Development of a Novel Therapeutic Against Coronaviruses

By

Yvonne Pho

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg

Copyright © 2019 by Yvonne Pho
Abstract

Although people around the world are commonly infected with coronaviruses, some of these pathogens are high on the list of public health concerns. The emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) in the last two decades has shown the pandemic potential of this rapidly adapting virus family. Treatment of MERS has been problematic as traditional antivirals were not shown to be effective against this pathogenic strain of coronavirus. Moreover, development of vaccines and therapeutics has been hindered by the lack of a commercially available permissive small animal for MERS-CoV. These circumstances contrast those seen in the emergence of SARS-CoV where some small animal models were susceptible to virus infection without aid, and moreover, with serial passaging of a SARS-CoV strain in mice a mouse-adapted variant was developed that was able to cause severe respiratory disease and death in mice. This work aims to develop and evaluate a therapeutic that specifically targets a coronavirus while developing a small animal model that allows MERS-CoV infection. I showed that the fusion of the ectodomain of the SARS-CoV host cell target receptor angiotensin converting enzyme 2 (ACE2) to mouse IgG Fc exhibits promising antiviral activity in vitro. This approach, if effective in subsequent in vivo evaluations, could be applied to in the generation of a treatment against MERS-CoV. Additionally, I was able to show that mice were permissive to MERS-CoV infection following transduction of their airways with an adenovirus vector expressing human host-cell receptor for MERS-CoV, dipeptidyl peptidase 4 (hDPP4), although I was not successful in further enhancing the efficacy of airway transduction using Adeno-
associated viral vector deliver of hDPP4. Altogether, this study contributes to the development and evaluation of therapeutics against current and potential emerging coronaviruses.
Acknowledgements

I would like to thank my advisor, Dr. Darwyn Kobasa, for mentoring me during my Master of Science program. He has provided me with the tools and knowledge to succeed in this field.

I would also like to thank my committee members, Dr. Michael Drebot and Dr. Shawn Babiuk, for their guidance and advice through these years.

To my lab members in the Special Pathogens group (especially Anders and Mable), thank you for always being available to answer all my many questions.

Lastly, I would like to thank my family and friends, whom I am grateful for their never-ending love and support. Thank you.
List of Tables

Table 1 - Primers used for amplification of DPP4 for cloning into pACAGGFP .......................... 36

Table 2 - Primers for generation of ACEP, 2ACEP, sACEP2 and eGFP fragments for cloning into pFUSE-mlG2a-Fc2........................................................................................................................................ 41

Table 3 - Primers and probes for detection and quantification of MERS-CoV RNA upE gene.. 63

Table 4 - Binding capacity of ACEP peptibodies to purified SARS spike protein ....................... 75

Table 5 - Protein concentration of purified Fc-ACE2 ....................................................................... 76

Table 6 - Inhibition of SARS-CoV infection in Vero E6 cells by Fc-sACE2........................................ 77

Table 7 - Stock titres of viral vectors AAV6-hDPP4, Ad5-hDPP4 and Ad5-ctrl .......................... 81
# List of Figures

**Figure 1.** Phylogenetic tree analysis of the 816 nucleotide RNA-dependent RNA polymerase gene sequence .......................................................... 7

**Figure 2.** Single stranded RNA genomes and structural diagram of SARS- and MERS-CoV ...... 10

**Figure 3.** Coronavirus replication strategy .......................................................... 13

**Figure 4.** Schematics of ACEP peptide gene and enhanced GFP constructs cloned into pFUSE-mlgG2a-Fc ........................................................................................................ 40

**Figure 5.** Schematic diagrams of generated peptibodies........................................... 64

**Figure 6.** Peptibody expression in 293T cell lysates and supernatants collected 48 hours post transfection ........................................................................................................... 66

**Figure 7.** Expression of eGFP in 293T cells 48 hours after transfection in 293T cells using different transfection reagents ......................................................................................... 68

**Figure 8.** Expression of eGFP in 293T cells 48 hours after transfection at different plasmid DNA concentrations ........................................................................................................... 70

**Figure 9.** Peptibody binding to 293T cells expressing SARS spike protein ......................... 72

**Figure 10.** Schematic of the transmembrane domain of ACE2 cloned into pFUSE-mlgG2a-Fc and generated fusion protein Fc-sACE2 ........................................................................................................... 73

**Figure 11.** Fc-sACE2 and Fc-ACEP-N peptibody detection in crude and purified supernatants .......................................................................................................................... 74

**Figure 12.** Fc-sACE2 and Fc-ACEP-N binding to 293T cells expressing SARS spike protein .... 75

**Figure 13.** Schematics of plasmids pACAGhDPP4 (A) and pDGM6 (B) used to produce AAV6 virus expressing hDPP4 ........................................................................................................... 79
**Figure 14.** Expression of human DPP4 from plasmid pACAGhDPP4 and a positive control plasmid expressing hDPP4.........................................................................................................................................................80

**Figure 15.** MERS-CoV RNA levels in mouse lungs following transduction of hDPP4 with AAV6-hDPP4 virus .................................................................................................................................................................................................................. 81

**Figure 16.** Fluorescence detection of mice transduced with Ad5-hDPP4, Ad5-ctrl, AAV9-hDPP4 and AAV9-ctrl ........................................................................................................................................................................................................................................... 84

**Figure 17.** MERS-CoV RNA levels in mouse lungs following transduction with hDPP4 using AAV9 and Ad5 viral vectors .................................................................................................................................................................................................................................................................................. 85

**Figure 18.** GFP gene expression *in vitro* by Adenovirus serotype 5 viral vectors ....................... 86

**Figure 19.** Reporter gene and human DPP4 expression *in vitro* by immunofluorescence assay .................................................................................................................................................................................................................................................................................. 87
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSL</td>
<td>Biosafety level</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine ligand (C-C motif)</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine ligand (C-X-C motif)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post infection</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEPA</td>
<td>High-efficiency particulate arrestance</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>nsp</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid, EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
</tbody>
</table>
# Table of Contents

(I) Abstract

(III) Acknowledgements

(IV) List of Tables

(V) List of Figures

(VII) List of Abbreviations

## Chapter One: Introduction

1.1 Coronaviruses ........................................................................................................ 4
1.2 SARS and MERS epidemiology ........................................................................... 5
1.3 Virus origins and transmission ........................................................................... 6
1.4 Clinical symptoms .......................................................................................... 9
1.5 Viral genome and structure ............................................................................. 10
1.6 Virus life cycle and replication ......................................................................... 12
1.7 Virus pathogenesis and immunopathology .................................................... 15
1.8 Coronavirus spike protein .............................................................................. 18
1.9 Treatments .................................................................................................. 21
1.10 Vaccines ..................................................................................................... 23
1.11 Animal models ............................................................................................ 26
   1.11.1 Non-human primate model .................................................................. 26
   1.11.2 Mouse model ....................................................................................... 27
   1.11.3 Syrian hamster and ferret model ................................................... 29

## Chapter Two: Research Goals

2.1 Rationale ..................................................................................................... 31
Chapter Three: Materials and Methods

3.1 Cell culture ........................................................................................................... 33
3.2 Virus strains and stock preparation ....................................................................... 33
  3.2.1 AAV6 ................................................................................................................. 33
  3.2.2 AAV9 and Ad5 .................................................................................................... 36
  3.2.3 SARS-CoV and MERS-CoV .............................................................................. 37

3.3 Peptibody production ........................................................................................... 40
  3.3.1 Peptibody design ............................................................................................... 40
  3.3.2 Polymerase chain reaction (PCR) ...................................................................... 44
  3.3.3 In-Fusion HD cloning ....................................................................................... 45
  3.3.4 Transformation into competent cells .................................................................. 46
  3.3.5 Transfection optimization ................................................................................ 47
  3.3.6 Peptibody expression ......................................................................................... 49
  3.3.7 SDS-polyacrylamide gel electrophoresis and Western blot ................................. 50
  3.3.8 Purification of peptibodies and Fc-conjugated proteins ..................................... 52
  3.3.9 Protein quantification ....................................................................................... 53

3.4 Immunofluorescence assay (IFA) ........................................................................... 54

3.5 Enzyme linked immunosorbent assay (ELISA) ..................................................... 55

3.6 Viral assays .......................................................................................................... 56
  3.6.1 AAV genome extraction ................................................................................... 56
  3.6.2 End point dilution assay ................................................................................... 57
  3.6.3 Plaque assay ..................................................................................................... 58
  3.6.4 Neutralization assay ......................................................................................... 58

3.7 Mice ........................................................................................................................ 59

3.8 Quantitative Real-Time PCR (qRT-PCR) .............................................................. 61

3.9 Statistical analysis .................................................................................................. 62

Chapter Four: Results ................................................................................................. 63

4.1 ACE2 peptide peptibodies and sACE2 fusion protein ......................................... 63
  4.1.1 Generation of ACE2 peptide and peptibodies ................................................... 63
  4.1.2 Evaluation of transfection efficiency of plasmid pFUSE-mlG2a-Fc .................. 67
  4.1.3 ACEP peptibody binding capacity to SARS-CoV S protein .............................. 70
4.1.4 Generation of sACE2 fusion protein .......................................................... 72
4.1.5 Binding capacity of Fc-sACE2 ................................................................... 74
4.1.6 Neutralization capacity of Fc-ACE2 .......................................................... 75

4.2 Generation of a MERS-CoV mouse model ......................................................... 78
  4.2.1 Generation of an AAV6-hDPP4 viral vector ................................................. 79
  4.2.2 Transduction of mouse airway with AAV6, AAV9 and Ad5 vectors ............... 81
  4.2.3 In vitro analysis of AAV9 and Ad5 reporter genes ....................................... 84

Chapter Five: Discussion ....................................................................................... 89

5.1 ACEP peptibody targeting SARS-CoV ............................................................ 89
5.2 Fc-sACE2 fusion protein ................................................................................. 93

5.3 Transduction of mouse airways ....................................................................... 100
  5.3.1 AAV6 ...................................................................................................... 100
  5.3.2 AAV9 & Ad5 .......................................................................................... 103

Chapter Six: Concluding remarks and future directions ....................................... 112

Chapter Seven: References .................................................................................. 113

Chapter Eight: Appendix ...................................................................................... 127

Chapter Nine: Copyright Permissions .................................................................. 128
Chapter One: Introduction

1.1 Coronaviruses

Coronaviruses are enveloped, single-stranded, positive sense RNA viruses belonging to the Coronaviridae family (order Nidovirales). These viruses are known to have some of the largest genomes among RNA viruses, ranging from 26-32 kilobases (kb) in size. Coronaviruses (CoV) can be phylogenetically separated into four genera: Alpha-CoV, Beta-CoV, Gamma-CoV and Delta-CoV\(^1\)\(^-\)\(^3\). Each group consists of a large variety of animal (bat, porcine, murine, etc.), avian and human coronaviruses, which is hypothesized to be a result of replication of a large genome with an error prone RNA-dependent RNA polymerase as well as high frequency of recombination allowing for rapid adaptation to novel hosts\(^2\),\(^4\),\(^5\). Coronaviruses have an exceptionally wide distribution in bats and birds and this diversity can be attributed to several unique properties of these two groups. Firstly, there are roughly 10,000 species of bird, while bats make up 900 of the 4800-mammalian species that are known. This large diversity potentially provides an abundant number of cell types for different coronaviruses. Secondly, the ability to fly has essentially eliminated geographical boundaries allowing for long distance migration and virus exchange with a variety of different animals. Furthermore, roosting and flocking habits result in the gathering of a large number of bats and birds, which would have also facilitated virus spread\(^6\).

Currently, there are six known coronaviruses able to infect humans: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU, Severe Acute Respiratory Syndrome CoV (SARS-CoV) and Middle East Respiratory Syndrome CoV (MERS-CoV). Strains 229E and OC43 are prototype viruses from the Alpha and Beta lineages, respectively, that are responsible for 15-29% of all common cold
cases\textsuperscript{2}. HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU are classified as low pathogenic human coronaviruses that infect the upper respiratory tract causing mild to moderate cold-like illness in healthy individuals. In contrast, highly pathogenic coronaviruses such as SARS-CoV and MERS-CoV can make their way into the lower respiratory tract causing severe pneumonia and respiratory tract damage resulting in high morbidity and mortality\textsuperscript{7}.

1.2 SARS and MERS epidemiology

SARS-CoV first appeared in November 2002 in the Guangdong province of China as an unusual epidemic of ‘infectious atypical pneumonia’ with a high rate of nosocomial transmission. A few months later in February 2003, an infected physician travelled from Foshan to Hong Kong, causing subsequent outbreaks in Hong Kong, that were further transmitted to Singapore and Toronto\textsuperscript{1}. Through international air travel, SARS managed to spread across the globe, affecting 25 countries with 8,098 cases and 774 fatalities by the end of the outbreak in July 2003\textsuperscript{8}. SARS was found to readily spread through close person-to-person contact via droplet transmission or fomites. This was reflected in a super-spreading event where 138 people were found to be infected two weeks after exposure to a hospitalized SARS patient\textsuperscript{1}. As one of the most severe diseases caused by a coronavirus at that time, the outbreak cost nearly $40 billion dollars in economic loss as SARS-CoV shut down a large number of activities for several months in China and Canada\textsuperscript{9}.

