcl-2 family was over-expressed, indicating interference of Bcl-X_L by 7a [102,103]. However, when reverse genetics was used to delete both 7a and 7b apoptosis was still seen in infected cells so it is not the only inducers of apoptosis during infection [104]. Over-expression of 7a has been shown to activate p38 mitogen-activated protein kinase (MAPK) [105]. The cellular protein small glutamine-rich tetratricopeptide repeat (SGT) has been reported to interact with 7a, the significance of this interaction is not known but could be important for viral interactions [101]. The transmembrane domain of the 7b protein has been shown to be essential for the localization of the protein to the Golgi compartment [106]. It has been reported that a mutant virus without the 7a/7b gene replicates as efficiently as the wild-type virus, indicating that 7a and 7b are dispensable for viral replication in cell culture and the mouse model [75].

1.7 REPLICATION

After the S protein has attached to the ACE2, the main receptor for SARS-CoV on the host cell, the virus is taken in by receptor-mediated endocytosis into endosomes [107]. The co-expression of ACE2 and the alternative receptor L-SIGN, mentioned above has been found in lung tissues from people who were infected with SARS-CoV and died [108].

In the endosomes the S protein is cleaved by acid-activated cathepsin L proteases [46,109]. The cleavage results in exposure of the fusion peptide in the S2 domain and the viral envelope and the cellular membrane fuses and the genome is released into the cell [44]. After the genome is released ORF1a and1b is translated into two polyproteins (pp) pp1a and pp1ab by a ribosomal frameshift into the –1 reading frame [74]. The polyproteins are autoproteolytically cleaved and processed by two proteinases (3CLpro and PLpro) encoded in ORF1a resulting in 16 nsps [110].

The function of some of the nsps has been identified. The nsp 1 can through the NF-κB pathway induce the chemokines CCL5, CXCL10 and CCL3 in human lung epithelial cells [111]. In SARS patients the level of certain chemokines are up-regulated and a dysregulation of chemokines could play a role in the pathogenesis of SARS [111]. Located in the nsp 3 domain is a papain-like accessory proteinase (PLpro) involved in processing of the polyproteins, together with protienase in nsp 5, a 3C-like main proteinase (3CLpro) [112]. RdRp resides in the nsp 12, necessary for replication of the genome. In nsp 13 is an RNA helicase, able to unwind both deoxyribonucleic acid (DNA) and RNA substrates [74,113]. Nidovirus uridylate-specific endoribonuclease (NendoU) is an enzyme conserved in all nidoviruses; *Coronaviriadae* being a family member of the order *Nidovirales*. Nendo U of SARS-CoV is located in nsp 15, and plays an important role in RNA synthesis [110,114].

The nsps form the RTC, critical for the replication of viral genome and transcription of the eight subgenomic mRNAs [74]. The replication of the genome takes place in the cytoplasm in what looks like double membrane vesicles (DMVs) suggested to be derived from the ER [115]. The RTC binds to the genome for replication and to transcribe the individual subgenomic RNAs (sgRNAs). All the sgRNAs have common 5’ and 3' sequences, the common 5’ sequence is derived from the 5’ end of the genome and is thought to be fused by discontinuous transcription (template switch) regulated by transcription-regulating sequences (TRSs) preceding each gene [74,116]. In order for the template switch to take place, base paring between the leader TRS and the newly formed minus RNA strand is required [74,117]. The base paring involves a conserved core sequence (CS) within the TRS which seem to be enough for directing transcription of subgenomic mRNAs, for SARS the CS is 5’ACGAAC 3’ [74].

The assembly of progeny virus involves packing of genome into viral particles, a procedure that involves interaction of the structural proteins and is taking place in the ER and Golgi compartments. The subgenomic mRNAs are translated into proteins, N protein binds to the genome and encapsulate it to become a nucleocapsid, while the S, E, M and 3a are directed to the ER and then transit to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). The nucleocapsid interacts with the rest of the

structural proteins in the ERGIC to form virions, which are transported in vesicles to the cell surface where they are released (Fig. 3) [3] .

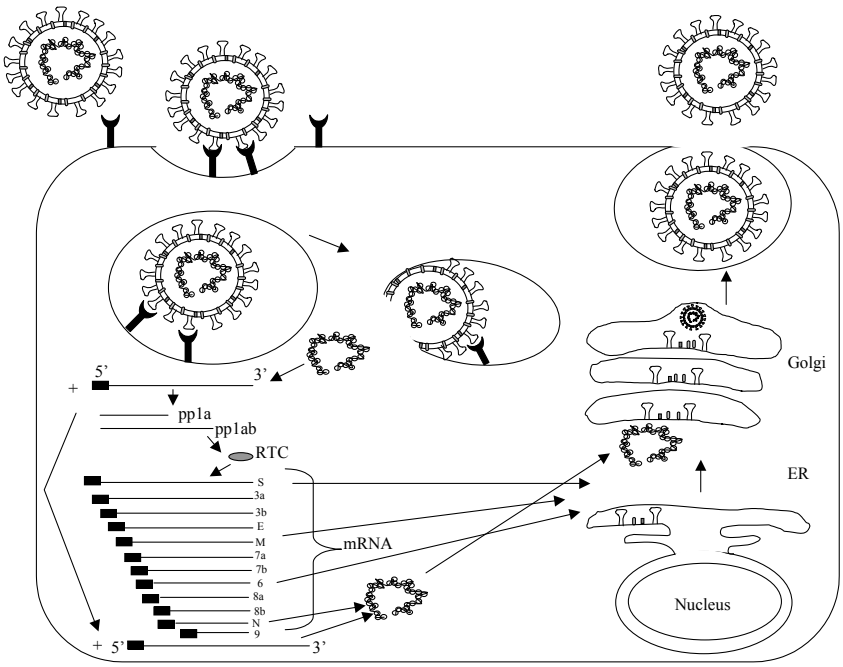

Figure 3. SARS-CoV replication cycle

2 PATHOGENESIS AND IMMUNE RESPONSE TO SARS COV

The pathogenesis of SARS-CoV is not fully understood, but suggested to be caused by direct injury of virus to target cells as well as causing injury by mediating immune system dysfunction [118].

2.1 INNATE IMMUNE RESPONSES

The main actors of the innate immunity are cytokines (pro-inflammatory, anti-inflammatory, chemokines, and interferons), NK cells, macrophages and DCs. They are the first line of defense after an infectious agent has breached the anatomic and physiological barriers (e.g skin, mucosa) [119]. Once a virus has entered a host cell, viral RNA and viral proteins can be recognized by pathogen recognition receptors (PRR) as pathogen-associated molecular patterns (PAMPs). The interaction initiates a signaling cascade that result in expression of cytokines known as interferons (IFNs) [120].

Production of IFNs is rapid and an essential part of the defense against virus infections. The IFNs can be divided into three types; type I consists of IFN-α with ~13 members and a single member of IFN-β, type II is represented by one member, IFN-γ, also important in the adaptive immunity. Type III contains three members of INF-λ [121-123]. Most cell types can produce type I IFNs while type II IFN is produced by T cells and NK cells [119].

Type I interferon (IFN-α, IFN-β) and type II interferon (IFN-γ) sensitivities have been shown for SARS-CoV when added exogenously both *in vitro* and *in vivo* [124-127]. According to one group the sensitivity towards IFN-β was much greater then for IFN-α or IFN-γ [125]. On the other hand another group showed that a combination of IFN-β and IFN-γ synergistically reduced replication in Vero E6 cells much more efficiently than either alone [128]. Nevertheless, SARS must somehow evade the IFN system in order to establish an infection. One way by which SARS-CoV is able to avoid the IFN system is by inhibiting the pathway of transcription factor interferon regulatory factor (IRF)-3. Viral infection or double stranded RNA (dsRNA) triggers kinases IKKε and TBK1 to phoshorylate IRF-3 residing in the cytoplasm. Upon phoshorylation IRF-3 is transported to the nucleus where it recruits coactivator protein histone acetyltransferases CREB-binding protein and p300, together activating the IFN-β promoter. However in SARS-CoV infected cells, IRF-3 has been observed in the nucleus early during infection but returns to the cytoplasm later in infection without activating the IFN-β promoter [129]. The viral factor inhibiting the IRF-3 pathway is unknown. For the influenza A virus the NS1 protein binds to dsRNA and consequently inhibits the IRF-3 activation [130]. For Ebola virus the inhibition of IRF-3 activation is due to the viral protein VP35 blocking the phosphorylation of IRF-3, thus inhibiting the translocation of IRF-3 to the nucleus for activation of the IFN-β promoter [131].

The transcription of the IFN-β promoter can also be activated by NF-κB. There are conflicting results regarding the initiation of NF-κB promoter activity *in vitro*. One group has reported a delayed activation of the NF-κB promoter in SARS-CoV infected HEK 293 cells stably expressing ACE2 [97]. While another study have reported no significant activation of NF-κB promoter in SARS-CoV infected 293 cells [132]. The inconsistency between the results might be due to the time at which they observed their results. The induction of NF-κB was observed at a late time point whereas the other study examined activation at an earlier time point post-infection.

Macrophages and DCs are two important cell types that secrete a range of cytokines, present antigens and are involved in phagocytosis in order to regulate the activity of innate and adaptive responses. A wide range of viruses affects macrophages and DCs in different ways contributing to pathogenesis seen in the host. Hence, widespread studies have been performed in order to look at the interplay between different viruses and macrophages or DCs in order to shed a light on viral infections involved in pathogenesis. For example, macrophages and DCs are able to mediate the spread of HIV to T helper (T_H) cells in lymphnodes [133].

SARS-CoV infection of macrophages and DCs has been reported by several groups to be non-productive [134-136]. However, one group demonstrated a low production of infectious virus in DCs [137]. Despite a non productive infection, certain cytokines and chemokines are produced such as IL-6 and IL-12, interferon induced protein of 10 kDa (IP-10), monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α) [7,136,138]. Though SARS-CoV infection was not able to induce IFN-α or IFN-β in macrophages or DCs, although another coronavirus HCoV-229E strongly induced IFN-β in macrophages [134,135]. Demonstration of induction of chemokines in an early stage in SARS-infected macrophages, as well as DCs showed an up-regulation of chemokines in immature DCs. The up-regulation of IP-10 and MCP-1 gene expression was strongest [134,138].

Analysis of cytokine and chemokine levels in serum and lung tissues from SARS patients demonstrate no increased levels of tumor necrosis factor (TNF)-α, IL-1α or type I IFN. The levels of IP-10, MCP-1, IL-6 and IL-8 in particular were up-regulated as was seen in infected macrophages and DCs except for IL-8 [7,139]. However, one group has reported a decrease in level for IL-8 in serum from infected patients [139]. Conflicting reports regarding elevated levels of IFN- γ have also been reported [139-142]. In fatal cases, increased levels of IL-8, monokine induced by IFN-γ (MIG), IP-10 and MCP-1 have been reported [7]. Influenza subtype H5N1 can also cause severe lung injury similar to SARS-CoV. Dysfunction of cytokines and chemokines has also been suggested to play a key role in the mechanism in the pathogenesis of H5N1. Cytokine and chemokine levels in patients infected with influenza H5N1 show an increase of IP-10, MCP-1 and IL-8, particularly in fatal cases as seen for SARS-CoV. However, increased levels of TNF-α are seen in infected patients with H5N1 but not in infected SARS patients [143,144].

It has been reported that viral replication is enhanced and prolonged in aged mice compared to that in young mice. Old mice also display indication of clinical illness and pneumonitis not seen in young mice [145]. One group has compared gene expression

from lungs of SARS-CoV infected young and old mice. High virus titers were observed at days 1 and 2 in young mice and at days 2 and 5 in aged mice [146]. During viral peak a down regulation of genes associated with cellular development, cellular growth and cell cycle were seen in young mice. However, when the virus was cleared the level of the regulatory genes returned to normal [146]. Aged mice showed an up regulation of cell-cell signaling and immune response genes. After viral clearance many genes were still up regulated [146].

2.2 ADAPTIVE IMMUNE RESPONSES

The adaptive immune response consists of the humoral response (antibodies) and the cell mediated response (effector T cells). In the humoral response, mature B cells expresses antibodies on its membrane. When membrane bound antibodies bind to antigens the cell divide and the new cells differentiate into memory B cells and plasma cells. Memory B cells can live for a long time, and can help in eliciting a faster humoral response next time the antibodies encounter the same antigen. Plasma cells secrete antibodies that can neutralize or help in the elimination of antigens [119]. Responsible for the cell mediated responses are the effector T cells generated in response to cytokines produced in response to antigens. T cells expressing CD4 on their surface generally function as T_H cells while CD8 expressing T cells generally function as cytotoxic T cells (CTLs). T_H cells recognize only antigens that are displayed by MHC class II molecules displayed on the surface of antigen presenting cells (APCs), such as DCs, macrophages and B cells. T_H cells can differentiate into a few different subsets of T_H cells; T_H1 and T_H2 being the two most well known. Most of the cytokines secreted by T_H 1 differ from those cytokines secreted by T_H 2. To simplify, one could say that T_H1 stimulates pro-inflammatory responses while T_H2 stimulates antibody response. CTLs recognize cells with antigens displayed by MHC class I. Memory T cells be generated by naïve T cells or effector T cells. T_H can activate different phagocytic cells by secreting cytokines while CTLs can kill virus-infected cells [119].

It appears as if both $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, NK, and DC cells are reduced during the acute phase of SARS.

A strong humoral immune response is elicited towards the N protein but without neutralizing properties. High prevalence of antibodies has been seen in sera from convalescent patients [99,147] and almost all patients have seroconverted by day 14 after onset of illness with serum IgM, IgG and IgA antibody responses against SARS-CoV. IgM and IgA antibodies against the N protein could be detected in patient sera as early as 2 days after onset of illness [147], and by day 4 after onset of illness IgG antibodies could be detected.

The S protein is highly immunogenic and elicits important neutralizing antibodies; three main domains have been localized as targets for neutralizing antibodies one is located in a region close to the carboxy terminal of the S protein, another one is located in the RBD, and one in the second heptad repeat regions [148]. All studies conducted in animal models indicate that S neutralizing antibodies play a vital role in the protective

immunity in those animal models [149-152]. S specific neutralizing antibodies has also been shown to persist for 1 year after onset of symptoms [153].

Antibodies against the 3a protein has been detected in sera and from convalescent SARS patients, and in tissues from infected patients [80]. Antibodies towards 3a have been studied in this thesis for their possible neutralizing properties against SARS-CoV in cell culture [154]. In sera from convalescent patients, antibodies towards all accessory proteins have been detected indicating that they could be expressed during infection *in vivo* [80,81,147].

So far, memory T cell responses to all four structural proteins have been documented. Memory T cells specific for the S and M protein have been observed to persist for over one year in recovered SARS patients [153,155-157]. E and N generating memory T cells have been observed to persist for two years [155,158].

3 NITRIC OXIDE

Another molecule used by the innate immune system is nitric oxide (NO). Numerous cells generate NO, an important signaling molecule involved in a wide range of processes. It can act as a vascular relaxing agent, a neurotransmitter and its antimicrobial activity have been described for numerous bacteria, protozoa and for some viruses. NO is a free radical with an unpaired electron but is not highly reactive in itself. However, NO can react rapidly with other free radicals with unpaired electrons forming "reactive nitrogen oxide species" (RNOS) [159,160]. Superoxide anion (O_2^-) and NO are considered to be the two most relevant radicals produced by the host during an infection [161]. But rather than NO or O_2^- being involved in the pathogenesis of various infectious diseases it seem to be due to the formation of nitrogen intermediates such as peroxynitrite ($ONOO^-$) [161]. Peroxynitrite is formed when NO reacts with O_2^- concurrently produced during an infection. At low production of peroxynitrite the oxidative damage to cells can be diminished by endogenous antioxidant defenses such as superoxide dismutase (SOD). Only a slight increase of both NO and O_2^- can lead to high output of peroxynitrite, potentially harmful both to virus and host cell [162].

NO is produced by three types of nitric oxide synthase (NOS) enzymes that catalyze the oxidation of L-arginine to NO and L-citrulline. Two of the enzymes are constitutively expressed and are calcium-dependent thus only activated in response to calcium signal. They were first characterized in neuronal and endothelial cells. Therefore the enzymes were named neuronal NOS (nNOS) and endothelial NOS (eNOS) but are now renamed NOS-1 and NOS-3 respectively since they are more broadly distributed. The production of NO by NOS-1 and NOS-3 is rapid and momentarily at low levels. The third enzyme is called inducible NOS (iNOS) or NOS-2 and is expressed only in activated cells and is calcium independent and therefore constantly active once it is expressed. The production of NO by NOS-2 is slower but produced for a longer time at larger amounts [160,163]. The up-regulation of NOS-2 is common during an infection and some viruses and bacteria are known to either be stimulated or inhibited by increased levels of NO. During a virus infection the induction of NOS-2 can be mediated by cytokines or by direct up-regulation by the virus. Both IL-1 and TNF-α are known to induce NOS-2. However, the T_H1 related cytokine IFN-γ is a major inducing NOS-2 cytokine. Other cytokines can down-regulate NOS-2 expression, like T_H2 associated IL-4, IL-10 and transforming growth factor (TGF)-β [163]. Direct up-regulation of NOS-2 expression has been seen for rotavirus, respiratory syncytial virus and HIV-1 [164-167].

High levels of NO output could be part of the pathogenic consequences of various diseases [162]. NO has been implicated to contribute in the pathogenesis of influenza virus-induced pneumonia [168]

NO has also been reported to have an antiviral effect on both DNA and RNA viruses, like Epstein-Barr virus (EBV) [169], Japanese encephalitis virus (JEV) [170], Crimean Congo hemorrhagic fever virus (CCHFV) [171], influenza virus [172] herpes simplex virus type 1 [173], coxsackievirus [174] and hantaviruses [175]. The mechanism behind the antiviral effect of NO is at present unknown. Still, there are a few reports that have

established the inhibition mechanism of NO. NO has been shown to inhibit the enzyme activity of the viral protease $3C^{Pro}$ by S-nitrosylation of the cysteine residues in the active site of the viral protease. $3C^{Pro}$ is critical for the viral life cycle of coxsackievirus [176]. The immediate early transactivator protein Zta is down regulated by NO which helps to maintain latency of EBV [169]. NO has been shown by indirect mechanism to reduce palmitoylation of rat myelin proteolipid protein [177].

In vivo models have shown the importance of NO in resistance towards a number of viruses. Mice infected with murine Friend leukemia virus, treated with the NO inhibitor L-NAME showed an increase in viral load in spleen cells as compared to control mice [178]. In another study, A/J mice, resistant to the coronavirus Murine hepatitis virus strain 3 (MHV-3), were treated with the NO inhibitor N^{G}-monomethyl-D-L-arginine (L-NMMA). Infection with MHV-3 resulted in 50% mortality and with liver pathology seen in MHV-3 infected BALB/cJ mice, which develop fulminant hepatitis [179]. Genetically deficient $NOS2^{-/-}$ mice infected with hantavirus showed higher viral titers compared to control mice, suggesting NO inhibit viral replication *in vivo* [175].