Almost a decade later in April 2012, 11 patients including 10 health care workers in Jordan came down with a severe lower respiratory tract infection from a cause that could not be identified at the time\textsuperscript{10}. Less than six months later in Saudi Arabia, an unidentified
coronavirus was detected in the sputum of a 60-year-old man presenting with acute pneumonia and subsequent renal failure\textsuperscript{11}. Thereafter in September 2012, the same virus was observed in a patient with severe respiratory illness who was transferred to the United Kingdom from the Middle East. Analysis of all of these cases recognized novel MERS-CoV as the cause of a new severe respiratory disease\textsuperscript{12}. The spread of MERS has been characterized by nosocomial outbreaks following sporadic zoonotic transmission. Super-spreading events reported in the Arabian Peninsula and South Korean hospitals have been a result of failure in infection control and prevention\textsuperscript{1,13}. Similar to SARS, MERS-CoV has managed to spread to 27 other countries via international air travel. While a smaller number of 2,144 cases have been reported, the mortality rate of MERS infection sits at approximately 35%, a significant increase from the SARS pandemic\textsuperscript{8,14}.

1.3 Virus origins and transmission

In contrast to some previous pandemics cause by the plague and influenza, the death toll of SARS infected individuals is relatively low; however, the rapid spread and prompt media coverage put a spotlight on SARS-CoV. This led to quick identification of the zoonotic virus and wild game from the wet markets as a potential source\textsuperscript{15}. SARS-CoV-like viruses with more than 99% nucleotide homology to SARS-CoV were found in Himalayan palm civets (Paguma larvata) and raccoon dogs (Nyctereutes procyonoides) indicating a route of interspecies transmission, but the natural reservoir could not be determined at that time\textsuperscript{16}. Eventually, these animals were determined to be only incidental intermediate hosts and the wet game markets provided an interface for human transmission. It is likely early interspecies transmission was inefficient
and eventual adaptation towards greater fitness allowed SARS to emerge\textsuperscript{15,17}. A spike in coronavirus research reported genetically diverse SARS-like-CoVs in different species of horseshoe bats (Rhinolophus genus). High sero-prevalence and wide distribution found in these animals are patterns consistent with a pathogen’s natural reservoir host\textsuperscript{4,5}. Subsequent phylogenetic analysis revealed human and civet isolates of SARS-CoV stemmed from SARS-like genomes found in bats\textsuperscript{18}.

\textbf{Figure 1.} Phylogenetic tree analysis of the 816 nucleotide RNA-dependent RNA polymerase gene sequence. \textit{Nycteris} and \textit{Pipistrellus} bat viruses are shown in red and blue, respectively. Data was analyzed with MrBayes version 3.1 (\url{http://mrbayes.sourceforge.net/}). Image from Annan A, Baldwin HJ, Corman V, et al. Human Betacoronavirus 2c EMC/2012–related Viruses in Bats, Ghana and Europe. Open access: Emerging Infectious Diseases. 2013;19(3):456-459. doi:10.3201/eid1903.121503.
When MERS-CoV emerged 10 years later, suspicion immediately fell onto bats as the primary reservoir. Initial phylogenetic analysis of viral genome fragments clustered MERS-CoV with known bat coronaviruses HKU4 and HKUS that belong to a subgroup of lineage Beta-CoV (Figure 1). Coronaviruses from this lineage have previously been recovered from Tylonycteris pachypus and Pipistrellus abramus species of bats. However, direct human contact with bats or bat secretions is rather rare thus, it was suspected, like palm civets during the SARS outbreak, an intermediate host was involved. Camels are a common animal species found in the Middle East and following serological studies found MERS-CoV neutralizing antibodies in a high percentage of dromedary camels. Additionally, viral RNA sequences isolated from a farm in Qatar were almost identical to viral sequences of two human MERS cases in the area. This data strongly suggested dromedary camels acquired the virus from bats some time ago and habitation in large groups has promoted subsequent circulation of MERS-CoV. While the removal of infected intermediate hosts discontinued the zoonotic transmission of SARS, the ubiquity and frequent contact with dromedary camels has allowed the persistence of MERS-CoV.

It has been proposed that transmission of SARS-CoV and MERS-CoV from bats to their respective intermediate hosts may have occurred via the fecal/oral route. Direct handling of civets and camels or consumption of uncooked meat may have then transmitted the viruses to humans. Secondary human-to-human transmission likely occurred through droplet transmission by either aerosolization or fomites. This led to a high number of nosocomial transmissions, which were responsible for 40-100% of individual cases. Spread between family members was substantially lower at 13-21% and 22-39% for MERS and SARS, respectively. The
predominance of transmission between patients and healthcare workers is likely due to viral shedding, which occurs after onset of symptoms, when patients are seeking medical care\textsuperscript{23}.

### 1.4 Clinical symptoms

Non-asymptomatic SARS infections symptom onset begins after an incubation of 2-16 days. Fever, chills, coughing, malaise, myalgia and headache, similar to that of an upper respiratory tract infection, were the most common signs of disease\textsuperscript{27}. About one third of patients show clinical improvements after one week, but some patients may develop a high fever and present with atypical pneumonia. Abnormal chest X-rays may be observed; however, this is more common in MERS infected patients. High morbidity and mortality rates are a result of further progression of disease to fatal acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) despite a decline in viral titres\textsuperscript{28,29}.

Clinical manifestation of MERS begins quite similar to SARS infection with fever, cough, sore throat, myalgia, and shortness of breath, however, rapid progression into pneumonia occurs at higher incidence, which is reflected by the case mortality rates. Gastrointestinal symptoms are also common, presenting as diarrhoea, vomiting and nausea\textsuperscript{30}. Several risk factors have been observed in association with fatal cases in both SARS and MERS. Individuals that were immunocompromised or had comorbidities such as diabetes mellitus, hypertension, cancer, renal and lung disease and other co-infections are more likely to progress to ARDS and therefore have increased odds of a poor disease outcome\textsuperscript{23,24,26}. 
1.5 Viral Genome and Structure

Figure 2. Single stranded RNA genomes (left) and structural diagram (right) of SARS- and MERS-CoV. Coronavirus genomes encode for two large polyproteins (pp1a and pp1b) which is cleaved into 16 non-structural proteins. Additional ORFs are transcribed through nested subgenomic RNA. Spherical virions of SARS- and MERS-CoV are formed by the envelope (E) and membrane (M) glycoproteins. Spike (S) proteins embedded in the envelope protrude out of the virion, while the viral positive sense RNA genome encapsidated by nucleocapsid (N) proteins reside within. Reprinted with permission of Springer Nature: de Wit, E., van Doremalen, N., Falzarano, D., & Munster, V. J. (2016). SARS and MERS: recent insights into emerging coronaviruses. Nature Reviews Microbiology, 14(8), 523.

The RNA genomes of Coronaviruses consist of at least six open reading frames (ORFs). The first ORF makes up almost two thirds of the genome encoding non-structural proteins including RNA-dependent RNA polymerase (Rdrp), helicase and others involved in replication (Figure 2). The rest of the genome translates into structural and accessory proteins that are transcribed from a nested set of subgenomic RNAs. The spike (S) protein, envelope (E) protein, transmembrane (M) glycoprotein and nucleocapsid (N) are structural proteins involved in the formation of viral particles\(^3\). Aside from these standard genes, all coronaviruses have additional ORFs that are interspersed across the genome. These “extra” genes can vary from
one to as many as eight (SARS-CoV) and can be found embedded in another ORF or extensively overlapped with another gene. Although these accessory genes were originally labelled non-structural, some gene products were observed to be components of the virion\textsuperscript{32,33}. For example, a subset of betacoronaviruses encode a haemagglutinin esterase protein that is not required for replication but may be involved in virus pathogenesis\textsuperscript{34}. Located at the 5’ end is a leader sequence and an untranslated region (UTR) with multiple stem loop structures necessary for RNA replication and transcription. Additionally, transcriptional regulatory sequences at the beginning of each structural and accessory gene are also required for expression. UTRs at the 3’ end contain RNA structures required for synthesis of viral RNA and replication\textsuperscript{9}.

Cryo-electron tomography and microscopy observed coronaviruses as spherical virions with diameters of approximately 125 nm\textsuperscript{9}. The shape of the viral envelope is the product of a small ~25-30 kDa transmembrane protein (M). Studies have shown that this abundant structural protein exists as a dimer in coronaviruses, promoting dual conformations that allow binding to the nucleocapsid\textsuperscript{35}.

The most defining features of these viruses are the club-shaped spike projections protruding from their surfaces. These S proteins are present as homotrimers that bear structural organization similar to class I viral fusion proteins that mediate attachment to host receptors\textsuperscript{36}. In a majority of coronaviruses, the spike is cleaved into two polypeptides: a S1 receptor-binding domain and S2 stalk, by host proteases. The S1 domain is variable across coronaviruses, while the S2 is the most conserved\textsuperscript{37}. Some coronaviruses have a set of hemagglutinin-esterase proteins that appear as a second row of protein structures beneath the large globular head of the spike\textsuperscript{32}. 
The interior of the virion consists of an E protein that is found in smaller quantities than both M and S proteins. This particular protein appears to be a transmembrane protein with ion channel activity that is involved with assembly and release of coronaviruses. While non-essential for replication or mRNA synthesis, deletion of the envelope protein has been observed to attenuate SARS-CoV\textsuperscript{38,39}.

The most abundantly expressed protein is the N protein. The primary function of this protein is to bind and package the viral RNA genome into a ribonucleoprotein complex called the capsid. Ribonucleocapsid formation is an essential part of viral assembly as well as replication by the RNA-dependent RNA complex where it serves as a template\textsuperscript{40}. N protein has also been shown to interact with cellular proteins that play a role in RNA replication and transcription. The important role of this protein is supported by evidence that inhibition of this protein decreases viral RNA synthesis by 90\%\textsuperscript{32}.

1.6 Virus life cycle and replication

Initial attachment of coronaviruses to host cells is mediated by S protein interaction with host cell receptor. This occurs via the receptor binding domain (RBD) located at varying sites of the S1 region depending on the virus\textsuperscript{41}. After attachment, some coronaviruses such as Mouse Hepatitis virus fuse with the plasma membrane at the surface of the host cell to gain access to the cytosol. However, other coronaviruses including SARS-CoV and MERS-CoV fuse via receptor-mediated endocytosis within acidified endosomes\textsuperscript{9}. The activation of S protein is accomplished through acid dependent proteolytic cleavage by a variety of host cell proteases.
This is similar to the mechanism exemplified by the hemagglutinin protein of influenza virus where cleavage events lead to the release of viral RNA into the cytoplasm\textsuperscript{42}.

Due to the 5’ cap and 3’ poly A tail found on the viral RNA, the genome can act as mRNA for immediate translation of polyproteins following release into the cytoplasm. The first gene to be translated is the viral replicase gene that encodes two large ORFs expressed as two co-terminal polyproteins. A slippery sequence (5’-UUUAAC-3’) and RNA pseudoknot causes a ribosomal frameshift responsible for the switch in ORFs\textsuperscript{43,44}. Coronaviruses encode a few proteases that cleave the replicase polyprotein into individual non-structural proteins, many of which are assembled into the replicase-transcriptase complex. In addition to their involvement in replication, some non-structural proteins function to block the innate immune response\textsuperscript{9,45}.

\textbf{Figure 3.} Coronavirus replication strategy: following translation of the replicase protein, a full copy complementary minus strand is generated. The replicase continues making minus-strand copies in a

Subsequently, genomic and subgenomic RNA are produced from viral RNA synthesis. The replicase-transcriptase complex synthesizes negative-sense RNA intermediates from genomic RNA (gRNA) to be used as templates for generation of more gRNA\textsuperscript{32}. Shorter minus-strand subgenomic RNA (sgRNA) are also generated as the replicase appears to recognize sites near transcription regulating sequences (TRS) that precede each ORF upstream of their respective start codons. This results in translocation of the replicase towards the 5’ end of the gRNA after copying the internal TRS, passing over large sections of the genome. These minus strand sgRNAs contain complementary leader sequences that are recognized by the viral transcriptase and consequentially are copied into subgenomic mRNA and genomes (Figure 3). Positive sense sgRNAs can serve as mRNA for production of structural and accessory genes downstream of the replicase as well as gRNA to be packaged into viral progeny\textsuperscript{46}.

Replicated genomic RNA is packaged by multiple N proteins into a helical structure (nucleocapsid) in the cytoplasm. Meanwhile, structural proteins S, E and M insert into the endoplasmic reticulum (ER) and are transported to the ER-Golgi intermediate compartment where they meet the nucleocapsids to form mature virions\textsuperscript{33}. Interactions between the M protein and all viral assembly partners and nucleocapsids are the main contributor to the formation of the viral envelope and subsequent assembly of the virion\textsuperscript{47}. Mature virions are transported to the cell surface through the constitutive secretory pathway and released via
exocytosis. A number of coronaviruses form giant multinucleated cells when free un-assembled S proteins are transited to the cell surface and fuse with adjacent uninfected cells. This allows the virus to spread without detection and avoid neutralization by virus-specific antibodies\textsuperscript{23,31}.