Inhalation of NO was approved by the US Food and Drug Administration 1999, and in 2001 by the European Medicine Evaluation Agency and European Commission [180]. Endogenous and exogenous produced NO results in an overabundance of responses in the lung, such as vasorealaxation and bronchodialation. The use of inhaled NO has resulted in improved oxygenation in newborn babies with persistent pulmonary hypertension. Pulmonary vasoconstriction was improved in lamb by inhalation of NO resulting in pulmonary vasodilatation [180]. In patients with acute respiratory distress syndrome (ARDS), treatment with inhaled NO improved oxygenation and reduced pulmonary arterial hypertension [181]. It was also tried in one patient with hantavirus pulmonary syndrome (HPS) and resulted in improved oxygenation [182]. Inhalation of NO has been tried in only a few patients diagnosed with SARS-CoV but with a favorable effect [183].

4 SPECIFIC AIMS

- To study the role of SARS-CoV accessory proteins, 3a/3b and 7a/7b in the replication cycle *in vitro* (Paper I).

- To compare the neutralizing properties of antibodies towards the ectodomain and the endodomain of the 3a protein (Paper II).

- To investigate the antiviral effect of nitric oxide against SARS-CoV (Paper III).

- To study the antiviral mechanism of the inhibition of the replication cycle by nitric oxide (Paper IV).

5 RESULTS AND DISCUSSION

5.1 INHIBITION OF SARS-COV REPLICATION CYCLE BY SMALL INTERFERENCE RNAS SILENCING SPECIFIC SARS PROTEINS, 7A/7B, 3A/3B AND S (PAPER I)

Small interfering RNA (siRNA) mediated knockdown is a process by which dsRNA can silence gene expression. Usually, siRNA is a 19-21 nt of dsRNA where one strand of RNA is complementary to the mRNA of interest. The double stranded siRNA unwind and one strand is incorporated into a protein complex called the RNA induced silencing complex (RISC). This single strand guides the RISC to the complementary target mRNA. RISC cleaves the target mRNA after the siRNA have base-paired with the target mRNA resulting in gene silencing [184-186].

Numerous groups have demonstrated inhibition of SARS-CoV in cell culture by using siRNAs designed to target different regions of the SARS-CoV genome. However most groups designed siRNAs targeting the first 2/3 of the genome and not the accessory proteins [187-191]. In vivo experiments using siRNA have been performed. In Rhesus macaque, siRNA targeting S and nsp 12 has shown antiviral effect without adverse effects when administered intranasal [192].

In this study we used siRNAs to investigate the importance of SARS-CoV 7a/7b and 3a/3b protein in the replication cycle. We designed three siRNAs targeting sgRNAs 2, 3 and 7, expressing the S, 3a/3b and 7a/7b protein respectively. The siRNAs were designed based on the differences in the junction between the CS and the sequence of the different ORFs. This design would exclude any effect on the translation of the full-length genomic mRNA. In order to achieve the best differentiation between the different sgRNAs, the sequence matching the CS was flanked by 5-7 nt before and 6-10 nt after each of the siRNA inserts. Vero E6 cells transiently transfected with siRNA or stable cell-lines expressing siRNA were infected with SARS-CoV. The yield of progeny virus was significantly reduced by all three siRNAs (Paper I). However, the reduction of progeny virus was more noticeable in cells transiently transfected with siRNAs compared to the stable cell-lines. The level of siRNA observed could explain this result. The levels of siRNA present were higher in cells transiently transfected with siRNA than in the stable cell-lines. The progeny virus was somewhat more reduced for the silencing by siRNA 7. This could be due to the fact that siRNA 7 was shown to silence both accessory proteins 7a/7b and 8a/8b due to a mismatch of only 1 nt between siRNA 7 and sgRNA8. This could also explain why a group using reverse genetics to create a recombinant virus lacking 7a/7b did not see a difference in virus yield compared to wild-type virus. However, they did see a reduction of virus yield from deletion of 3a [75].

Evaluation of the knockdown efficiency of the proteins translated from the sgRNAs targeted by the siRNAs was performed. The vectors contain a green fluorescence protein (GFP) marker under the CMV promoter facilitating tracking of transfected cells. Indirect immunofluorescence assay (IFA) showed that cells expressing the

siRNAs specifically silenced the expression of targeted proteins without affecting the infection shown by expression of the N protein (Paper I).

5.2 AMINO ACID 15-28 IN THE ECTODOMAIN OF SARS CORONAVIRUS 3A PROTEIN INDUCES NEUTRALIZING ANTIBODIES (PAPER II)

Humoral responses are a significant part of the immune response against viruses. Neutralizing antibodies can block virus from binding to and entering cells, and are an important part of the adaptive immunity. Antibodies towards 3a have been detected in SARS patients, and 3a has also been detected in tissues from infected patients. 3a has been shown to be a minor structural protein with three transmembrane domains, with an N-terminal ectodomain and a C-terminal endodomain.

In this study we used antibodies raised towards amino acids 15-28 in the ectodoamin, and antibodies raised towards amino acid 134-274 in the endodomain of 3a to investigate and compare potential neutralizing properties. Western blot showed that both antibodies towards the N-terminal and the C-terminal could detect 3a in SARS-CoV infected cells (Paper II). Neutralization assay showed a neutralizing titer of 1:80 for anti-3a N-terminal antibodies in the absence of the complement system. In contrast, no neutralizing properties were shown for anti-3a C-terminal antibodies. However the binding affinity was stronger for antibodies towards the C-terminal than for antibodies towards the N-terminal (Paper II). Our results suggest that antibodies targeting the N-terminal of 3a could probably stimulate a protective humoral response during SARS-CoV infection. It has been reported that 48.8 % of patients recovering from SARS displayed antibodies towards the N-terminal of 3a as compared to only 7.4% of deceased persons [91]. In another study, 40% of plasma samples from convalescent SARS patients showed antibodies against the N-terminal of 3a [193]. With help from the complement system, antibodies against the ectodomain of 3a are also able to bind and kill 3a expressing cells [91]. Several groups have studied the prophylactic effect of passive transfer of antibodies towards different epitopes of the S protein against challenge with SARS-CoV in mice. The passive transfer of antibodies did provide an effective immunoprohylaxis in mice [151,194,195].

Both a strong humoral and cellular response in humans seems to be positively related with a less severe disease and survival. However, the relative contribution of the cellular and the humoral immune responses are not known. Antibodies against 3a could be a good complement to neutralizing antibodies against the S protein and anti-N antibodies in protection against SARS.

5.3 NITRIC OXIDE INHIBITS THE REPLICATION CYCLE OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS (PAPER III)

Nitric oxide is involved in a wide range of processes and is an important signaling molecule between cells. NO has been described to have an antiviral activity against some viruses, and an up-regulation of NOS-2 is common during an infection. Some viruses can be inhibited whereas other viruses can be stimulated by increased levels of NO.

We used both exogenous and endogenous NO to investigate the antiviral effect on SARS-CoV. After Vero E6 cells had been infected with SARS-CoV, an exogenous NO donor called S-nitroso-N-acetylpenicillamine (SNAP) or the control, N-acetylpenicillamine (NAP) was used to treat the cells. The amount of progeny virus for SNAP-treated cells was reduced by almost 4 log compared to NAP-treated cells (Paper III). The inhibitory effect of NO was also demonstrated by Western blot and IFA (Paper III). A real time PCR was performed showing that NO inhibit viral RNA. To investigate the effect of NOS-2, cells were stimulated with IL-1β and IFN-γ together with L-NMMA, a NOS-2 inhibitor to confirm that NO production was mediated by NOS-2. The inhibitory effect of NOS-2 corresponded to approximately the same level of inhibition seen with 50μM SNAP. L-NMMA did inhibit the production of NOS-2 to a great extent. However, a reduction of virus titer was still observed. This observed inhibition could probably be due to an incomplete inhibition of NOS-2 by L-NMMA as shown in paper III; hence some NO could still be produced to inhibit the virus.

NO can be both harmful and protective. Elevated levels of NO have been suggested for some viruses to play part in pathogenesis. It is not known whether SARS patients show elevated levels of NO. It would be interesting to examine whether NOS-2 is part of the antiviral response against SARS-CoV by infecting $NOS2^{-/-}$ mice. Most studies have shown an increase in viral replication in $NOS2^{-/-}$ mice [175,178,179]. Inhalation of NO has been approved as a treatment both in the U.S and in Europe [180]. This method has been tried in patients with ARDS, in newborn babies with persistent pulmonary hypertension and in one patient with HPS with positive outcomes [182]. Inhalation of NO has been tried in a small group of SARS patients with a favorable effect [183]. Arterial oxygenation and clinical signs were improved as well as chest radiograph findings. Perhaps inhalation of NO could be used as a complementary treatment if SARS were to appear again.

5.4 NITRIC OXIDE INHIBITS SARS COV REPLICATION DIRECTLY AND DECREASES THE FUSION ACTIVITY OF THE SPIKE PROTEIN (PAPER IV)

NO has been shown to have an antiviral effect on some viruses. In addition to NO, O_2^- is also produced during an infection. When NO and O_2^- interact with each other peroxynitrite ($ONOO^-$) is formed. Peroxynitrite has also been shown to have an inhibitory effect on viruses.

In this study we further investigated the antiviral mechanism behind NO on SARS-CoV. We also investigated whether NO per se inhibits SARS-CoV or if peroxynitrite could be involved. We used the chemical 3-Morpholinosydnonimine hydrochloride (SIN-1) producing peroxynitrite, and a superoxide scavenger MnTBAP. SIN-1 had no effect on the replication of SARS-CoV (Paper IV). Infected cells were treated with SIN-1 together with different concentrations of MnTBAP. SIN-1 produces both NO and O_2^-, which react to form peroxynitrite. The use of MnTBAP in combination with SIN-1 removes the superoxide. As a result the amount of NO is increased, and the amount of peroxynitrite is decreased. Different concentrations of MnTBAP were used together with SIN-1 which resulted in a decrease of progeny virus. The inhibition of progeny virus was dependent on the concentration of MnTBAP (Paper IV). The results show that NO inhibits SARS-CoV directly, and that peroxynitrite has no effect on SARS-CoV replication. Interestingly for hantavirus, both NO and peroxynitrite showed an inhibitory effect, but on different parts of the replication cycle. NO showed an antiviral effect on the replication *in vitro*, while peroxynitrite had little effect. The opposite was observed for the effect on free viruses, where peroxynitrite showed a strong antiviral effect compared to NO [175]. NO was not observed to have any antiviral effect on free viruses of SARS, though we did not try the effect of peroxynitrite, and can not state whether what was observed for the hantavirus applies to SARS-CoV.

The S protein involved in receptor binding and membrane fusion, undergoes posttranslational modifications in order to attain proper functionality. S-palmitoylation of the endodomain of the S protein has been shown to be important for mediating cell fusion. S-palmitoylation is a posttranslational process by which palmitate is added to cysteine residues through thioester linkage.

After establishing the direct effect of NO on SARS-CoV replication, we examined whether NO nitrates SARS-CoV S protein. Vero E6 cells were infected with a recombinant vaccinia virus carrying the S gene (rVV-L-S), and treated with SNAP (NO donor) or the control NAP. Immunoprecipitation was performed using beads specifically binding to nitrated proteins. Detection of S by western blot analysis showed that S had been nitrated after stimulation with SNAP. Since S-palmitoylation of S was shown to be important for mediating cell fusion, we wanted to determine whether NO might have any effect on the S-palmitoylation of the S protein. The results showed that NO reduced S-palmitoylation of the S protein to a great extent, and subsequently also reduced S-mediated cell-cell fusion as shown by a membrane fusion assay (Paper IV).

We demonstrated that one antiviral mechanism of NO is to affect the post-translational modification of the S protein of SARS-CoV. The structural protein E has also been shown to be S-palmitoylated for both SARS-CoV and MHV. It was shown for MHV that the viral production was affected when the cysteine residues in the carboxy domain were substituted. It would be interesting to investigate whether NO also affects the S-palmitoylation of E.

6 ACKNOWLEDGEMENTS

Many people have contributed to the work and I would especially like to thank:

Ali Mirazimi, my supervisor for taken me on as a student. For sharing your knowledge with me, and teaching me a lot about science. For your generosity, and your encouragements. For always have been there when I needed you, and for being so patient with me even though I am to quick sometimes.

Yee-Joo Tan, my co-supervisor for good advice, teaching me new methods, and for your hospitality during my stay in Singapore.

Åke Lundkvist, my co-supervisor who suggested I started to work on SARS-CoV for Ali. For good comments, and for always encouraging me before presentations.

Former and present heads of the department **Gunnar Sandström, Johan Struwe, Anders Tegnell,** for letting me conduct research at KCB.

All the **co-authors**

Helen Karlberg, for brightening up the long days at work. For your support, and consideration. For being a great friend at the office and outside. For trusting my sense of stroller friendly pathways. **Ida Andersson,** for all the long hours together in P4. For being a friend, and thankfully keeps calm when I once in a while scream NEJ! over the radio. For sharing your wonderful pantomime performances. **Jonas Hardestam,** for all the fun at and outside the office. For sharing all your interesting hobbies, and for always remembering my birthday. **Jonas Klingström,** for your endless stream of new ideas, funny facts and for always being in a happy mood.

Melinda Simon, for all the peptalking, and great tasting pastries. **Vithia Gunalan,** for our SARS discussions, and for that thing with V and amp. **Monika Hammarberg,** for always taking time to help me with anything and everything I might need. **Mikael Nilsson,** for keeping me safe in the P4 lab, and for all the interesting discussions. **Gunnel Lindegren,** for your down to earth spirit, good luck with your licentiate.

Lindvi Gudmundsdotter, a great roommate and friend. For fun cooking, magnificent dinner parties, and port wine. For always willing to help me out. **Susanne Johansson,** a patient friend who is never getting tired of answering and explaining all the immunological questions I ask you. When are we going to the West Indies again? Next time I will take a later bus to the airport. **Malin Karlsson,** for all the fun when making Christmas candies. You are the only one I know to make liquid mint kisses.

Andreas Bråve, for great discussions, fabulous partying, and for luring out Shannon at special occasions. **Kristian Hallermalm,** for giving good advice, and a helping hand whenever needed. **Andreas Boberg,** for our brief collaboration, and introduction to Elispot. **Andreas Mörner,** for great partying. **David Hallengärd,** for contributing to a

good atmosphere in the office. **Gunnel Engström,** for your tips about tulips and amaryllis.

All present and former colleagues at KCB and VZO. **Anne-Marie Connelly Andersen,** for budding up with me in P4 with short notice. **Mehrdad,** for helping me with RT-PCR. **Cecilia Johansson,** for always being updated on everytning imaginable. **Annette Kraus,** for always being enthusiastic and encouraging ever since the first day you moved into the office. **Marie,** for all fun discussions. **Elisabeth Gustafsson,** for being a fun roommate at numerous conferences. **Sirkka Vene,** for your great knowledge about so many things. **Kerstin Falk,** for nice company at the Steamboat conference. **Malin S,** for having as heavy eyelids as I do. **Jolle,** for entertaining company in Steamboat. **Maria, Sofia, Karin, Angerd Berndtson, Kirill, Kjell-Olof Hedlund, Kicki, Sofia, Henrik, Tuija, Sándor, Sven Löfdahl, Talar, Tara, Linda B, Linda T, Benjamin, Daniel,** for creating a good atmosphere in the office.

The Lab in Singapore
Choong Tat, for a great tasting steamboat, and for introducing me to all kinds of dishes I would have missed while being in Singapore. **Sujit,** for showing me and accompany me at new eating-places, and for showing me short cuts with the possibility of encountering spitting cobras. **Yook Wah,** for showing me the "proper" way to eat with shop sticks. For filling in any missing dishes Choong Tat might have missed. **Timothy,** for introducing me to Laksa

Joo-Cheng Tan for showing me all the good shopping places in Singapore.

Patricia Burge and Dick Vessel, for all wonderful times at the Lost Creek ranch in Colorado. For all your support and big-heartedness, I have experienced some unforgettable memories together with you. Pat, I wish I had half the energy you have. **Xiaoyan,** for your support thousands of miles away. For your generosity, all the fun and for being a great friend.

Helene, what should I do without you. **Peter,** for the time we all lived together at 202 Maryland Ave in D.C. I miss our lunches. **Charlotte,** for always making me feel welcome at your home. **Jenny G,** for offering me a well needed sanctuary on a horseback, and for all the fun over the years. Keep an eye on the oat bag. **Krille,** for all your pranks. **Jenny J,** and **Jon,** for fun trips to castles, tasting evenings with themes from different countries, and for keeping the whiskey cabinet ful. **Jesper,** for being my personal banker.

My mother and father, for all the love and support during the years.
My brother **Jimmy** his wife **Iwona** and their kids **Isabella** and **Kristofer**. You will all soon be invited to my place.

7 REFERENCES

1 Pyrc, K., Berkhout, B. & van der Hoek, L. Identification of new human coronaviruses. *Expert Rev Anti Infect Ther* 2007, **5**(2), 245-253.

2 Rota, P.A., Oberste, M.S., Monroe, S.S. *et al.* Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003, **300**(5624), 1394-1399.

3 Masters, P.S. The molecular biology of coronaviruses. *Adv Virus Res* 2006, **66**, 193-292.

4 McIntosh, K., Dees, J.H., Becker, W.B., Kapikian, A.Z. & Chanock, R.M. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc Natl Acad Sci U S A* 1967, **57**(4), 933-940.

5 McIntosh, K., Kapikian, A.Z., Turner, H.C., Hartley, J.W., Parrott, R.H. & Chanock, R.M. Seroepidemiologic studies of coronavirus infection in adults and children. *Am J Epidemiol* 1970, **91**(6), 585-592.

6 Woo, P.C., Lau, S.K., Chu, C.M. *et al.* Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* 2005, **79**(2), 884-895.

7 Chen, J. & Subbarao, K. The Immunobiology of SARS*. *Annu Rev Immunol* 2007, **25**, 443-472.

8 Shi, Z. & Hu, Z. A review of studies on animal reservoirs of the SARS coronavirus. *Virus Res* 2007.

9 Zhong, N.S., Zheng, B.J., Li, Y.M. *et al.* Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *Lancet* 2003, **362**(9393), 1353-1358.

10 Guan, Y., Zheng, B.J., He, Y.Q. *et al.* Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* 2003, **302**(5643), 276-278.

11 Song, H.D., Tu, C.C., Zhang, G.W. *et al.* Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. *Proc Natl Acad Sci U S A* 2005, **102**(7), 2430-2435.

12 Wang, L.F., Shi, Z., Zhang, S., Field, H., Daszak, P. & Eaton, B.T. Review of bats and SARS. *Emerg Infect Dis* 2006, **12**(12), 1834-1840.

13 Wu, D., Tu, C., Xin, C. *et al.* Civets are equally susceptible to experimental infection by two different severe acute respiratory syndrome coronavirus isolates. *J Virol* 2005, **79**(4), 2620-2625.