1.7 Virus pathogenesis and immunopathology

SARS and MERS are fairly similar in terms of clinical presentation, however, due to the sparsity of data on human infections, the pathogenesis of the causative viruses is poorly understood. The receptors for SARS-CoV and MERS-CoV are angiotensin-converting enzyme 2 (ACE2)\textsuperscript{48,49} and dipeptidyl peptidase 4 (DPP4)\textsuperscript{10}, respectively. ACE2 is an 805-amino acid type I integral membrane protein that plays a role in the renin-angiotensin pathway that regulates blood pressure homeostasis and fluid and salt balance\textsuperscript{50}. Although it’s exact role in the lungs is unknown, data from animal studies suggest a protective role in acute lung injury\textsuperscript{51,52}. ACE2 has been identified on the surface of type 1 and 2 pneumocytes, enterocytes of all parts of the small intestine and proximal tubular cells of the kidney. Accordingly, SARS-CoV has been isolated from the lungs, gastrointestinal tract and urine, as well as the colon despite the lack of ACE2 protein expression in colonic enterocytes. ACE2 is also strongly expressed in endothelial cells of the small and large arteries, veins of the aforementioned tissues and smooth muscle cells of the intestinal tract, but virus infection has yet to be seen at any of these sites\textsuperscript{53,54}. Two other receptors have also been identified as alternative SARS-CoV receptors: liver/lymph node-specific ICAM3-grabbing nonintegrin (L-SIGN) and dendritic-cell-specific DC-SIGN. L-SIGN receptors are mainly found on lymph nodes and liver sinusoidal cells, while DC-SIGN is mainly found on certain dendritic cells (DCs) and alveolar macrophages\textsuperscript{55,56}. However, in vitro studies
have shown that L-SIGN and DC-SIGN expressing cells that do not also express the ACE2 receptor are only partially or not at all susceptible to SARS-CoV infection. This may suggest that the role of these two receptors may merely be to enhance infection of permissive cells\textsuperscript{57,58}. Although ACE2 functions as the binding receptor for SARS-CoV, it also has a role in viral pathogenesis. The binding of SARS-CoV spike protein to ACE2 impairs its ability to cleave and inactivate angiotensin II (Ang II) leading to increased levels in the lungs and plasma. Studies have shown the raised levels of the Ang II and it stimulation of its receptor (Ang II type 1a) lead to increased pulmonary vascular permeability, lung oedemas and impaired lung function, all contributing factors to ARDS\textsuperscript{51,52,59}.

The DPP4 receptor is a transmembrane glycoprotein primarily expressed as a homotrimer in epithelial cells in the kidneys, small intestine and liver. It is also expressed on B and T cells and exists in a soluble form that circulates the body. DPP4 is considered a housekeeping protein that functions as a serine protease, receptor, costimulatory protein, adhesion molecule and is involved in apoptosis\textsuperscript{60}. Wide spread expression of this receptor in the body corresponds to systemic dissemination of MERS-CoV observed in clinically infected patients\textsuperscript{61}. In other respects, DPP4 does not seem to play as direct of a role in pathogenesis as seen by the SARS-CoV ACE2 receptor, but our understanding of MERS-CoV pathogenesis is still greatly lacking.

Perhaps the most significant factor in pathogenesis and subsequent progression to ALI and ARDS revolves around the host immune system. A strong antiviral response by the innate immune system is able to limit viral replication and subsequent spread. Host pattern recognition receptors (PRRs) are proteins expressed on a variety of cells whose role is to
identify invading pathogens and mount a defensive response. PRRs recognize pathogen-associated molecular patterns (PAMPs) such as glycoproteins, lipopolysaccharides and nucleic acid motifs that are vital to the survival or infection process of a microbe. This interaction triggers a series of complex signalling cascade events that lead to a production of type I interferons, transcription of pro-inflammatory cytokines and activation of the adaptive immune system\textsuperscript{62}. While this response is able to limit invading pathogens to some extent, SARS-CoV and MERS-CoV have various strategies in delaying and dis-regulating the immune system.

Rapid viral replication results in massive inflammatory cell infiltration and elevated chemokine/cytokine responses. Both SARS and MERS coronaviruses encode several structural and non-structural proteins that inhibit or dampen the immune response and by doing so allows for rapid viral replication. SARS-CoV nsp1, nsp3-macromdomain, nsp3-deubiquitinase (DUB), and ORF3b, ORF6, and ORF9b proteins and MERS-CoV accessory proteins orf3, orf4a, and orf4b antagonize IFN and IFN-stimulated gene (ISG) expression. Structural proteins M and N of SARS-CoV and MERS-CoV also contribute to dampening of the IFN response\textsuperscript{7,63}. The mechanisms by which these proteins inhibit the immune response, some of which are unknown, apply to different stages in the immune response. For example, the SARS-CoV nsp1 protein inhibits the STAT while MERS-CoV ORF4b blocks activation of RNAse L\textsuperscript{64}. It is important to note that many of these putative antiviral proteins and their mechanisms were evaluated via transient expression assays and the significance of their roles in infection are yet to be determined.

A rather high rate of immune cells including DCs, macrophages, monocytes and T lymphocytes have been observed to be infected during the early phase of SARS disease\textsuperscript{65,66}.
Although infection of these cells by SARS-CoV is abortive, a large up-regulation of cytokines and chemokines is caused\textsuperscript{67,68}. In contrast, MERS-CoV is able to replicate in monocyte-macrophages, DCs and T cells and infection also induces high levels of pro-inflammatory chemokines and cytokines\textsuperscript{61}. SARS patients with severe clinical presentation of disease exhibited high serum levels of pro-inflammatory cytokines (IFN-\(\gamma\), IL-1, IL-6, IL-12, and TGF\(\beta\)) and chemokines (CCL2, CXCL10, CXCL9, and IL-8) compared to those with uncomplicated infections. These same patients also exhibited relatively elevated levels if IFN (IFN-\(\alpha\) and IFN-\(\gamma\)) and IFN-stimulated genes\textsuperscript{7}. Similarly, elevated levels of serum pro-inflammatory cytokines (IL-6 and IFN-\(\alpha\)) and chemokines (IL-8, CXCL-10, and CCL5) were seen in severely infected MERS individuals\textsuperscript{69,70}.

The initial consequences of high viral load and exuberant immune response is apoptosis of lung epithelial and endothelial cells. Death of these cells compromises the microvascular and alveolar epithelial cell barrier resulting in vascular leakage and alveolar edema. Up-regulated inflammatory responses also induce T cell apoptosis which are crucial in viral clearance and limitation of host cell damage. Along with uncontrolled inflammation, these events contribute to induction of ALI and ARDS, a devastating end-stage lung disease\textsuperscript{7,63}.

1.8 Coronavirus spike protein

A key determinant to host specificity for coronaviruses is the surface spike protein. This glycoprotein consists of three segments: an ectodomain, a single pass membrane anchor and a short intracellular tail. The ectodomain can be further divided into a N-terminal S1 subunit and a C-terminus membrane embedded S2 region\textsuperscript{71}. The S1 segment in itself contains two independent domains (N-terminal domain and C terminal domain) and is found to be more
variable in sequence and is involved in recognizing host cell receptors\textsuperscript{72}. For most coronaviruses, the RBD is found in the C-terminus of the S1 domain. However, the N-terminus may promote initial attachment to host cells by recognizing sugar moieties. Overall, coronaviruses have a rather complex receptor recognition pattern; coronaviruses with highly similar S1 C-terminal domains within the same genus are able to recognize completely different receptors. Vice versa, coronaviruses with different S1 C-terminal domains from different genera can recognize the same receptor\textsuperscript{73}.

Following binding of the S1 protein to the target receptor, S2 mediates fusion between viral and cellular membranes. S2 functions similar to type I fusion proteins, with at least one fusion peptide and conserved heptad repeats to drive membrane penetration and virus-cell integration\textsuperscript{72}. It is suggested that the fusion peptides insert into the target membranes while the heptad repeats trimerize to bring the virus envelope and host cell surface closer together. However, in order for this to take place the S2 subunit needs to be cleaved by host cell proteases, illustrating another key factor in cell tropism\textsuperscript{9,37}.

The 1255 residue SARS-CoV S glycoprotein is typically synthesized in an inactive form that requires activation by host cell proteases to transition into a fusion active state\textsuperscript{74}. SARS-CoV spike protein is largely un-cleaved after biosynthesis due to the lack of furin-recognizable sites, but it can be later cleaved by endosomal cathepsin L allowing infection via endocytosis. Additionally, extracellular enzymes such as trypsin, thermolysin and elastase can activate viral S and induce syncytia formation and virus entry\textsuperscript{42}. SARS spike has two distinct cleavage sites, one at the S1/S2 boundary (residue 667) and another upstream of the putative fusion peptide in the S2 domain. Both cleavage sites are crucial in the role of spike protein activation and viral entry.
and cleavage at each is believed to occur sequentially. Cleavage at the first S1/S2 site enhances subsequent cleavage at S2, which is believed to be vital for fusion activation\textsuperscript{74,75}.

Crystal structures of SARS-CoV spike protein and ACE2 interaction have been resolved and a 193-amino acid RBD has been found in the C-terminal portion of S1. Studies have found that this small fragment within residues 318 to 510 is able to bind to ACE2 with greater affinity than the full S1 domain (residues 12-672)\textsuperscript{76}. Specifically, residues 424-494, referred to as the receptor binding motif (RBM), make all the contact with the ACE2 receptor. Spike protein sequences from SARS-CoV isolated from civets during the 2002-2003 epidemic only differ at four positions (residues 344, 360, 479 and 487) compared to viruses isolated from humans. Despite the small divergence, human viral spike protein is able to bind human ACE2 almost $10^4$ times tighter than the civet counterpart\textsuperscript{77}. Most SARS-like coronaviruses isolated from bats contain deletions in the RBM and are unable to interact with human ACE2. However, Ge et al. isolated a strain from Chinese horseshoe bats able to recognize both bat and human ACE2 receptors, further validating suggested bat origins of SARS-CoV. Although palm civets and raccoon dogs were postulated as intermediate hosts, this data suggests transmission from bats directly to humans may be a potential scenario\textsuperscript{78}.

MERS-CoV S protein is a 1353 residue glycoprotein that bears limited sequence identity to the SARS-CoV spike protein. Another key difference between them is the processing of MERS spike into S1 and S2 subunits after protein biosynthesis. Furin cleavage to separate the two segments occurs between residues 751 and 752. A second furin cleavage site was identified in S2 between R887 and S888, upstream of the putative fusion peptide corresponding to the second cleavage seen for SARS-CoV priming\textsuperscript{42,79}. The MERS viral RBD has been narrowed down
to residues 377 to 588 of the S1 subunit and crystallographic analyses has indicated that the RBM is specifically located within residues 484-567\textsuperscript{80,81}.

1.9 Treatments

Currently, there are no licensed therapies able to specifically treat human coronavirus infections. Patients mainly received supportive care often supplemented by various types of antivirals, interferons, broad spectrum antibiotics and oxygen\textsuperscript{23}. During the SARS outbreak, ribavirin was the most frequently used medication and was often combined with corticosteroids for its anti-inflammatory properties. Interferon (IFN) alpha was also administered and in some cases in conjunction with ribavirin, however, retrospective analysis did not determine whether any of these treatment combinations were effective. Some data even showed significant toxicity associated with the use of ribavirin\textsuperscript{28}.

Ribavirin and IFNs yielded good results against MERS-CoV in cell culture and the combination of the two were able to reduce disease severity in a rhesus macaque model by reducing host inflammatory response and viral replication. However, because of potential side effects, these antivirals are not considered first-line treatments against MERS\textsuperscript{23,82}. In one human cohort study of five infected MERS patients from Saudi Arabia, clinical outcomes did not improve after patients were administered ribavirin and IFNα2b 10 days after admission. Another study saw improvement when treatment was administered 14 days after diagnosis, but not 28 days\textsuperscript{30}.

High-throughput screening of large libraries has identified previously approved pharmaceutical drugs among several classes with \textit{in vitro} activity against SARS-CoV and MERS-
CoV. One of these classes includes neurotransmitter receptor agonists, ordinarily used for psychiatric help by blocking the dopamine/anti-histamine receptor. Drugs like chloroquine and chlorpromazine have been previously approved for human use and has shown to inhibit MERS-CoV infection \textit{in vitro} \textsuperscript{83}. Protease inhibitors such as lopinavir and ritonavir are used in combination to treat HIV positive patients. However, when combined with ribavirin, these two drugs were observed to improve the outcome of SARS infected individuals\textsuperscript{84}. Despite lack of clear antiviral activity against MERS-CoV \textit{in vitro}, marmosets treated with lopinavir and ritonavir 6 hours post infection also saw better outcomes\textsuperscript{85}.

Another alternative treatment used in subsequent outbreaks was convalescent plasma collected from previously infected patients. Passive immunotherapy is a practice that has been used for over a century that takes advantage of high neutralizing antibody titres present in human sera. Antibodies produced by stimulated B lymphocytes are able to neutralize viruses by either blocking virus-host cell interactions, aggregation and/or recognition that leads to antibody-dependent cytotoxicity (ADCC) or complement-mediated lysis\textsuperscript{86}. Analysis of 32 studies using convalescent plasma to treat severe acute respiratory infections showed a reduction in mortality rates. These studies, however, appeared to be of low quality, lacked control groups and were at risk of bias\textsuperscript{87}. A similar protocol was developed for MERS-CoV but was ultimately scrapped due to the lack of convalescent sample donors\textsuperscript{88}.

Polyclonal and monoclonal antibodies have a broad range of applications including therapeutic intervention against virus infections. With the ability to direct these antibodies to a specific antigen, many studies have aimed to block the interaction between coronavirus spike protein and their respective host cell receptor. Those that have been developed for potential
treatment of MERS-CoV were found to be potently neutralizing in vitro and were able to reduce lung pathology in vivo, but viral replication could not be completely inhibited\(^{89-93}\).