14 Kan, B., Wang, M., Jing, H. *et al.* Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in

palm civets at an animal market and on farms. *J Virol* 2005, **79**(18), 11892-11900.

15 Li, W., Shi, Z., Yu, M. *et al.* Bats are natural reservoirs of SARS-like coronaviruses. *Science* 2005, **310**(5748), 676-679.

16 Lau, S.K., Woo, P.C., Li, K.S. *et al.* Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* 2005, **102**(39), 14040-14045.

17 Ren, W., Li, W., Yu, M. *et al.* Full-length genome sequences of two SARS-like coronaviruses in horseshoe bats and genetic variation analysis. *J Gen Virol* 2006, **87**(Pt 11), 3355-3359.

18 Woo, P.C., Lau, S.K., Li, K.S. *et al.* Molecular diversity of coronaviruses in bats. *Virology* 2006, **351**(1), 180-187.

19 Poon, L.L., Chu, D.K., Chan, K.H. *et al.* Identification of a novel coronavirus in bats. *J Virol* 2005, **79**(4), 2001-2009.

20 Tang, X.C., Zhang, J.X., Zhang, S.Y. *et al.* Prevalence and genetic diversity of coronaviruses in bats from China. *J Virol* 2006, **80**(15), 7481-7490.

21 Lam, C.W., Chan, M.H. & Wong, C.K. Severe acute respiratory syndrome: clinical and laboratory manifestations. *Clin Biochem Rev* 2004, **25**(2), 121-132.

22 Cheng, V.C., Lau, S.K., Woo, P.C. & Yuen, K.Y. Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin Microbiol Rev* 2007, **20**(4), 660-694.

23 Choi, K.W., Chau, T.N., Tsang, O. *et al.* Outcomes and prognostic factors in 267 patients with severe acute respiratory syndrome in Hong Kong. *Ann Intern Med* 2003, **139**(9), 715-723.

24 Ding, Y., He, L., Zhang, Q. *et al.* Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. *J Pathol* 2004, **203**(2), 622-630.

25 Gu, J., Gong, E., Zhang, B. *et al.* Multiple organ infection and the pathogenesis of SARS. *J Exp Med* 2005, **202**(3), 415-424.

26 Ye, J., Zhang, B., Xu, J. *et al.* Molecular pathology in the lungs of severe acute respiratory syndrome patients. *Am J Pathol* 2007, **170**(2), 538-545.

27 Nicholls, J.M., Poon, L.L., Lee, K.C. *et al.* Lung pathology of fatal severe acute respiratory syndrome. *Lancet* 2003, **361**(9371), 1773-1778.

28 Hsu, L.Y., Lee, C.C., Green, J.A. *et al.* Severe acute respiratory syndrome (SARS) in Singapore: clinical features of index patient and initial contacts. *Emerg Infect Dis* 2003, **9**(6), 713-717.

29 Xu, R.H., He, J.F., Evans, M.R. *et al.* Epidemiologic clues to SARS origin in China. *Emerg Infect Dis* 2004, **10**(6), 1030-1037.

30 Prevalence of IgG antibody to SARS-associated coronavirus in animal traders--Guangdong Province, China, 2003. *MMWR Morb Mortal Wkly Rep* 2003, **52**(41), 986-987.

31 Reilley, B., Van Herp, M., Sermand, D. & Dentico, N. SARS and Carlo Urbani. *N Engl J Med* 2003, **348**(20), 1951-1952.

32 Chan, K.H., Poon, L.L., Cheng, V.C. *et al.* Detection of SARS coronavirus in patients with suspected SARS. *Emerg Infect Dis* 2004, **10**(2), 294-299.

33 Peiris, J.S., Guan, Y. & Yuen, K.Y. Severe acute respiratory syndrome. *Nat Med* 2004, **10**(12 Suppl), S88-97.

34 Tan, C.C. SARS in Singapore--key lessons from an epidemic. *Ann Acad Med Singapore* 2006, **35**(5), 345-349.

35 Poon, L.L., Guan, Y., Nicholls, J.M., Yuen, K.Y. & Peiris, J.S. The aetiology, origins, and diagnosis of severe acute respiratory syndrome. *Lancet Infect Dis* 2004, **4**(11), 663-671.

36 Lim, P.L., Kurup, A., Gopalakrishna, G. *et al.* Laboratory-acquired severe acute respiratory syndrome. *N Engl J Med* 2004, **350**(17), 1740-1745.

37 Normile, D. Infectious diseases. Mounting lab accidents raise SARS fears. *Science* 2004, **304**(5671), 659-661.

38 Marra, M.A., Jones, S.J., Astell, C.R. *et al.* The Genome sequence of the SARS-associated coronavirus. *Science* 2003, **300**(5624), 1399-1404.

39 Lai, M.M. & Holmes, K.V. Coronaviridae: The Viruses and Their Replication. In *Field's Virology*, Vol. 1 (Eds. Knipe, D.M. & Howley, P.M.) Lippincott Williams & Wilkins, Philadelphia, 2001.

40 Bartlam, M., Yang, H. & Rao, Z. Structural insights into SARS coronavirus proteins. *Curr Opin Struct Biol* 2005, **15**(6), 664-672.

41 Li, F., Li, W., Farzan, M. & Harrison, S.C. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 2005, **309**(5742), 1864-1868.

42 Li, W., Moore, M.J., Vasilieva, N. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003, **426**(6965), 450-454.

43 Jeffers, S.A., Tusell, S.M., Gillim-Ross, L. *et al.* CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci U S A* 2004, **101**(44), 15748-15753.

44 Wentworth, D.E. & Holmes, K.V. Coronavirus Binding and Entry. In *Coronaviruses: Molecular and Cellular Biology* (Ed. Thiel, V.) Caister Academic Press, Norfolk, 2007.

45 Yao, Y.X., Ren, J., Heinen, P., Zambon, M. & Jones, I.M. Cleavage and serum reactivity of the severe acute respiratory syndrome coronavirus spike protein. *J Infect Dis* 2004, **190**(1), 91-98.

46 Bosch, B.J., Bartelink, W. & Rottier, P.J. Cathepsin L functionally cleaves the SARS-CoV class I fusion protein upstream of rather than adjacent to the fusion peptide. *J Virol* 2008.

47 Smotrys, J.E. & Linder, M.E. Palmitoylation of intracellular signaling proteins: regulation and function. *Annu Rev Biochem* 2004, **73**, 559-587.

48 Resh, M.D. Palmitoylation of ligands, receptors, and intracellular signaling molecules. *Sci STKE* 2006, **2006**(359), re14.

49 Greaves, J. & Chamberlain, L.H. Palmitoylation-dependent protein sorting. *J Cell Biol* 2007, **176**(3), 249-254.

50 Petit, C.M., Chouljenko, V.N., Iyer, A. *et al.* Palmitoylation of the cysteine-rich endodomain of the SARS-coronavirus spike glycoprotein is important for spike-mediated cell fusion. *Virology* 2007, **360**(2), 264-274.

51 Bos, E.C., Heijnen, L., Luytjes, W. & Spaan, W.J. Mutational analysis of the murine coronavirus spike protein: effect on cell-to-cell fusion. *Virology* 1995, **214**(2), 453-463.

52 Thorp, E.B., Boscarino, J.A., Logan, H.L., Goletz, J.T. & Gallagher, T.M. Palmitoylations on murine coronavirus spike proteins are essential for virion assembly and infectivity. *J Virol* 2006, **80**(3), 1280-1289.

53 Liao, Y., Yuan, Q., Torres, J., Tam, J.P. & Liu, D.X. Biochemical and functional characterization of the membrane association and membrane permeabilizing activity of the severe acute respiratory syndrome coronavirus envelope protein. *Virology* 2006, **349**(2), 264-275.

54 Corse, E. & Machamer, C.E. The cytoplasmic tail of infectious bronchitis virus E protein directs Golgi targeting. *J Virol* 2002, **76**(3), 1273-1284.

55 Boscarino, J.A., Logan, H.L., Lacny, J.J. & Gallagher, T.M. Envelope protein palmitoylations are crucial for murine coronavirus assembly. *J Virol* 2008, **82**(6), 2989-2999.

56 Lopez, L.A., Riffle, A.J., Pike, S.L., Gardner, D. & Hogue, B.G. Importance of conserved cysteine residues in the coronavirus envelope protein. *J Virol* 2008, **82**(6), 3000-3010.

57 Rousso, I., Mixon, M.B., Chen, B.K. & Kim, P.S. Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity. *Proc Natl Acad Sci U S A* 2000, **97**(25), 13523-13525.

58 Nal, B., Chan, C., Kien, F. *et al.* Differential maturation and subcellular localization of severe acute respiratory syndrome coronavirus surface proteins S, M and E. *J Gen Virol* 2005, **86**(Pt 5), 1423-1434.

59 Torres, J., Wang, J., Parthasarathy, K. & Liu, D.X. The transmembrane oligomers of coronavirus protein E. *Biophys J* 2005, **88**(2), 1283-1290.

60 Helenius, A. Unpacking the incoming influenza virus. *Cell* 1992, **69**(4), 577-578.

61 DeDiego, M.L., Alvarez, E., Almazan, F. *et al.* A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. *J Virol* 2007, **81**(4), 1701-1713.

62 Dediego, M.L., Pewe, L., Alvarez, E., Rejas, M.T., Perlman, S. & Enjuanes, L. Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 transgenic mice. *Virology* 2008, **376**(2), 379-389.

63 Voss, D., Kern, A., Traggiai, E. *et al.* Characterization of severe acute respiratory syndrome coronavirus membrane protein. *FEBS Lett* 2006, **580**(3), 968-973.

64 Ma, H.C., Fang, C.P., Hsieh, Y.C., Chen, S.C., Li, H.C. & Lo, S.Y. Expression and membrane integration of SARS-CoV M protein. *J Biomed Sci* 2008, **15**(3), 301-310.

65 Fang, X., Ye, L., Timani, K.A. *et al.* Peptide domain involved in the interaction between membrane protein and nucleocapsid protein of SARS-associated coronavirus. *J Biochem Mol Biol* 2005, **38**(4), 381-385.

66 Fang, X., Gao, J., Zheng, H. *et al.* The membrane protein of SARS-CoV suppresses NF-kappaB activation. *J Med Virol* 2007, **79**(10), 1431-1439.

67 Chan, C.M., Ma, C.W., Chan, W.Y. & Chan, H.Y. The SARS-Coronavirus Membrane protein induces apoptosis through modulating the Akt survival pathway. *Arch Biochem Biophys* 2007, **459**(2), 197-207.

68 Huang, Y., Yang, Z.Y., Kong, W.P. & Nabel, G.J. Generation of synthetic severe acute respiratory syndrome coronavirus pseudoparticles: implications for assembly and vaccine production. *J Virol* 2004, **78**(22), 12557-12565.

69 Chen, C.Y., Chang, C.K., Chang, Y.W. *et al.* Structure of the SARS coronavirus nucleocapsid protein RNA-binding dimerization domain suggests a mechanism for helical packaging of viral RNA. *J Mol Biol* 2007, **368**(4), 1075-1086.

70 Hsieh, P.K., Chang, S.C., Huang, C.C. *et al.* Assembly of severe acute respiratory syndrome coronavirus RNA packaging signal into virus-like particles is nucleocapsid dependent. *J Virol* 2005, **79**(22), 13848-13855.

71 Lam, W.Y., Tang, J.W., Yeung, A.C., Chiu, L.C., Sung, J.J. & Chan, P.K. Avian influenza virus A/HK/483/97(H5N1) NS1 protein induces apoptosis in human airway epithelial cells. *J Virol* 2008, **82**(6), 2741-2751.

72 Diemer, C., Schneider, M., Seebach, J. *et al.* Cell type-specific cleavage of nucleocapsid protein by effector caspases during SARS coronavirus infection. *J Mol Biol* 2008, **376**(1), 23-34.

73 Eleouet, J.F., Slee, E.A., Saurini, F. *et al.* The viral nucleocapsid protein of transmissible gastroenteritis coronavirus (TGEV) is cleaved by caspase-6 and -7 during TGEV-induced apoptosis. *J Virol* 2000, **74**(9), 3975-3983.

74 Thiel, V., Ivanov, K.A., Putics, A. *et al.* Mechanisms and enzymes involved in SARS coronavirus genome expression. *J Gen Virol* 2003, **84**(Pt 9), 2305-2315.

75 Yount, B., Roberts, R.S., Sims, A.C. *et al.* Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. *J Virol* 2005, **79**(23), 14909-14922.

76 Schaecher, S.R., Mackenzie, J.M. & Pekosz, A. The ORF7b protein of severe acute respiratory syndrome coronavirus (SARS-CoV) is expressed in virus-infected cells and incorporated into SARS-CoV particles. *J Virol* 2007, **81**(2), 718-731.

77 Ito, N., Mossel, E.C., Narayanan, K. *et al.* Severe acute respiratory syndrome coronavirus 3a protein is a viral structural protein. *J Virol* 2005, **79**(5), 3182-3186.

78 Shen, S., Lin, P.S., Chao, Y.C. *et al.* The severe acute respiratory syndrome coronavirus 3a is a novel structural protein. *Biochem Biophys Res Commun* 2005, **330**(1), 286-292.

79 Huang, C., Ito, N., Tseng, C.T. & Makino, S. Severe acute respiratory syndrome coronavirus 7a accessory protein is a viral structural protein. *J Virol* 2006, **80**(15), 7287-7294.

80 Qiu, M., Shi, Y., Guo, Z. *et al.* Antibody responses to individual proteins of SARS coronavirus and their neutralization activities. *Microbes Infect* 2005, **7**(5-6), 882-889.

81 Chow, S.C., Ho, C.Y., Tam, T.T. *et al.* Specific epitopes of the structural and hypothetical proteins elicit variable humoral responses in SARS patients. *J Clin Pathol* 2006, **59**(5), 468-476.

82 Pewe, L., Zhou, H., Netland, J. *et al.* A severe acute respiratory syndrome-associated coronavirus-specific protein enhances virulence of an attenuated murine coronavirus. *J Virol* 2005, **79**(17), 11335-11342.

83 Geng, H., Liu, Y.M., Chan, W.S. *et al.* The putative protein 6 of the severe acute respiratory syndrome-associated coronavirus: expression and functional characterization. *FEBS Lett* 2005, **579**(30), 6763-6768.

84 Tangudu, C., Olivares, H., Netland, J., Perlman, S. & Gallagher, T. Severe acute respiratory syndrome coronavirus protein 6 accelerates murine coronavirus infections. *J Virol* 2007, **81**(3), 1220-1229.

85 Kumar, P., Gunalan, V., Liu, B. *et al.* The nonstructural protein 8 (nsp8) of the SARS coronavirus interacts with its ORF6 accessory protein. *Virology* 2007, **366**(2), 293-303.

86 Keng, C.T., Choi, Y.W., Welkers, M.R. *et al.* The human severe acute respiratory syndrome coronavirus (SARS-CoV) 8b protein is distinct from its counterpart in animal SARS-CoV and down-regulates the expression of the envelope protein in infected cells. *Virology* 2006, **354**(1), 132-142.

87 Meier, C., Aricescu, A.R., Assenberg, R. *et al.* The crystal structure of ORF-9b, a lipid binding protein from the SARS coronavirus. *Structure* 2006, **14**(7), 1157-1165.

88 Moshynskyy, I., Viswanathan, S., Vasilenko, N. *et al.* Intracellular localization of the SARS coronavirus protein 9b: evidence of active export from the nucleus. *Virus Res* 2007, **127**(1), 116-121.

89 Oostra, M., de Haan, C.A., de Groot, R.J. & Rottier, P.J. Glycosylation of the severe acute respiratory syndrome coronavirus triple-spanning membrane proteins 3a and M. *J Virol* 2006, **80**(5), 2326-2336.

90 Tan, Y.J., Lim, S.G. & Hong, W. Understanding the accessory viral proteins unique to the severe acute respiratory syndrome (SARS) coronavirus. *Antiviral Res* 2006, **72**(2), 78-88.

91 Zhong, X., Guo, Z., Yang, H. *et al.* Amino terminus of the SARS coronavirus protein 3a elicits strong, potentially protective humoral responses in infected patients. *J Gen Virol* 2006, **87**(Pt 2), 369-373.

92 Tan, Y.J., Teng, E., Shen, S. *et al.* A novel severe acute respiratory syndrome coronavirus protein, U274, is transported to the cell surface and undergoes endocytosis. *J Virol* 2004, **78**(13), 6723-6734.

93 Huang, C., Narayanan, K., Ito, N., Peters, C.J. & Makino, S. Severe acute respiratory syndrome coronavirus 3a protein is released in membranous structures from 3a protein-expressing cells and infected cells. *J Virol* 2006, **80**(1), 210-217.

94 Yuan, X., Yao, Z., Wu, J. *et al.* G1 phase cell cycle arrest induced by SARS-CoV 3a protein via the cyclin D3/pRb pathway. *Am J Respir Cell Mol Biol* 2007, **37**(1), 9-19.

95 Law, P.T., Wong, C.H., Au, T.C. *et al.* The 3a protein of severe acute respiratory syndrome-associated coronavirus induces apoptosis in Vero E6 cells. *J Gen Virol* 2005, **86**(Pt 7), 1921-1930.

96 Kanzawa, N., Nishigaki, K., Hayashi, T. *et al.* Augmentation of chemokine production by severe acute respiratory syndrome coronavirus 3a/X1 and 7a/X4 proteins through NF-kappaB activation. *FEBS Lett* 2006, **580**(30), 6807-6812.

97 Narayanan, K., Huang, C. & Makino, S. SARS coronavirus accessory proteins. *Virus Res* 2008, **133**(1), 113-121.

98 Kopecky-Bromberg, S.A., Martinez-Sobrido, L., Frieman, M., Baric, R.A. & Palese, P. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. *J Virol* 2007, **81**(2), 548-557.

99 Guo, J.P., Petric, M., Campbell, W. & McGeer, P.L. SARS corona virus peptides recognized by antibodies in the sera of convalescent cases. *Virology* 2004, **324**(2), 251-256.

100 Fielding, B.C., Tan, Y.J., Shuo, S. *et al.* Characterization of a unique group-specific protein (U122) of the severe acute respiratory syndrome coronavirus. *J Virol* 2004, **78**(14), 7311-7318.

101 Fielding, B.C., Gunalan, V., Tan, T.H. *et al.* Severe acute respiratory syndrome coronavirus protein 7a interacts with hSGT. *Biochem Biophys Res Commun* 2006, **343**(4), 1201-1208.

102 Tan, Y.J., Fielding, B.C., Goh, P.Y. *et al.* Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis via a caspase-dependent pathway. *J Virol* 2004, **78**(24), 14043-14047.

103 Tan, Y.X., Tan, T.H., Lee, M.J. *et al.* Induction of apoptosis by the severe acute respiratory syndrome coronavirus 7a protein is dependent on its interaction with the Bcl-XL protein. *J Virol* 2007, **81**(12), 6346-6355.