1.10 Vaccines

Several strategies have been employed to generate a vaccine against coronaviruses such as inactivated viruses, live-attenuated viruses, subunit vaccines, recombinant proteins, viral vectors and DNA vaccines. Most studies focus on vaccine development against SARS-CoV and use non-lethal animal models for evaluation\(^{25}\). Inactivated viruses have a few advantages as a vaccine candidate. They are easy to manufacture and inactivation by radiation or chemicals renders the virus non-infectious but still antigenically similar to live virus\(^{94}\). Previous studies demonstrated that administration of inactivated SARS virus with or without the use of adjuvants was able to induce neutralizing antibodies that protected against viral replication\(^{95-97}\). Use in humans was well tolerated and elicited neutralizing antibodies, however, the absence of natural infection leaves no data on vaccine efficacy\(^{98}\).

Live attenuated SARS-CoV was generated by removing the E protein or exonuclease (ExoN) from the nsp14 coding region in ORF1B which is involved in proofreading for the viral RdRp. A deletion mutant of SARS-CoV lacking the envelope protein was found to protect against virus challenge in hamsters, but only partially in the human ACE2 transgenic mouse model\(^{38,99}\). Removal of these genes in mouse-adapted strain 15 (MA15) and subsequent challenge in aged mice elicited high titres of neutralizing antibodies\(^{100}\). Another attenuated MA-SARS virus, with inactivated ExoN, was observed to elicit large numbers of neutralizing antibodies. This strain is highly resistant to reversion back to virulence due to the increased mutation frequency that
introduces more attenuating/neutral mutations. Live attenuation may be a prime candidate for the design of a MERS-CoV vaccine as genes such as ExoN are conserved across all coronaviruses\textsuperscript{25,101}.

Viral vector vaccines function as gene delivery systems that encode antigenic components of a virus of interest to elicit a targeted immune response. Adenovirus vectors expressing SARS S and N proteins yielded varying results depending on a multitude of factors: preparation, the route of administration and the animal model used\textsuperscript{94,97}. Compared to inactivated virus vaccines, neutralizing antibody titres were significantly lower and protective abilities were also diminished\textsuperscript{102}. Subunit vaccines containing SARS-CoV S protein and DNA-Spike vaccines have also been found to elicit high neutralizing antibody responses but in vivo challenges have yet to be performed\textsuperscript{103,104}.

Although a lot of progress has been made since the emergence of MERS in 2012, there are currently no vaccines available for human use. As the approaches for SARS-CoV were promising, the same methods were used in the development of a MERS-CoV vaccine. While both the S and N proteins showed high immunogenicity, current MERS vaccine candidates mainly make use of all or parts of the spike glycoprotein, especially the RBD\textsuperscript{105}. Predominantly, current vaccines under development are based on viral vectors (modified vaccinia Ankara, adenovirus, measles virus, rabies virus, and Venezuelan equine encephalitis replicons) expressing MERS S/S1 proteins, nanoparticles, subunit, DNA and DNA/protein. Viral vectors are able to induce a strong protective immune response, but there are limitations. In addition to possible pre-existing immunity, neutralizing antibodies can be produced when the viral vectors are administered\textsuperscript{106–110}. Protein based platforms such as virus-like particles (VLPs),
nanoparticles, peptide-based, and subunit vaccines directed against various regions of the spike protein S1, N-terminal domain and RBD have the highest safety profiles but fluctuate in immunogenicity. However, immunogenicity of these approaches could be significantly improved by optimization of dosage, site of administration and use of adjuvants\textsuperscript{105}. Current versions of DNA vaccines expressing the full-length S protein have been observed to induce potent immune responses in both mice and rhesus macaques, which later protected non-human primates (NHPs) after immunization. Additionally, S protein DNA vaccine in combination with recombinant protein S1 in a prime/boost strategy was able to induce higher immune responses than when used alone\textsuperscript{111}. One of the main concerns with using the full-length spike protein with any of the mentioned methods is possibility of enhancement of viral infection or harmful immune responses as seen in the evaluation of SARS-CoV s protein viral vector vaccines, therefore special attention should be paid in development of these vaccines\textsuperscript{112}.

As dromedary camels are the intermediate hosts for MERS, some studies have involved using vector-based vaccines in these animals to potentially reduce horizontal and zoonotic transmission. During the outbreak of SARS, civets were culled, which may have limited the outbreak though widespread as it was. The modified vaccinia Ankara vaccine against MERS has been shown to elicit neutralizing antibodies and reduce virus shedding in camels\textsuperscript{13}. This approach could be useful in problematic regions where people regularly come in close contact with these animals and warrants further studies.
1.11 Animal models

1.11.1 Non-human primate model

Rhesus macaques, African Green monkeys (AGMs), cynomolgus macaques and common marmosets have all been shown to support SARS-CoV replication, however, significant variation in viral replication, pathology and clinical disease symptoms has been noted among these models\textsuperscript{113}. Virus was detected in lung samples and nasal secretions but, disease observed in infected rhesus and cynomolgus macaques were mild and self-limiting\textsuperscript{114,115}. Moderate to high viral titres can be detected in AGMs, but these titres usually peak after 2 day and virus cleared from the respiratory tract 8-10 days post infection\textsuperscript{116}. Marmosets also exhibited only mild respiratory illness, but evidence of interstitial pneumonia and hepatic pathology was observed\textsuperscript{117}. Despite susceptibility to infection, none of these NHPs reproduced severe clinical disease or mortality observed in humans.

Rhesus macaques and common marmosets also support MERS-CoV replication, but as seen with SARS-CoV, disease presentation varied among the animals. Rhesus macaques infected with MERS-CoV isolate HCoV-EMC/2012 via multiple routes (oral, ocular, intranasal and intratracheal) developed transient lower respiratory tract infections with mild clinical signs, virus shedding and viral replication peaking early in infection. Not surprisingly, all infected animals survived until the designated end point of 6 days post infection\textsuperscript{118}. The most severe signs of disease and highest viral loads were observed in MERS-CoV infected marmosets in a study conducted by Falzarano et al\textsuperscript{119}. While post mortem analysis of macaques revealed pathology restricted to the lungs, viral RNA was detected in the blood, kidney, liver and heart of infected marmosets indicating viral dissemination. Furthermore, due to severe disease
progression, a few animals required euthanization\textsuperscript{119}. Because the common marmoset reproduced several features of MERS-CoV infection seen in humans, this model could potentially be used to evaluate novel therapeutics. On the other hand, the cumbersome nature of using such a large animal model has warranted studies for a smaller alternative.

1.11.2 Mouse model

Several mouse models have been established for SARS-CoV. Despite human ACE2 receptor only sharing 83% identity with mouse ACE2, SARS-CoV isolated from infected patients was able to replicate in several strains of inbred mice (BALB/c, C57BL6 and 129S)\textsuperscript{120}. Symptoms exhibited in these mice, however, did not reflect clinical disease observed in humans, showing minimal respiratory illness and weight loss (<5%). Furthermore, younger mice rapidly cleared the virus and did not present with any symptoms of disease. Two approaches were utilized to enhance infection in mice: development of transgenic mice expressing human ACE2 and viral adaptation via serial passaging in mice\textsuperscript{121}. Transgenic mice expressing human ACE2 developed by Tseng \textit{et al.}\textsuperscript{122} showed strong viral replication, clinical manifestation and tissue pathology after infection with SARS-CoV. Of the two lineages generated by this group, AC70 and AC63, one yielded a lethal mouse model (AC70). Several mouse-adapted strains of SARS-CoV were propagated by passaging the Urbani strain in the respiratory tract of young mice. These viruses replicated to high titres and disease observed in mice resembled that seen in severe human cases. Mutations found among the genomes of mouse adapted SARS-CoV strains were found in the non-structural protein nsp9 and spike glycoprotein, both of which are important in viral pathogenesis\textsuperscript{121,123}.
In contrast, mice do not naturally support MERS-CoV replication as murine DPP4 receptors differ from human DPP4 (hDPP4) in areas critical for interaction with viral spike protein, thus similar approaches have been utilized to improve viral susceptibility. In initial studies to generate a model by Zhao et al., mice were transduced by delivering an adenoviral vector expressing hDPP4 by the intranasal route. This approach yielded mice that supported viral replication associated with interstitial pneumonia and signs of disease including weight loss, but mortality was not observed. Later on, another group adopted the method used to generate the MERS transgenic mouse model and globally expressed hDPP4 in all tissues under transcriptional control of a CAG promoter. These mice were fully susceptible and exhibited a robust infection with severe respiratory disease that was eventually lethal. Additionally, MERS-CoV was found to have disseminated to other organs such as the heart spleen and intestines likely because the hDPP4 was overexpressed in all tissues. Although this model elicited a lethal respiratory disease, overexpression of the hDPP4 may complicate the study of MERS-CoV pathogenesis and host immune response.

More recently, two separate studies modified the murine DPP4 gene within the genome to generate knock in mice susceptible to MERS-CoV infection. One study used a RNA-guided genome editing tool, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9), to insert human codons for amino acids 288 and 330, while the other utilized a plasmid-mediated gene targeting strategy to replace murine exons 10-12 with human equivalents. In both transgenic animals the distribution of DPP4 remained

---

\[a\] The CAG promoter consists of the cytomegalovirus (CMV) immediate-early enhancer element, the chicken \(\beta\)-actin promoter and rabbit globulin splicing and polyadenylation sites.
the same as in the wildtype mouse and MERS-CoV was able to establish an infection. However, viral replication was limited by other factors including the inability of the virus to suppress the murine innate immune response, so the virus was further passaged in the lungs of the engineered animals to increase replication efficiency. Modified mice infected with the adapted virus exhibited symptoms such as decreased pulmonary function, significant weight loss, pathological signs indicative of ARDS and mortality, aligning with severe clinical disease seen in humans\textsuperscript{110,127}.

1.11.3 Syrian hamster and ferret model

Golden Syrian hamsters have been found to be highly permissive to infection by SARS-CoV Urbani strain, and subsequently shown to be susceptible to other strains as well. Infection results in high viral titres, transient viremia and pulmonary pathology. Despite the fact that mortality was not a prominent feature among this model, hamsters were initially deemed more suitable than wild type mice for evaluation of therapeutics and vaccines against SARS-CoV\textsuperscript{123,128}.

Based on the success of hamsters as a model for SARS-CoV, these animals were evaluated for MERS-CoV susceptibility. Despite high expression levels of the DPP4 receptor, clinical signs of disease and weight loss were not observed after intratracheal inoculation by strain EMC-2012. Additionally, qRT-PCR analysis of nasal swabs taken daily post infection and post-mortem tissue samples came back negative for viral RNA. Similar to mice, these results show that Syrian hamsters are not naturally permissive to MERS-CoV infection and is therefore not an adequate animal model for this virus\textsuperscript{129}.
The same trend was observed in the ferret model for both SARS-CoV and MERS-CoV. Ferrets have presented as excellent models for influenza and when evaluated for SARS-CoV, supported viral replication and showed varying signs of illness, but mortality was not observed\textsuperscript{130,131}. In MERS-CoV infected ferrets, virus was not recovered from nasal and throat swabs at any time point post infection and only low levels of viral RNA were detected 1-2 days after intratracheal inoculation. Later studies found that ferret kidney cells were resistant to infection despite high levels of expression of DPP4. Subsequent transfection with a human DPP4 expressing plasmid permitted viral replication in the cells, demonstrating again that animal DPP4 is a major host restriction factor\textsuperscript{132}. 
Chapter Two: Research Goals

2.1 Rationale

Taking into consideration the SARS-CoV outbreak in 2003 and the ongoing outbreak of MERS-CoV, therapeutic development against coronaviruses is a vital area of research. With the many factors contributing to mutations in the replication cycle, it is almost inevitable that another coronavirus will cross the species barrier and potentially cause another deadly outbreak. Previously approved drugs that are non-specific to coronaviruses have had little to no effect on clinical disease or patient outcomes. This study aims to develop a therapeutic able to specifically target and neutralize SARS-CoV and MERS-CoV by concentrating on the viral spike protein which is essential for viral entry. The novel treatment will be derived from the fusion of spike protein specific peptide and Fc domain of an IgG antibody in a construct called a peptibody. This synthetic alternative to monoclonal antibodies has been previously developed as a therapeutic to modulate biological processes. Compared to monoclonal antibodies and other treatments used against infecting pathogens, the design of peptibodies provide some distinct advantages. Their synthetic nature allows for high specificity through the peptide domain, while their small size allows for efficient production. Additionally, the Fc portion of the peptibody is able to prolong its half-life by recycling via the FcRn salvage pathway and potentially mediate ADCC and complement-dependent cytotoxicity (CDC).

Initially, I targeted SARS-CoV as a proof-of-concept model as a lethal animal is available for subsequent in vivo evaluation. Based on the expectation of a successful outcome with the SARS-CoV model I proposed to generate a peptibody against MERS-CoV. As a small animal model for MERS-CoV is not commercially available, I also proposed to develop a MERS-CoV
susceptible mouse model for evaluation of our novel therapeutic. Mice are not naturally susceptible to infection by MERS-CoV due to the lack of the viral target human DPP4 receptor. However, transduction of this receptor with a viral vector into the airways has been shown to generate a susceptible model. In this study I used viral vectors Adenovirus (Ad) serotype 5, Adeno-associated virus (AAV) serotype 6 and AAV serotype 9 to transduce the hDPP4 receptor into a murine model to allow MERS-CoV to provide human DPP4 in murine airway cells to establish an infection.