104 Schaecher, S.R., Touchette, E., Schriewer, J., Buller, R.M. & Pekosz, A. Severe acute respiratory syndrome coronavirus gene 7 products contribute to virus-induced apoptosis. *J Virol* 2007, **81**(20), 11054-11068.

105 Kopecky-Bromberg, S.A., Martinez-Sobrido, L. & Palese, P. 7a protein of severe acute respiratory syndrome coronavirus inhibits cellular protein synthesis and activates p38 mitogen-activated protein kinase. *J Virol* 2006, **80**(2), 785-793.

106 Schaecher, S.R., Diamond, M.S. & Pekosz, A. The Transmembrane Domain of the SARS-CoV ORF7b Protein is Necessary and Sufficient for Its Retention in the Golgi Complex. *J Virol* 2008.

107 Wang, H., Yang, P., Liu, K. *et al.* SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. *Cell Res* 2008, **18**(2), 290-301.

108 Chan, V.S., Chan, K.Y., Chen, Y. *et al.* Homozygous L-SIGN (CLEC4M) plays a protective role in SARS coronavirus infection. *Nat Genet* 2006, **38**(1), 38-46.

109 Simmons, G., Gosalia, D.N., Rennekamp, A.J., Reeves, J.D., Diamond, S.L. & Bates, P. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. *Proc Natl Acad Sci U S A* 2005, **102**(33), 11876-11881.

110 Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C. *et al.* Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. *J Mol Biol* 2003, **331**(5), 991-1004.

111 Law, A.H., Lee, D.C., Cheung, B.K., Yim, H.C. & Lau, A.S. Role for nonstructural protein 1 of severe acute respiratory syndrome coronavirus in chemokine dysregulation. *J Virol* 2007, **81**(1), 416-422.

112 Ziebuhr, J. & Snijder, E.J. The Coronavirus Replicase Gene: Special Enzymes for Special Viruses. In *Coronaviruses: Molecular and Cellular Biology* (Ed. Thiel, V.) Caister Academic Press, Norfolk, 2007.

113 Ivanov, K.A., Thiel, V., Dobbe, J.C., van der Meer, Y., Snijder, E.J. & Ziebuhr, J. Multiple enzymatic activities associated with severe acute respiratory syndrome coronavirus helicase. *J Virol* 2004, **78**(11), 5619-5632.

114 Ivanov, K.A., Hertzig, T., Rozanov, M. *et al.* Major genetic marker of nidoviruses encodes a replicative endoribonuclease. *Proc Natl Acad Sci U S A* 2004, **101**(34), 12694-12699.

115 Snijder, E.J., van der Meer, Y., Zevenhoven-Dobbe, J. *et al.* Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. *J Virol* 2006, **80**(12), 5927-5940.

116 Sawicki, S.G., Sawicki, D.L. & Siddell, S.G. A contemporary view of coronavirus transcription. *J Virol* 2007, **81**(1), 20-29.

117 Enjuanes, L., Sola, I., Zuniga, S. & Moreno, J.L. Coronavirus RNA Synthesis:Transcription. In *Coronaviruses: Molecular and Cellular Biology* (Ed. Thiel, V.) Caister Academic Press, Norfolk, 2007.

118 Guo, Y., Korteweg, C., McNutt, M.A. & Gu, J. Pathogenetic mechanisms of severe acute respiratory syndrome. *Virus Res* 2008, **133**(1), 4-12.

119 Goldsby, R.A., Kindt, T.J. & Osborne, B.A. *Kuby Immunology*, W. H. Freeman and Company, New York, 2000.

120 Saito, T. & Gale, M., Jr. Principles of intracellular viral recognition. *Curr Opin Immunol* 2007, **19**(1), 17-23.

121 Kotenko, S.V., Gallagher, G., Baurin, V.V. *et al.* IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003, **4**(1), 69-77.

122 Sheppard, P., Kindsvogel, W., Xu, W. *et al.* IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003, **4**(1), 63-68.

123 Ank, N., West, H. & Paludan, S.R. IFN-lambda: novel antiviral cytokines. *J Interferon Cytokine Res* 2006, **26**(6), 373-379.

124 Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H. & Doerr, H.W. Treatment of SARS with human interferons. *Lancet* 2003, **362**(9380), 293-294.

125 Spiegel, M., Pichlmair, A., Muhlberger, E., Haller, O. & Weber, F. The antiviral effect of interferon-beta against SARS-coronavirus is not mediated by MxA protein. *J Clin Virol* 2004, **30**(3), 211-213.

126 Stroher, U., DiCaro, A., Li, Y. *et al.* Severe acute respiratory syndrome-related coronavirus is inhibited by interferon- alpha. *J Infect Dis* 2004, **189**(7), 1164-1167.

127 Haagmans, B.L., Kuiken, T., Martina, B.E. *et al.* Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. *Nat Med* 2004, **10**(3), 290-293.

128 Sainz, B., Jr., Mossel, E.C., Peters, C.J. & Garry, R.F. Interferon-beta and interferon-gamma synergistically inhibit the replication of severe acute respiratory syndrome-associated coronavirus (SARS-CoV). *Virology* 2004, **329**(1), 11-17.

129 Spiegel, M., Pichlmair, A., Martinez-Sobrido, L. *et al.* Inhibition of Beta interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3. *J Virol* 2005, **79**(4), 2079-2086.

130 Talon, J., Horvath, C.M., Polley, R. *et al.* Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* 2000, **74**(17), 7989-7996.

131 Basler, C.F., Mikulasova, A., Martinez-Sobrido, L. *et al.* The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. *J Virol* 2003, **77**(14), 7945-7956.

132 Frieman, M., Heise, M. & Baric, R. SARS coronavirus and innate immunity. *Virus Res* 2008, **133**(1), 101-112.

133 Wu, L. & KewalRamani, V.N. Dendritic-cell interactions with HIV: infection and viral dissemination. *Nat Rev Immunol* 2006, **6**(11), 859-868.

134 Cheung, C.Y., Poon, L.L., Ng, I.H. *et al.* Cytokine responses in severe acute respiratory syndrome coronavirus-infected macrophages in vitro: possible relevance to pathogenesis. *J Virol* 2005, **79**(12), 7819-7826.

135 Ziegler, T., Matikainen, S., Ronkko, E. *et al.* Severe acute respiratory syndrome coronavirus fails to activate cytokine-mediated innate immune responses in cultured human monocyte-derived dendritic cells. *J Virol* 2005, **79**(21), 13800-13805.

136 Tseng, C.T., Perrone, L.A., Zhu, H., Makino, S. & Peters, C.J. Severe acute respiratory syndrome and the innate immune responses: modulation of effector cell function without productive infection. *J Immunol* 2005, **174**(12), 7977-7985.

137 Spiegel, M., Schneider, K., Weber, F., Weidmann, M. & Hufert, F.T. Interaction of severe acute respiratory syndrome-associated coronavirus with dendritic cells. *J Gen Virol* 2006, **87**(Pt 7), 1953-1960.

138 Law, H.K., Cheung, C.Y., Ng, H.Y. *et al.* Chemokine up-regulation in SARS-coronavirus-infected, monocyte-derived human dendritic cells. *Blood* 2005, **106**(7), 2366-2374.

139 Zhang, Y., Li, J., Zhan, Y. *et al.* Analysis of serum cytokines in patients with severe acute respiratory syndrome. *Infect Immun* 2004, **72**(8), 4410-4415.

140 Wong, C.K., Lam, C.W., Wu, A.K. *et al.* Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin Exp Immunol* 2004, **136**(1), 95-103.

141 Huang, J.L., Huang, J., Duan, Z.H. *et al.* Th2 predominance and CD8+ memory T cell depletion in patients with severe acute respiratory syndrome. *Microbes Infect* 2005, **7**(3), 427-436.

142 Tang, N.L., Chan, P.K., Wong, C.K. *et al.* Early enhanced expression of interferon-inducible protein-10 (CXCL-10) and other chemokines predicts adverse outcome in severe acute respiratory syndrome. *Clin Chem* 2005, **51**(12), 2333-2340.

143 Korteweg, C. & Gu, J. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. *Am J Pathol* 2008, **172**(5), 1155-1170.

144 de Jong, M.D., Simmons, C.P., Thanh, T.T. *et al.* Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 2006, **12**(10), 1203-1207.

145 Roberts, A., Paddock, C., Vogel, L., Butler, E., Zaki, S. & Subbarao, K. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. *J Virol* 2005, **79**(9), 5833-5838.

146 Baas, T., Roberts, A., Teal, T.H. *et al.* Genomic Analysis Reveals Age Dependent Innate Immune Responses to SARS Coronavirus. *J Virol* 2008.

147 Tan, Y.J., Goh, P.Y., Fielding, B.C. *et al.* Profiles of antibody responses against severe acute respiratory syndrome coronavirus recombinant proteins and their potential use as diagnostic markers. *Clin Diagn Lab Immunol* 2004, **11**(2), 362-371.

148 Rockx, B. & Baric, R.S. Grand Challenges in Human Coronavirus Vaccine Development. In *Coronaviruses: Molecular and Cellular Biology* (Ed. Thiel, V.) Caister Academic Press, Norfolk, UK, 2007. 350.

149 Bukreyev, A., Lamirande, E.W., Buchholz, U.J. *et al.* Mucosal immunisation of African green monkeys (Cercopithecus aethiops) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. *Lancet* 2004, **363**(9427), 2122-2127.

150 Buchholz, U.J., Bukreyev, A., Yang, L. *et al.* Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. *Proc Natl Acad Sci U S A* 2004, **101**(26), 9804-9809.