2.2 Hypotheses

i. A peptibody based on peptides that represent the viral spike binding site of the cellular receptor will target the spike protein of coronavirus to effectively bind and neutralize the virus.

ii. AAV and Ad vectors will provide an effective delivery system to transduce the airway of mice with hDPP4, permitting mice to be effectively infected with MERS-CoV. Furthermore, AAV vectors may provide additional advantages over Ad vectors by permitting longer and stronger transduction of airway cells.
Chapter Three: Materials and Methods

3.1 Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC). Human embryonic kidney (HEK) 293 cells that contain the SV40 T antigen (293T) and African green monkey kidney (Vero and Vero E6) cells were cultured in Dulbecco’s modified Eagle medium (DMEM, HyClone for Vero E6) or Minimal Essential Medium (MEM, HyClone for Vero) supplemented with 5% heat inactivated (56°C for 30 min) bovine growth serum (BGS, HyClone) and 2 mM L-glutamine. HEK 293 GripTite™ (293GT) cells were cultured in DMEM supplemented with 1X non-essential amino acids, 5% BGS, 2 mM L-glutamine and geneticin at a concentration of 600 μg/ml. Baby Hamster Kidney (BHK) cells were cultured in MEM supplemented with 5% BGS, 2mM L-glutamine, and 1x Tryptose Phosphate Buffer. Cells were grown in a 37°C humidified incubator with 5% CO₂. Passaging of cells was performed by first removing old medium, followed by a brief rinse with phosphate buffered saline (PBS). One ml of 0.05-0.25% trypsin-EDTA (HyClone) was then added and cells were incubated room temperature until they detached from the bottom of the flask. Fresh culture medium was used to resuspend the cells. For routine maintenance, cells were passaged when they reached ~90% confluency.

3.2 Virus strains and stock preparation

3.2.1 Adeno-associated virus serotype 6 (AAV6)

A human DPP4 expressing AAV6 virus was produced by cloning the hDPP4 sequence using primers AAV6-DPP4F and AAV6-DPP4R described in Table 1. These primers were designed to contain adjacent sequences flanking the SacI and HindIII cut sites of the pACAGGFP plasmid.
provided by Dr. Wootton of the University of Guelph. The hDPP4 gene sequence was PCR amplified using a commercially sourced plasmid containing the full gene sequence (pCMV-MERS-Spike ORF, Sino Biological) as template, ligated by using the InFusion kit protocol (Takara) into pACAGGFP, as described in detail below. The ligated vector was transformed into Stellar™ competent E. coli cells as described in section 3.3.4. Primers AAV6 seq1-5 were used to screen for presence of the DPP4 insert via sequencing (Table 1). As the inverted terminal repeat sequences are prone to deletion and rearrangement, plasmid pACAGhDPP4 was propagated in SURE2 Supercompetent cells (Agilent Technologies) rather than Stellar™ competent E. coli cells described in section 3.3.4. Transformation, however, was performed in the same manner.

To produce the AAV virions, plasmid pACAGhDPP4 was co-transfected with a packaging plasmid (pDMG6) that encodes and expresses the AAV6 capsid, Rep 68 and 78 proteins as well as Adenovirus helper proteins. For transfection, 293T cells were seeded onto forty 10 cm diameter poly-lysine coated tissue culture dishes to a confluency of ~75% for the day of transfection. Transfection mixtures were prepared using the CalPhos™ Mammalian Transfection Kit (Clontech) as described in the manual. Briefly, solution A was prepared by mixing 400 µl of 1 µg/µl concentrated pACAGhDPP4 plasmid, 800 µl of 1 µg/µl concentrated pDMG6, 3.5 ml of 2 M calcium solution in 23.3 ml of tissue culture grade H₂O. While vortexing solution A, 28 ml of 2X HEPES buffered saline (HBS) was added dropwise. After a 15-minute incubation at room temperature, 1.4 ml of transfection solution was added dropwise to each culture plate. Cells were incubated for 24 hours in a 37°C humidified chamber containing 5% CO2. After 24 hours, the transfection solution was aspirated from each dish and replaced with 10 ml of DMEM without BGS and incubated again in the humidified chamber. Cells were then
dislodged and harvested 48 hours later and subjected to three rounds of freeze thawing in a dry ice and ethanol bath and 37°C water bath, and vortexed after each thaw. To remove cell debris, crude lysate was passed through a Stericup-GP (0.22 μm) and SteriTop vacuum-filtration system (Millipore Sigma).

As AAV6 is known to bind to heparin and is not inhibited by it (Halbert 2001), 5 ml HiTrap Heparin HP columns (GE Healthcare Life Sciences) were used to purify the AAV6-DPP4 virions. Masterflex platinum-cured tubing (L/S 14) (Cole-Parmer) was threaded through a MasterFlex® Easy-Load Pump Head (Thermo Fisher Scientific) attached to a MasterFlex® P/S Stainless Peristaltic Pump (Thermo Fisher Scientific) and attached to the inlet of the heparin column via a Union 1/16” male/female luer coupler. Additional tubing was attached to the flow through end of the column. Plain DMEM was pumped through the column to flush out storage medium and to examine for leaks. Filtered sample lysate was applied to the column at a rate of 2 mls/min and washed with the following solutions: 50 ml of Hank’s Balanced Salt Solution (HBSS) buffer (without Mg²⁺/Ca²⁺), 15 ml of 0.5% N-Lauroylsarcosine (Gibco)/HBSS (without Mg²⁺/Ca²⁺), 50 ml of HBSS (without Mg²⁺/Ca²⁺), followed by 50 ml of HBSS (with Mg²⁺/Ca²⁺) and finally 50 ml of 200 mM NaCl/HBSS (with Mg²⁺/Ca²⁺). Virus was then eluted with 25 ml of 300 mM NaCl/HBSS (with Mg²⁺/Ca²⁺) in aliquots of 5 ml.

Eluted fractions 2 and 3 were concentrated using two Amicon Ultra 15 Centrifugal Filter Units (Millipore Sigma) with a membrane nominal molecular weight limit of 100 kDa.

pretreated overnight at 4°C and over day at room temperature with 5 ml of HBSS (with Mg²⁺/Ca²⁺)/5% Tween® 20 (Sigma). Prior to loading virus samples, HBSS/Tween solution was removed and Amicon columns were washed twice via centrifuging 2 ml of HBSS (with
Mg\(^{2+}/Ca^{2+}\)) through the membrane for 2 minutes at 900g. Virus fractions were spun in the Amicon columns at 900xg at 2-minute intervals until approximately 500 µl of sample was left in the upper part of the column. Virus was then removed and placed in a clean tube, while 500 µl of HBSS without NaCl was added into each Amicon column and pipetted up and down to dislodge virus that may have stuck to the filter. This buffer was added to the concentrated AAV6-hDPP4 stock resulting in a final NaCl concentration 150 mM, which is optimal for in vivo transduction. Stock solution was divided into 500 µl aliquots and stored at -80°C.

Table 1. Primers for amplification of DPP4 for cloning into plasmid pACAGGFP. Primers used for sequencing are also listed below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPP4 fragment</td>
<td></td>
</tr>
<tr>
<td>AAV6-DPP4F</td>
<td>TCCTCGACCGAGCTATGAAGACACGTGGAGGT</td>
</tr>
<tr>
<td>AAV6-DPP4R</td>
<td>CGGTATCGATAAGCTTAAGGAAAAACATTTTT</td>
</tr>
<tr>
<td>Sequencing</td>
<td></td>
</tr>
<tr>
<td>AAV6seq1</td>
<td>CCATGTTCATGCTTTCTTTT</td>
</tr>
<tr>
<td>AAV6seq2</td>
<td>CAGAATCAGATCCAGGAGG</td>
</tr>
<tr>
<td>AAV6seq3</td>
<td>CTGCACATTATTACAAAAAGCA</td>
</tr>
<tr>
<td>AAV6seq4</td>
<td>GTTGATTGCATGCATGATCTTA</td>
</tr>
<tr>
<td>AAV6seq5</td>
<td>CCAGACCATGATAAGATACATTTG</td>
</tr>
</tbody>
</table>

3.2.2 AAV9 and Adenovirus serotype 5 (Ad5)

AAV9 encoding human DPP4, AAV blank control encoding luciferase, Ad5 control encoding GFP and Ad5 encoding human DPP4 were purchased from Applied Biological Materials Inc. (Abm Inc.) at titres of 10\(^{12}\) genome copies per ml (GC/ml), 10\(^{12}\) GC/ml, 10\(^{6}\) pfu/ml and 10\(^{5}\) pfu/ml, respectively. Seed stocks were frozen in -80°C. Viral stocks of Ad5-ctrl and Ad5-hDPP4 were prepared in T150 cm\(^2\) tissue culture flasks (Corning) seeded with 293GT cells. Higher titre virus stocks were prepared in the same manner using ten T150 cm\(^2\) flasks instead of
one. When cells reached a confluency of ~80%, old culture medium was replaced with DMEM/1% BGS/2mM L-glutamine and 100 μl of seed virus stock. Cells and medium were collected when over 90% of cells detached from the bottom of the flask (approximately 8-9 days post infection). Collected cells and supernatants were frozen in a dry ice/ethanol bath and thawed in a 37°C water bath a minimum of 3 times to lyse cells. To harvest virus, cell debris was pelleted by a 10-minute centrifugation at 3,000 rpm at room temperature and supernatants were collected. Supernatants were further subjected to high-speed ultracentrifugation at 25,000 rpm for 1 hour at 4°C using a SW32Ti rotor and Beckman-Coulter L90-K ultracentrifuge. Supernatants were discarded, and 1 ml of PBS was added to the viral pellets and incubated overnight at 4°C. After 24 hours, viral pellets were re-suspended by pipetting up and down and combined, aliquoted and stored at -80°C. Final stock of Ad5 viruses were quantified using plaque assays as described in section 3.6.3. All experiments involving AAV and Adenovirus were performed under biosafety level 2 (BSL-2) conditions at the National Microbiology Laboratory (NML) in Winnipeg, Canada.

3.2.3 SARS-CoV and MERS-CoV

According to the World Health Organization (WHO), the handling of live SARS-CoV and MERS-CoV and inoculation of animals must be performed in BSL-3 facilities or higher\textsuperscript{133}. Therefore, all experiments involving SARS-CoV and MERS-CoV were performed in BSL-3 or BSL-4 conditions at the NML. SARS-CoV (Tor 2 strain) was produced by reverse genetics from a full genomic clone, pBAC/SARS-CoV, that had previously been generated in our lab in the Bacterial Artificial Chromosome vector pBAC-TrueBlue. It was generated by transfection of the plasmid
into Vero E6 cells as described in detail below. Similarly, MERS-CoV was also rescued by reverse genetics by transfection of BHK cells with the full genome plasmid pBAC/MERS-CoV that had previously been generated in our lab. Each of these cell lines was seeded into a 6 well dish a day prior to transfection to reach a confluency of ~90%. On the day of transfection, old growth medium was removed, and cells were washed with PBS and overlaid with 1 ml of Opti-MEM per well. Transfection solutions were set up by first adding 10 μl of LT1 reagent to 200 of Opti-MEM for each reaction. 2 μg of plasmid DNA was then added to the Opti-MEM/LT1 solution and gently stirred with the pipet tip to disperse. Both solutions were incubated at room temperature for 15 minutes and added drop wise to the corresponding cells for rescue of each virus. Cells were then incubated in a 37°C humidified chamber with 5% CO₂ in BSL-3 containment. After 48 hours, supernatants were harvested and placed in 2 ml tubes. Prior to -80°C storage, 2 μl of tosyl phenylalanyl chloromethyl ketone treated-trypsin (TPCK-trypsin; 1 μg/μl stock in H₂O; Sigma) was added to 1 ml of the MERS-CoV supernatant and incubated at 37°C for 5 minutes. Trypsin treatment of the MERS-CoV cleave activates the Spike protein, and subsequently increases efficiency of propagation from the virus produced from transfection of the BHK cells although it is not needed for further propagation of the virus when producing a larger stock from the rescued seed stock.

To blind passage the virus in the transfection supernatants, Vero E6 cells and Vero cells were seeded onto 12-well plates to a confluency of ~70% for infection. On the day of infection, old media was removed and replaced with fresh cell culture medium. Media used to culture the MERS-CoV virus on Vero cells contained TPCK-trypsin at a concentration of 1 μg/ml. SARS-CoV supernatant harvested from the transfection was added to each well of the Vero E6 cells at a
volume of 100 μl, while MERS-CoV supernatant was added to the Vero cells at the same volume. Plates were incubated in a 37°C humidified chamber with 5% CO₂. Supernatants were harvested 72 hours later if CPE was observed.

Larger viral stocks were prepared by seeding Vero E6 and Vero cells in T75 cm² flasks to a confluency of ~90% for infection of SARS-CoV and MERS-CoV, respectively. On the day of infection, old medium on Vero E6 cells was replaced with fresh DMEM/1% BGS or on Vero cells with MEM/1%BGS and 10 μl of viral supernatant collected from the 12 well dishes were added. Supernatants were collected 72 hours later and frozen at -80°C for storage.
3.3 Peptibody production

3.3.1 Peptibody design
Figure 4. Schematics of ACEP peptide gene and enhanced GFP constructs cloned into pFUSE-mlG2a-Fc: pFc-ACEP-N (A), pFc-ACEP-C (B), pFc-ACEP-NC (C), pFc-2ACEP-N (D), pFc-2ACEP-C (E), and pFc-eGFP (F). Peptide ACEP encodes for amino acids 22-44 and 351-357 joined by a glycine amino acid as described by Han et al. 2ACEP is a combination of two ACEP linked together with 7 glycine amino acids. Peptides cloned into the N terminus of pFUSE-mlG2a-Fc were PCR amplified with primers encoding EcoRI and EcoRV restriction enzyme recognition sites to flank either side of the gene sequence, while peptides cloned into the C terminus of mlG2a-Fc2 used unique cut sites Scal and Nhel. Enhanced GFP gene was cloned into the N terminus using primers containing EcoRV cut sites (G).