151 Bisht, H., Roberts, A., Vogel, L., Subbarao, K. & Moss, B. Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein. *Virology* 2005, **334**(2), 160-165.

152 Yang, Z.Y., Kong, W.P., Huang, Y. *et al.* A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature* 2004, **428**(6982), 561-564.

153 Li, T., Xie, J., He, Y. *et al.* Long-term persistence of robust antibody and cytotoxic T cell responses in recovered patients infected with SARS coronavirus. *PLoS ONE* 2006, **1**, e24.

154 Akerstrom, S., Tan, Y.J. & Mirazimi, A. Amino acids 15-28 in the ectodomain of SARS coronavirus 3a protein induces neutralizing antibodies. *FEBS Lett* 2006, **580**(16), 3799-3803.

155 Peng, H., Yang, L.T., Wang, L.Y. *et al.* Long-lived memory T lymphocyte responses against SARS coronavirus nucleocapsid protein in SARS-recovered patients. *Virology* 2006, **351**(2), 466-475.

156 Yang, L.T., Peng, H., Zhu, Z.L. *et al.* Long-lived effector/central memory T-cell responses to severe acute respiratory syndrome coronavirus (SARS-CoV) S antigen in recovered SARS patients. *Clin Immunol* 2006, **120**(2), 171-178.

157 Tsao, Y.P., Lin, J.Y., Jan, J.T. *et al.* HLA-A*0201 T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus nucleocapsid and spike proteins. *Biochem Biophys Res Commun* 2006, **344**(1), 63-71.

158 Peng, H., Yang, L.T., Li, J. *et al.* Human memory T cell responses to SARS-CoV E protein. *Microbes Infect* 2006, **8**(9-10), 2424-2431.

159 Fang, F.C. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* 2004, **2**(10), 820-832.

160 Coleman, J.W. Nitric oxide in immunity and inflammation. *Int Immunopharmacol* 2001, **1**(8), 1397-1406.

161 Akaike, T. Role of free radicals in viral pathogenesis and mutation. *Rev Med Virol* 2001, **11**(2), 87-101.

162 Pacher, P., Beckman, J.S. & Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007, **87**(1), 315-424.

163 Akaike, T. & Maeda, H. Nitric oxide and virus infection. *Immunology* 2000, **101**(3), 300-308.

164 Borghan, M.A., Mori, Y., El-Mahmoudy, A.B. *et al.* Induction of nitric oxide synthase by rotavirus enterotoxin NSP4: implication for rotavirus pathogenicity. *J Gen Virol* 2007, **88**(Pt 7), 2064-2072.

165 Hori, K., Burd, P.R., Furuke, K., Kutza, J., Weih, K.A. & Clouse, K.A. Human immunodeficiency virus-1-infected macrophages induce inducible nitric oxide synthase and nitric oxide (NO) production in astrocytes: astrocytic NO as a possible mediator of neural damage in acquired immunodeficiency syndrome. *Blood* 1999, **93**(6), 1843-1850.

166 Adamson, D.C., Wildemann, B., Sasaki, M. *et al.* Immunologic NO synthase: elevation in severe AIDS dementia and induction by HIV-1 gp41. *Science* 1996, **274**(5294), 1917-1921.

167 Kao, Y.J., Piedra, P.A., Larsen, G.L. & Colasurdo, G.N. Induction and regulation of nitric oxide synthase in airway epithelial cells by respiratory syncytial virus. *Am J Respir Crit Care Med* 2001, **163**(2), 532-539.

168 Akaike, T., Noguchi, Y., Ijiri, S. *et al.* Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A* 1996, **93**(6), 2448-2453.

169 Mannick, J.B., Asano, K., Izumi, K., Kieff, E. & Stamler, J.S. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* 1994, **79**(7), 1137-1146.

170 Lin, Y.L., Huang, Y.L., Ma, S.H. *et al.* Inhibition of Japanese encephalitis virus infection by nitric oxide: antiviral effect of nitric oxide on RNA virus replication. *J Virol* 1997, **71**(7), 5227-5235.

171 Simon, M., Falk, K.I., Lundkvist, A. & Mirazimi, A. Exogenous nitric oxide inhibits Crimean Congo hemorrhagic fever virus. *Virus Res* 2006, **120**(1-2), 184-190.

172 Rimmelzwaan, G.F., Baars, M.M., de Lijster, P., Fouchier, R.A. & Osterhaus, A.D. Inhibition of influenza virus replication by nitric oxide. *J Virol* 1999, **73**(10), 8880-8883.

173 Adler, H., Beland, J.L., Del-Pan, N.C. *et al.* Suppression of herpes simplex virus type 1 (HSV-1)-induced pneumonia in mice by inhibition of inducible nitric oxide synthase (iNOS, NOS2). *J Exp Med* 1997, **185**(9), 1533-1540.

174 Zaragoza, C., Ocampo, C.J., Saura, M., McMillan, A. & Lowenstein, C.J. Nitric oxide inhibition of coxsackievirus replication in vitro. *J Clin Invest* 1997, **100**(7), 1760-1767.

175 Klingstrom, J., Akerstrom, S., Hardestam, J. *et al.* Nitric oxide and peroxynitrite have different antiviral effects against hantavirus replication and free mature virions. *Eur J Immunol* 2006, **36**(10), 2649-2657.

176 Saura, M., Zaragoza, C., McMillan, A. *et al.* An antiviral mechanism of nitric oxide: inhibition of a viral protease. *Immunity* 1999, **10**(1), 21-28.

177 Bizzozero, O.A., Bixler, H., Parkhani, J. & Pastuszyn, A. Nitric oxide reduces the palmitoylation of rat myelin proteolipid protein by an indirect mechanism. *Neurochem Res* 2001, **26**(10), 1127-1137.

178 Akarid, K., Sinet, M., Desforges, B. & Gougerot-Pocidalo, M.A. Inhibitory effect of nitric oxide on the replication of a murine retrovirus in vitro and in vivo. *J Virol* 1995, **69**(11), 7001-7005.

179 Pope, M., Marsden, P.A., Cole, E. *et al.* Resistance to murine hepatitis virus strain 3 is dependent on production of nitric oxide. *J Virol* 1998, **72**(9), 7084-7090.

180 Bloch, K.D., Ichinose, F., Roberts, J.D., Jr. & Zapol, W.M. Inhaled NO as a therapeutic agent. *Cardiovasc Res* 2007, **75**(2), 339-348.

181 Hsu, C.W., Lee, D.L., Lin, S.L., Sun, S.F. & Chang, H.W. The initial response to inhaled nitric oxide treatment for intensive care unit patients with acute respiratory distress syndrome. *Respiration* 2008, **75**(3), 288-295.

182 Rosenberg, R.B., Waagner, D.C., Romano, M.J., Kanase, H.N. & Young, R.B. Hantavirus pulmonary syndrome treated with inhaled nitric oxide. *Pediatr Infect Dis J* 1998, **17**(8), 749-752.

183 Chen, L., Liu, P., Gao, H. *et al.* Inhalation of nitric oxide in the treatment of severe acute respiratory syndrome: a rescue trial in Beijing. *Clin Infect Dis* 2004, **39**(10), 1531-1535.

184 Pellish, R.S., Nasir, A., Ramratnam, B. & Moss, S.F. Review article: RNA interference--potential therapeutic applications for the gastroenterologist. *Aliment Pharmacol Ther* 2008, **27**(9), 715-723.

185 Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001, **411**(6836), 494-498.

186 Stram, Y. & Kuzntzova, L. Inhibition of viruses by RNA interference. *Virus Genes* 2006, **32**(3), 299-306.

187 He, M.L., Zheng, B., Peng, Y. *et al.* Inhibition of SARS-associated coronavirus infection and replication by RNA interference. *Jama* 2003, **290**(20), 2665-2666.

188 Lu, A., Zhang, H., Zhang, X. *et al.* Attenuation of SARS coronavirus by a short hairpin RNA expression plasmid targeting RNA-dependent RNA polymerase. *Virology* 2004, **324**(1), 84-89.

189 Ni, B., Shi, X., Li, Y., Gao, W., Wang, X. & Wu, Y. Inhibition of replication and infection of severe acute respiratory syndrome-associated coronavirus with plasmid-mediated interference RNA. *Antivir Ther* 2005, **10**(4), 527-533.

190 Wang, Z., Ren, L., Zhao, X. *et al.* Inhibition of severe acute respiratory syndrome virus replication by small interfering RNAs in mammalian cells. *J Virol* 2004, **78**(14), 7523-7527.

191 Elmén Joacim, W.C., Brytting Maria, Wahren Britta, and Ljungberg Karl. SARS Virus Inhibiton by siRNA. *Preclinica* 2004, **2**(2), 135-142.

192 Li, B.J., Tang, Q., Cheng, D. *et al.* Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat Med* 2005, **11**(9), 944-951.

193 Zhong, X., Yang, H., Guo, Z.F. *et al.* B-cell responses in patients who have recovered from severe acute respiratory syndrome target a dominant site in the S2 domain of the surface spike glycoprotein. *J Virol* 2005, **79**(6), 3401-3408.

194 Greenough, T.C., Babcock, G.J., Roberts, A. *et al.* Development and characterization of a severe acute respiratory syndrome-associated coronavirus-neutralizing human monoclonal antibody that provides effective immunoprophylaxis in mice. *J Infect Dis* 2005, **191**(4), 507-514.

195 Sui, J., Li, W., Roberts, A. *et al.* Evaluation of human monoclonal antibody 80R for immunoprophylaxis of severe acute respiratory syndrome by an animal study, epitope mapping, and analysis of spike variants. *J Virol* 2005, **79**(10), 5900-5906.