ACE2-derived peptibodies were generated by inserting the coding sequence for the ACE2 peptide into plasmid pFUSE-mlG2a-Fc2 (InvivoGen) containing the Fc portion of a mouse IgG2a antibody by InFusion ligation. Based on the study done by Han et al. 2006., the amino acid segments 22-44 and 351-357 of ACE2 were codon optimized (mammalian codon specificity) and linked together by a single glycine amino acid to generate peptide ACEP (Table 2). The pFUSE-mlG2a-Fc1 vector contains a multiple cloning site (MCS) for insertion of the ACEP coding sequence at the amino end of the Fc domain (Figure 4G). This concept was designed by Akeel Baig of the National Microbiology Laboratory. In this construct the ACEP coding sequence is flanked at the amino end by the high efficiency IL-2 secretion signal that is cleaved off the final Fc-ACEP product during intracellular processing of the protein before being secreted from the cell. The position of the peptide at the amino end of the Constant Heavy Chain domain 2 (CH2) is analogous to the natural configuration of an antibody which would have the variable heavy chain domain (VH) attached to the CH1 domain in the configuration N-VH1-CH1-C attached to the amino end of CH2.
Table 2. Primers for generation of ACEP, 2ACEP, sACEP2 and eGFP fragments for cloning into pFUSE-mIgG2a-Fc2. Sequencing primers used for confirmation of insertions are also listed below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACEP fragment in N terminus</strong></td>
<td></td>
</tr>
<tr>
<td>MCSACEPF</td>
<td>CTTCGACTTGCAGATTTCCGGAGGAGCAGGCCAAGACC</td>
</tr>
<tr>
<td>MCSACEPR</td>
<td>AGATCTAACCATGGCGATATCCTGAAATCCTGCCGCTTGC</td>
</tr>
<tr>
<td><strong>Linker sequence</strong></td>
<td></td>
</tr>
<tr>
<td>LinkF</td>
<td>AGGCGGAGGCGGAGGCGGAGGAGGAGAGGAGGCAAGACC</td>
</tr>
<tr>
<td>LinkR</td>
<td>CCCGCTCTGGCCTCGGCTCGGCTCGGCTCGCAGGAGGCAAGACC</td>
</tr>
<tr>
<td><strong>ACEP fragment</strong></td>
<td></td>
</tr>
<tr>
<td>ACEPF</td>
<td>GAGGAGCAAGCAGGCAAGACCTTTCTGGACAAG</td>
</tr>
<tr>
<td>ACEPR</td>
<td>CCGCCCTGAAGTCGCCCTTGCCCAGGCCGC</td>
</tr>
<tr>
<td><strong>mlG2a-Fc2 fragment</strong></td>
<td></td>
</tr>
<tr>
<td>C-Fc-F</td>
<td>GAGGATTTACACAGTACTTCTT</td>
</tr>
<tr>
<td>C-Fc-R</td>
<td>TTTACCGGAGTCCGGGAGGAG</td>
</tr>
<tr>
<td><strong>ACEP fragment for C terminus</strong></td>
<td></td>
</tr>
<tr>
<td>C-ACEPF</td>
<td>CTTCGACCTTGCAGATTTCCGGAGGAGGAGGCAAGACC</td>
</tr>
<tr>
<td>C-ACEPR</td>
<td>TTATCGTGGCCAGCTAGCTGAGCTCGCACTGAAAGTCCTGCCGCTT</td>
</tr>
<tr>
<td><strong>eGFP fragment for MCS</strong></td>
<td></td>
</tr>
<tr>
<td>eGFPF</td>
<td>CGCCCTGGGGAGATCTACCATCGCTTGGAGTCATCTGACAG</td>
</tr>
<tr>
<td>eGFPR</td>
<td>CTGGTCTGGGAGTCTACCATCGCTTGGAGTCATCTGACAG</td>
</tr>
<tr>
<td><strong>sACE2 fragment for MCS</strong></td>
<td></td>
</tr>
<tr>
<td>sACE2F</td>
<td>CTTGCACCTTGCACAGATTTCCGGATGTCAGCTTTCGCCGCTT</td>
</tr>
<tr>
<td>sACE2R</td>
<td>GCCCTCTGAGGAGGATCTACCATGCGGGAAGTATCCATTCAT</td>
</tr>
<tr>
<td><strong>Sequencing</strong></td>
<td></td>
</tr>
<tr>
<td>pFUSEseqF</td>
<td>CTGTTTCTGCAGGCGCTGACAG</td>
</tr>
<tr>
<td>pFUSEseqR</td>
<td>ATGCAAACTTATAATGGAGTTAACA</td>
</tr>
</tbody>
</table>

To generate a plasmid that would express ACEP at the amino-end of the Fc domain, restriction sites EcoRI and EcoRV present at the MCS of pFUSE-mlG2a-Fc2 were utilized. Primers MCSACEPF and MCSACEPR were designed to amplify ACEP flanked by EcoRI (N terminus), EcoRV (C terminus) and 15 bp overhangs adjacent to these cut sites in the vector (Table 2). This resulted in complete removal of the MCS and an ACEP peptide with overlaps complementary to pFUSE-mlG2a-Fc2 upstream and downstream the restriction sites which is required for insertion via In-fusion HD Cloning. These primers were constructed to allow
insertion of the ACEP sequence in-frame following the IL2 secretion signal at the N terminus to the Fc hinge region that attaches ACEP to the Fc domain (Figure 4A).

To determine whether a tandem duplicated ACEP would have better binding activity than a single copy of the peptide, two ACEP peptides were joined together with a six-glycine amino acid linker to generate 2ACEP, which was cloned in a 2-step PCR process. First, ACEP was amplified by PCR to attach the linker sequence (5'–GGCGGAGGCGAGCGGG–3') to the N terminus using primers LinkF and ACEPR. The linker sequence was also attached to the C terminus of ACEP using primers LinkR and ACEPF. The overlapping PCR products from both reactions were then added to a single PCR mixture and amplified with primers MCSACEPF and MCSACEPR (Table 2) to add flanking restriction sites. The annealing of linker sequences from each product allowed amplification of the larger joint peptide 2ACEP, which was subsequently cloned into the vector in a similar manner as single peptide ACEP (Figure 4D).

Alternatively, peptibodies were also generated where ACEP and 2ACEP were attached at the C terminus of the mouse IgG Fc. As there was no MCS at this end of the vector, a unique Scal site located approximately 270 bp within the Fc gene and a Nhel site a few base pairs downstream of the Fc were used. Due to the location of the Scal site within the Fc domain, ~440 bps of the Fc downstream the cut site was amplified using primers C-Fc-F and C-Fc-R (Table 2). This was done to link ACEP and 2ACEP to this Fc segment so that it is not removed during cloning. Linking of the two fragments was carried out in a similar manner as when generating 2ACEP. ACEP and 2ACEP were amplified using primers C-ACEPF and C-ACEPR (Table 2), where primer C-ACEPF was constructed to overlap the Fc domain at the C terminus. ACEP and 2ACEP were then each assembled with the 440-bp Fc fragment by putting in a single PCR
reaction using primers C-Fc-F and C-ACEPR. The overhang generated by C-ACEPF provides a complementary sequence at the N terminus of ACEP and 2ACEP, thus allowing binding to the C terminus of the Fc fragment and subsequent amplification as a single, larger single segment to be cloned into pFUSE-mlgG2A-Fc2 (Figure 4B and 4E). Additionally, a peptibody was generated where ACEP was cloned into both the N and C terminus of mouse IgG Fc (Figure 4C).

An eGFP gene was amplified off a pCAGGS/eGFP plasmid (NML) using primers eGFPF and eGFPR (Table 2), and similar to the primers used to clone ACEP, contain overhang sequences adjacent to the restriction enzymes EcoRI and EcoRV found in the MCS of plasmid pFUSE-mlgG2a-Fc2 (Figure 4F).

While the coding sequences of the peptides ACEP and 2ACEP were inserted using the EcoRI and EcoRV restriction sites, the EcoRV enzyme recognition site is found within the sequence of the soluble domain of ACE2. Cloning this portion of ACE2 required primers composed of the EcoRI and BglII cut sites of the plasmid and adjacent 15 bps flanking the outer limits of these sites. Primers sACE2F and sACE2R (Table 2) were used to amplify the soluble portion ACE2 (sACE2) from amino acids 1-740 off plasmid pMD-ACE2 (Sino Biological) for cloning into the MCS of pFUSE-mlgG2a-Fc2 to generate pFc-sACE2.

3.3.2 Polymerase chain reaction (PCR)

In general, PCR amplification reactions for ACE2 peptide fragment clones were set up using the following formula: 12.5 μl of Takara PrimeSTAR Max 2X mix, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 10.5 μl of PCR grade H₂O (Qiagen) for a final volume of 25 μl. Thermocycler parameters used for this reaction were: 10 cycles of 98°C for 10 sec., 50°C for
5 sec. 72°C for 15 sec., followed by 10 cycles of 98°C for 10 sec., 55°C for 5 sec., and 72°C for 15 sec. PCR reactions for amplification of a plasmid encoded gene as template were set up as follows: 12.5 μl of Takara PrimeSTAR Max 2X mix, 1 μl forward primer (10 μM stock), 1 μl reverse primer (10 μM stock), 10 ng plasmid template and 9.5 μl of PCR grade H₂O for a final volume of 25 μl. Thermocycler parameters used were: 98°C for 10 sec., 55°C for 5 sec., and 72°C for 15 sec., cycled 35 times with a final extension at 72°C for 30 sec. Assembly of the ACE2 linker fragments and Fc portion PCR fragments were carried out using a reaction set up of: 12.5 μl Takara PrimeSTAR Max 2X mix, 1 μl DNA fragment #1 (gel-purified), 1 μl DNA fragment #2 (gel-purified) and 8.5 μl of PCR grade H₂O with a thermocycler program of 98°C for 10 sec., 50°C for 5 sec., and 72°C for 20 sec. for 5 cycles with an extension of 72°C for 30 sec., which allows the two DNA segments to anneal to one another. Subsequently 1 μl of forward primer and 1 μl of reverse primer were added to the reaction and placed in the thermocycler again for 35 cycles of 98°C for 10 sec., 60°C for 5 sec., and 72°C for 20 sec., with a final extension time of 30 sec. at 72°C to generate the larger assembled DNA fragment. Prior to proceeding to gel electrophoresis, 2.5 μl of 10X FastDigest Green Buffer (Thermo Fisher Scientific) was added to the PCR product. A 1.0% agarose gel made up of 0.40 g of UltraPure Agarose (Invitrogen, Thermo Fisher Scientific) dissolved in 40 ml of 1X TAE buffer and 1 μl of GelGreen™ DNA dye (Biotium, VWR) was used to run PCR products alongside a 1 kb DNA ladder (Invitrogen, Thermo Fisher Scientific). The gel was placed in an electrophoresis chamber and run at 100V for 30 minutes. A SafeImager™ (Invitrogen) was used to visualize the bands via Blue light. Bands of interest were excised and placed in a 1.5 ml tube for DNA extraction using the QIAquick Gel Extraction Kit (Qiagen) as described in the manual.
3.3.3 In-Fusion HD Cloning

To prepare the vectors for In-Fusion cloning, restriction enzyme digestion reactions were set up by adding 1 μl of vector to a mixture of 1 μl 10X FastDigest buffer, 1 μl Calf Intestinal Alkaline Phosphatase (New England Biolabs, NEB), 1 μl restriction enzyme 1 (FastDigest Enzymes, ThermoFisher), 1 μl restriction enzyme 2 and 5 μl of PCR grade H₂O. Following an incubation period of 1 hour at 37°C, digested vectors were separated by electrophoresis on an agarose gel and isolated as previously described. DNA inserts were generated as described above with appropriate inserts for Infusion cloning into the digested vector and were purified by extraction for agarose gels following electrophoresis to separate the PCR fragments and reaction components such as primers.

The In-Fusion HD Cloning Kit (Takara, Clontech) requires a 15 bp overlap between the insert DNA and the vector. All primers used to amplify the ACE2 peptides, full ACE2 sequence and eGFP contained at least a 15 bp overlap with adjacent sequences of the vectors of interest from the point that the vector is cut by the restriction enzyme. In-Fusion reaction mixtures were set up as follows: 1 μl digested vector, 1 μl 5X In-Fusion Enzyme mix, 1.5 μl DNA insert and 1.5 μl PCR grade H₂O and incubated at 50°C for 15 minutes before proceeding to transformation.

3.3.4 Transformation into competent cells

For bacterial transformation, Stellar™ competent E. coli cells (Clontech) or SURE2 cells (for AAV6) were thawed on ice and aliquoted into 30 μl volumes in 1.5 ml tubes per reaction.
Two microliters of In-Fusion reaction was added to competent cells and incubated on ice for 20 minutes. Cells were then heat shocked in a 42°C water bath for 40 seconds and 150 μl of Super Optimal broth with Catabolite repression (SOC) medium was added to each tube. Cells were next placed in a thermal mixer and incubated at 37°C for 1 hr while being shaken at 900 rpm. Luria-Bertani (LB) agar plates containing 100 μg/ml of zeocin (peptibody vectors) or ampicillin (AAV6) were used to plate 50-100 μl of transformed cells, which were then incubated overnight at 33-37°C. If colonies were present the next morning, 3-6 individual colonies were selected and grown overnight in 3 ml Terrific broth (TB) with 100 μg/ml zeocin (peptibodies) or LB broth with 100 μg/ml ampicillin (AAV6) with shaking at 225 rpm at 33-37°C. Plasmids were isolated from bacteria cultures via the QIAprep Spin Miniprep Kit (Qiagen) or the Zippy™ Plasmid Miniprep Kit (Zymo Research) via procedures described in the manuals. To confirm the presence of the inserts of interest, extracted plasmids were sequenced by the DNA Core Facility at the NML using sequencing primers described in Table 2. All DNA samples were sequenced using the Sanger method and results were analyzed using DNASTAR Lasergene 11 SeqMan Pro software. Once plasmids and inserts were confirmed, 0.5 ml of bacteria culture was mixed with 0.5 ml of a 1:1 ratio of LB broth to glycerol for storage at -80°C.

For the generation of higher concentrations of plasmid DNA, bacteria cultures were grown up in 100-500 ml of TB with 100 μg/ml of zeocin overnight with shaking at 225 rpm at 37°C. Plasmid DNA was then isolated using the Qiagen Plasmid Midi kit (≤100 ml cultures) or Maxi kit (≤500 ml cultures) as described in the manuals.

3.3.5 Transfection optimization
To optimize expression efficiency of ACE2 incorporated peptibodies, the eGFP peptibody was used for evaluation of a variety of transfection reagents and plasmid DNA concentrations. The following transfection reagents were examined: Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific), Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific), TransIT® LT1 (Mirusbio), CalPhos™ (Clontech) and FuGENE®6 (Promega). A concentration of 2.5 μg of plasmid DNA was used with each transfection reagent per well of a 6 well dish. Ratios of transfection reagent to 1 μg of plasmid DNA were 3:1 for Lipofectamine 2000, Lipofectamine 3000, LT1 and FuGene®6. CalPhos™ protocol recommends 12.4 μl of calcium solution per 1-3 μg of DNA, thus a ratio of 12.5:2.5 was used.

In preparation for transfection, mammalian 293T cells were seeded onto poly-D-lysine (Sigma) coated 6-well plates (Corning) one day before needed for ~70-80% confluency on the day of transfection. On the day of transfection, cell culture medium was removed, and cells were washed with PBS. Medium was replaced with enough Opti-MEM so that the final volume after the addition of transfection solution would equal 1 ml. Transfection reactions were prepared as per manufacturer’s guidelines. Briefly, a tube containing 150 μl of Opti-MEM and 7.5 μl of Lipofectamine 2000 was combined with a tube containing equal amounts Opti-MEM and 2.5 μg of eGFP peptibody plasmid DNA and incubated for 5 minutes. In another reaction, 7.5 μl of Lipofectamine 3000 in 125 μl Opti-MEM was combined with a tube containing 115 μl Opti-MEM, 10 μl P3000™ reagent and plasmid DNA and incubated for 10-15 minutes. LT1 reagent was added to a mixture of 250 μl Opti-MEM and plasmid DNA and incubated for 15-30 minutes. Fugene®6 protocol indicates an incubation period of 5 minutes at room temperature after the addition of 7.5 μl of reagent to 300 μl Opti-MEM. Subsequently, DNA was then added.
to the tube and incubated an additional 15 minutes. Finally, CalPhos™ utilized 12.4 μl of a 2M calcium solution combined with 2.5 μg plasmid DNA and sterile H₂O to a final volume of 100 μl. This solution was vortexed while 100 μl of 2X HBS was added dropwise. This mixture was then incubated for 15 minutes. All incubations were carried out at room temperature. Following each incubation period, transfection reaction mixtures were added drop-wise into their respective wells and incubated in a 37°C chamber with 5% CO₂ for 72 hours. GFP fluorescence was examined at 24, 48 and 72 hours by fluorescent microscopy using an EVOS FL Cell Imagine System (Life Technologies, Thermo Fisher Scientific).

The effect of plasmid DNA concentration on transfection efficiency was measured by using Lipofectamine 2000 with either 1 μg, 2 μg, 2.5 μg, 3 μg or 4 μg per well of a 6 well dish. A ratio of 3 μl transfection reagent per 1 μg of DNA was used per reaction. Transfection of 293T cells were performed as previously described and GFP expression was examined 24, 48 and 72 hours later via fluorescent microscopy.

3.3.6 Peptibody expression

Expression of the ACE2 incorporated peptibodies were carried out in mammalian 293T cells following transfection optimization. Briefly, 293T cells were seeded onto poly-D-lysine coated 6 well plates. For each ACE2 peptibody transfection reaction, 150 μl Opti-MEM and 2 μg plasmid DNA solution was added drop-wise to a tube of 150 μl Opti-MEM and 6 μl Lipofectamine 2000. Mixtures were then incubated at room temperature for 5 minutes and again, added drop wise to the respective 6 well plate. Plates were incubated in a humidified 37°C chamber with 5% CO₂ for 72 hours. Supernatants were collected and centrifuged at
1000xg for 5 minutes to remove cell debris and stored at -20°C for later analysis. Remaining cells of each well were collected after 5-10 minutes of incubation in 250 μl of 4X sodium dodecyl sulfate (SDS) gel loading buffer (Appendix) at room temperature. Cells and SDS were transferred to 1.5 ml tubes where 50 μl of 2-Mercaptoethanol (BME) was added. Mixtures were boiled for 10 minutes at 99°C and then placed in a -20°C freezer for storage.

For larger peptibody preparations, T150 cm² or Corning™ Falcon™ Cell Culture T875 cm² Multi Flasks (Thermo Fisher Scientific) were coated with poly-D-lysine and seeded with 293T cells. When cells reached a confluency of ~80% they were transfected with a solution prepared as previously described at a ratio of 6 μl of Lipofectamine 2000 per 2 μg plasmid DNA per approximately 1.6x10⁶ cells. This scaled up to 66.6 μl of Lipofectamine 2000 per 33.3 μg plasmid DNA per T150 cm² flask or 583.3 μl of Lipofectamine 2000 per 194.4 μg plasmid DNA per T875 cm² flask. Cells were incubated for a total of 144 hours; however, supernatants were collected from the flasks at 72 hours post transfection. Fresh medium (DMEM/1% BGS/2mM L-glutamine) was then added to the multi-flasks and supernatants were collected again 72 hours later.

3.3.7 SDS-polyacrylamide gel electrophoresis and Western blot

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins based on molecular weight. In-house made gels and NuPAGE™ 10% Bis-Tris Protein gels (Thermo Fisher Scientific) used consisted of a 10% resolving gel and 4% stacking gel. Prior to loading, samples were thawed to room temperature. PageRuler™ Prestained NIR Protein Ladder (Life Technologies, Thermo Fisher Scientific) was loaded with each SDS-PAGE run and used as a
standard to confirm protein size. Generally, 10-20 μl of each sample was loaded into each lane and gels were run at 120V for 1.5 hours using either a mini-PROTEAN 3 electrophoresis system (Bio-Rad) or the Mini Gel Tank (Life Technologies, Thermo Fisher Scientific).

Following electrophoresis, protein gels were transferred to membranes and blotted using one of two methods. The first method utilizes polyvinylidene fluoride membranes (PVDF) (Millipore Sigma). Prior to transfer, PDVF membranes were soaked in methanol for 5 minutes and then washed, along with filter papers and fibre pads, in transfer buffer (Appendix). Each component was stacked between the transfer cell from bottom to top as follows: fibre pads, PVDF membrane, protein gel and fibre pad. After placement of each item, a roller was used to remove trapped air bubbles. The Trans Blot SD Semi-dry Transfer Cell was run at 120 mA for 1.5 hours. After completion of transfer, PVDF membranes were incubated in Odyssey Blocking Buffer (OBB) (LI-COR, Mandel Scientific Company) at room temperature for 1 hour or overnight at 4°C. To blot, primary antibody was diluted in 5 ml of OBB as recommended by product data sheets and incubated with the membrane at room temperature on a rotator for 1 hour. After incubation, membrane was washed 3X for 5 minutes each with PBS-0.1% Tween. This step was omitted when detecting ACE2 peptibodies as they did not require a primary peptibody. Secondary antibody was diluted in the same manner and incubated with the membrane at room temperature on a rotator for 1 hour in the dark. Membranes were again washed 3X with PBS-0.1% Tween. A final wash in PBS for 5 minutes was carried out prior to visualization with a LI-COR Odyssey Infrared Imaging System.

The second method utilizes the iBlot™ 2 Dry Blotting System (Life Technologies, Thermo Fisher Scientific). After electrophoresis, protein gels were rinsed in Milli-Q (Millipore Sigma)
filtered H₂O and placed on top of the transfer membrane of the Bottom Stack in the iBlot 2 Gel Transfer Device. Pre-soaked (in Milli-Q filtered H₂O) iBlot™ filter paper was placed on top of the gel and then covered with the Top Stack. A Blotting Roller was used to remove any air bubbles trapped between the layers. Next, an Absorbent Pad was placed on top of the Top Stack. The gel transfer stack was then run under the following conditions: 20V for 1 min., 23V for 4 min. and 25V for 2 min. When the run was finished, membranes were placed into distilled water until detection of transferred proteins was done.

Prior to detection, membranes were soaked in 1X iBind™ Flex FD Solution on a rocker for a minimum of 10 minutes. Primary antibody solutions were prepared following manufacturers guidelines to a final volume of 2 ml in the iBind™ Flex FD Solution, while secondary antibody dilutions were prepared adding 1 μl of antibody to 1999 μl of iBind™ Flex FD Solutions. Next, an iBind™ Flex Card was placed onto the stage of the iBind™ Flex Western Device and saturated with 10 ml of 1X iBind™ Flex SD Solution. The protein membrane was placed protein-side down with the lower molecular weight region closest to the stack of the card. A roller was then used to remove trapped air bubbles. After the lid was closed and locked on the device, primary, secondary and iBind™ Flex SD solutions were loaded into the well inserts and incubated for 2.5 hours or overnight. All membranes were stained using Odyssey Infrared secondary dyes and visualized using a LI-COR Odyssey Infrared Imaging System.

3.3.8 Purification of Peptibodies and Fc-conjugated proteins

HiTrap™ Protein G HP 5 ml columns (GE Healthcare Life Sciences) were chosen for peptibody purification due to the high relative mouse IgG2a binding strength of the Protein G
Sepharose™ High Performance beads. Purification was set up by threading Masterflex platinum-cured tubing (L/S 14) through a MasterFlex® Easy-Load Pump Head attached to a MasterFlex® P/S Stainless Peristaltic Pump. One end of the tubing was placed in buffer or sample, while the other end was attached to the protein G column by a Union 1/16” male/female luer connector. Prior to purification, supernatants were filtered through a Stericup-GP (0.22 μm) and SteriTop vacuum-filtration system to remove cell debris and then diluted in binding buffer (20 mM sodium phosphate pH 7.4, made in house) until a pH of approximately 7.0 was reached.

The column was first washed with 50 ml of binding buffer. Next, supernatant was applied to the column and then washed again with 50 ml of binding buffer. Each step was performed at a flowrate of 5 ml/min as recommended by manufacturer’s protocol. Peptibodies were eluted using 25 ml of elution buffer (0.1 M glycine-HCl) at a rate of 1 ml/min in 2 ml aliquots into tubes containing 200 μl of 1M Tris-HCl pH 8.0.

3.3.9 Protein quantification

Purified peptibodies were quantified one of two ways. The first utilized the Invitrogen Qubit® Fluorometer and Quant-i™ assay kit. Reagents were allowed to come to room temperature prior to quantification. Qubit™ working solution was prepared by adding 1 μl of Qubit™ Reagent to 199 μl of Qubit™ Buffer per reaction. Next, 190 μl of working solution was aliquoted into Qubit™ Assay Tubes. Samples and Standards were added in 10 μl quantities for a final volume of 200 μl and incubated at room temperature for 15 minutes. Tubes were then
read in a Qubit® 2.0 Fluorometer which provides an instrument calculated concentration for the unknown based on the fluorescence of the standard values that were read.

The second method allowed for assessment of purity as well as quantity using the 2100 Bioanalyzer instrument (Agilent Technologies) and Agilent protein kit. Kit reagent Protein 230 sample buffer was allowed to come to room temperature prior to the start of the assay. For reducing conditions, 7 μl of BME was added to 200 μl of Protein 230 sample buffer and vortexed to generate a denaturing solution. Next, 4 μl of protein sample was combined with 2 μl of denaturing solution in a 0.5 ml microcentrifuge tube and boiled at 100°C for 5 minutes. Samples were allowed to cool and briefly centrifuged to collect the condensate before the addition of 84 μl of deionized water. A Protein chip was then primed using a Gel-Dye mix that was prepared by adding 25 μl of Protein 230 dye concentrate to 650 μl of Protein 230 gel matrix. Samples were added in 6 μl volumes to the wells of the chip. A Protein 230 ladder was run alongside the samples as a standard and to confirm sample protein size. The chip was then inserted into the Agilent 2100 Bioanalyzer and results were analyzed using 2100 Expert Software. Proteins were detected automatically by laser-induced fluorescence and translated to gel-like imagine and electropherograms. Sample size and quantification were determined using a standard curve calculated using the ladder.

3.4 Immunofluorescence assay (IFA)

IFAs were used to evaluate the binding capacity of peptibodies and Fc-sACE2 to the SARS spike protein. Depending on the number of samples and available volume of peptibody or other Fc-conjugate, cells were split into a 6, 12 or 24-well dishes to a confluency of ~80% for the
day of transfection. Transfections of plasmid constructs, such as that for expression of the SARS spike protein, were carried out as previously described using Lipofectamine 2000 in Opti-MEM and scaled down depending on the tissue culture dish used. Cell culture medium was removed prior to the addition of transfection media and cells were incubated for 48 hours. After two days, transfection media was removed, and cells were washed once with PBS. Cells were then fixed with methanol free 16% formaldehyde (Fischer Scientific) for 10 minutes at room temperature or overnight at 4°C. Following fixation, formaldehyde was removed, and cells were washed 3 times for 3 minutes per wash with PBS. To prevent non-specific binding, cells were blocked with 1% bovine serum albumin (BSA) in PBS for 45 minutes at room temperature prior to the primary antibody binding step. Peptibodies were added directly to the wells after the removal of blocking buffer and incubated for 1 hour at room temperature. After incubation, cells were washed 3 times with PBS. Secondary antibodies, carrying fluorescent detection tags, were diluted in blocking buffer according to manufacturer suggested concentrations and aliquotted into each well. Incubation took place in the dark at room temperature for 1 hour. Cells then underwent 3 final washes with PBS prior to imaging on an EVOS FL Cell Imagine System.

Visualization of hDPP4 receptor expression in 293T cells that have been infected by Ad5 an AAV9 vectors was carried out in a similar matter described above. Cells (293GT) seeded into 6-well dishes to a confluency of ~80% were infected at a multiplicity of infection (MOI) of 0.1-100. Infected cells were fixed with methanol as described at 4-7 days post infection. After washing, cells were further permeabilized by incubating with 0.1% Triton X-100 (Fisher Scientific) in PBS for 10 minutes at room temperature. Cells were washed again, 3 times with
PBS before proceeding to the remaining steps which were similar to those described above. Primary antibodies specific to hDPP4 were used instead of peptibodies and were diluted in blocking buffer according to the suggested manufacturer concentrations.

3.5 Enzyme linked immunosorbent assay (ELISA)

This assay was used to confirm the binding capacity of peptibodies to highly purified SARS spike protein to demonstrate antigen specificity. SARS spike protein (Sino Biological) diluted in 5% skim milk in PBS to a concentration of 1.2 ng per well was aliquoted and used to coat a Corning® 96 Well Half-Area Microplate and incubated overnight at 4°C. Plates were then blocked with 5% skim milk in PBS overnight at 4°C. Plates were washed three times with 0.5% Tween in PBS using a microplate washer (BioTek). Crude supernatants and purified peptibodies/Fc conjugated proteins were then added directly into each well at a volume of 50 μl and incubated for 1 hour at 37°C. Plates were washed again using a microplate washer three times with 0.5% Tween in PBS and incubated with secondary peroxidase conjugated anti-mouse IgG (KPL) for 1 hour at 37°C. After the incubation, 96-well plates were washed a final three times with PBS using a microplate washer. ABTS® (C_{18}H_{16}N_{4}O_{6}S_{4}·(NH_{4})_{2}) 2-component peroxidase substrate (KPL) was mixed at a 1:1 ratio and then added to each well at a volume of 60 μl per well. After 30 minutes, plates were read using a BioTek Synergy HTX multi-mode plate reader at a wavelength of 405 nm.
3.6 Viral assays

3.6.1 AAV genome extraction

To determine the titre of the AAV6-hDPP4 virus stock, AAV genomic DNA needed to be extracted for quantitative real-time PCR (qRT-PCR). A TURBO DNA-free™ kit (Invitrogen) was used to remove possible DNA contamination from the purified virus stock. Briefly, 5 μl from the concentrated AAV6-hDPP4 stock was mixed with 1 μl TURBO DNase, 5 μl 10X TURBO DNase Buffer and 39 μl of nuclease free H$_2$O and incubated for 30 minutes at 37°C. After the incubation, 5 μl of DNase Inactivation Reagent was added and the solution incubated for an additional 5 minutes at room temperature. The suspension was then brought up to a final volume of 450 μl using nuclease free H$_2$O.

To extract the encapsidated viral DNA, 1 μl of 10 mg/ml concentrated carrier RNA, 10 μl of 5M NaCl and 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the suspension and spun at 14,000 rpm for 5 minutes to separate the mixture into layers. The upper aqueous layer containing the DNA was removed (approximately 400 μl) and aliquoted into a clean tube. Another 400 μl of nuclease free water was added back to the phenol:chloroform solution and the last step was repeated. Again, the aqueous layer was removed and combined with the previous aqueous phase. The solution was then mixed and separated into two microcentrifuge tubes. One ml of 100% ethanol was added to each tube and DNA was allowed to precipitate over a 72-hour period at -80°C. Following precipitation, tubes were spun at 14,000 rpm for 20 minutes at 4°C to pellet the DNA. Ethanol was removed and 10 μl of nuclease free water was added to each tube to resuspend the DNA and then combined for
storage at -20°C. Extracted DNA was sent to Dr. Wootton at the University of Guelph for qRT-PCR.

3.6.2 End point dilution assay to determine virus titers

End point dilution assays are used to quantify the amount of virus able to kill 50% of infected hosts or cause cytopathic effect in 50% of tissue culture cells. This is also known as the 50% tissue culture infectious dose (TCID$_{50}$) concentration. This assay was carried out by first seeding cells into a 96 well plate to a confluency of ~80-90% for the day of infection. Vero cells were used for titration of MERS-CoV, Vero E6 cells for SARS-CoV and 293GT for Ad5. Virus stocks were serially diluted 1:10 starting at $10^{-1}$ to $10^{-8}$ in low serum (1-2%) media. Prior to the addition of virus to cells, old medium was removed. Then 100 μl of each virus dilution was added to the cells and all dilutions were plated in 3-6 replicates each. Plates were incubated for 72 hours in a 37°C humidified chamber with 5% CO$_2$ or until CPE was observed in the control wells. The highest dilution that caused CPE was recorded for each replicate and the TCID$_{50}$/ml concentration was calculated using the Reed-Muench method$^{134}$.

3.6.3 Plaque assay

To measure the number of plaque forming units in a virus sample, plaque assays were carried out. First cells were seeded in a 12 well dish to reach 100% confluency on the day of infection. Cells were then washed with plain medium without serum. Virus was serially diluted at a ratio of 1:10 from $10^{-1}$ to $10^{-6}$ in low serum medium and 200 μl from each dilution was added to the wells in duplicate. Plates were placed at 37°C/5% CO$_2$ for 1 hour with intermittent
rocking every 15 minutes to prevent the cells from drying. A mixture of 2% SeaPlaque low melting point agar in tissue culture grade water (Lonza) and 2X MEM was prepared for a final concentration of 1% agar/1X MEM (Appendix). After the 1-hour incubation, virus medium was removed from the cells and 1 ml of overlay was added to each well. Overlay was allowed to solidify before incubation upside down in a 37°C humidified chamber with 5% CO₂. Viral plaques were counted 72-148 hours post infection.

3.6.4 Neutralization assay

Neutralization assays were used to determine whether Fc-sACE2 have the capacity to prevent viral infectivity and if so, at what magnitude based on the amount of antibody required to fully neutralize a set amount of SARS-CoV. For this assay, Vero E6 cells were seeded into a 96 well plate to ~80-90% confluency for the day of infection. Fc-ACE2 was serially diluted at a ratio of 1:2 from purified stock preparation up to 1/128 in DMEM (1% BGS/1% L-Glu). A volume of 50 μl was taken from each dilution and mixed with 50 μl of SARS-CoV from a viral stock concentration of 500 TCID₅₀/ml and incubated at 37°C for 1 hour. After the incubation, media was removed from the Vero E6 cells and 100 μl of the peptibody/virus mixture was added to each well in triplicate and incubated again at 37°C to allow for virus absorption. After 30 minutes, an additional 50 μl of DMEM (1% BGS/1% L-Glu) was added to each well and cells were incubated in a 37°C humidified chamber with 5% CO₂ until CPE was observed in the control wells. The highest dilution of Fc-ACE2 that was able to completely inhibit CPE was recorded. Based on triplicate repeats of the assay the percentage of wells where CPE was not
observed was calculated, and the median amount of Fc-sACE2 required to inhibit 50% of SARS-CoV virus infection of Vero E6 cells (IC$_{50}$) was determined using the Reed-Muench method.

3.7 Mice

Experiments conducted with live mice were approved by the Animal Care Committee from the Canadian Science Centre for Human & Animal Health, as described in protocol H-14-005 according to guidelines provided by the Canadian Council on Animal Care. BALB/c mice (Charles River) were initially housed in BSL-2 facilities in HEPA filtered air caging units. Prior to SARS-CoV or MERS-CoV infection, mice were transferred to the BSL-4 facilities at the NML where each caging unit is kept under negative pressure and air transfer is HEPA filtered.

Mice to be transduced with AAV6-hDPP4, Ad5-hDPP4 and Ad5-ctrl were first anesthetized, using 5% isoflurane in O$_2$ for anesthesia induction and 3% isoflurane for maintenance, before virus infection via the intranasal route. AAV9-hDPP4 and AAV9-ctrl were transduced by tail vein injection in similarly anesthetized mice. AAV6-hDPP4, Ad5-hDPP4, Ad5-ctrl, AAV9-hDPP4, AAV9-ctrl were inoculated at concentrations of 4.2x10$^{11}$ genome equivalents/ml ( GE/ml), 1.12 x10$^8$ pfu/ml, 1.12 x10$^8$ pfu/ml, 10$^{12}$ GC/ml and 10$^{12}$ GC/ml, respectively. Since Ad5 and AAV9 control viral vectors encoded fluorescing genes GFP and luciferase, respectively, mice were anesthetized and imaged for fluorescence/chemiluminescence using an UVP iBox® Scientia™ small animal imaging system. Ad5-hDPP4 and Ad5-ctrl mice were imaged 1, 2, 3 and 4 days post transduction. AAV9-hDPP4 and AAV9-ctrl mice were further injected with 100 μl of VivoGlo luciferin (2 mg) via tail vein 15
minutes prior to imaging at days 0, 4, 7 12 and 14 post-transduction. Imaging of these mice was done in darkness without a filter to capture normal white light.

AAV6 inoculated mice were divided into 3 groups of 6 mice, anesthetize using isoflurane and challenged via intranasal instillation with a clinical strain of MERS-CoV at $10^5$ PFU in 50 µl PBS per mouse 7-, 14- and 21-days post transduction alongside a size-matched control group of mice that were not transduced. Infected animals were monitored daily and clinical signs of disease including weight loss were recorded daily for the duration of the infection using an approved scoring sheet for the assessment of clinical signs that also indicates criteria for humane euthanasia in animals exhibiting signs of severe disease, as detailed in animal protocol H-14-005. Mice (n=2) were euthanized for scheduled necropsy by the method of isofluorane overdose and cervical dislocation at 4- and 7-days post MERS virus challenge. Lung, liver, heart, spleen and kidney tissues were harvested for virus detection via quantitative RT-PCR.

In a similar manner, Ad5 and AAV9 transduced mice were challenged with a clinical strain of MERS-CoV at $10^5$ PFU per mouse at 4 and 14 days post transduction, respectively. Following daily monitoring for clinical signs of weight loss and disease, mice were sacrificed at 2- and 4-days post MERS-CoV challenge. Again, lung, liver, heart, spleen and kidney tissues were collected for quantitative RT-PCR evaluation of viral titres. TCID$_{50}$ assays to quantify recoverable replicating virus were carried out, as described in section 3.6.2, on lung homogenates of all mice.
3.8 Quantitative Real-Time PCR (qRT-PCR)

Prior to qRT-PCR, RNA was extracted from mouse tissue (lung, liver, heart, spleen and kidneys) using the Qiagen QIAamp Viral RNA Mini Kit. Briefly, organs from the AAV6 transduced mice were homogenized in 1 ml of MEM and centrifuged for 10 minutes at 1500xg to pellet cell debris. A volume of 140 μl was taken from the supernatant added to 560 μl Buffer AVL containing carrier RNA in a 1.5 ml tube. Subsequent RNA extraction was carried out as described per the QIAamp Viral RNA Mini Handbook.

In a similar manner, organs from mice infected with AAV9 and Ad5 were homogenized in Buffer RLT and centrifuged for 10 minutes at 1500xg. Subsequent RNA extraction was carried out on the supernatant using the Qiagen RNeasy Mini Kit according to the RNeasy Mini Handbook.

According to the protocol described by Corman et al. in 2012, MERS-CoV was detected using primers upE-Fwd, upE-Rev and probe upE-Prb (Table 3). Generally, qRT-PCR reactions were set up using LightCycler® 480 RNA Master Hydrolysis Probes kit as follows: 7.4 μl 2.7X Master, 5.62 μl PCR grade H2O, 1.28 μl 5 mM Activator, 0.8 μl 20X Enhancer, 0.3 μl primer 1 (10 μM stock), 0.3 μl primer 2 (10 μM stock), 0.3 μl probe and 4 μl extracted RNA. A standard curve was generated using a 1:10 serial dilution of MERS-CoV construct. LightCycler parameters were as follows: 61°C for 3 min., 95°C for 30 sec. followed by 45 cycles of 95°C for 15 sec., 60°C for 40 sec. and 72°C for 1 sec. with a final step of 40°C for 10 sec.
Table 3. Primers and probes for detection and quantification of MERS-CoV RNA upE gene.

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>upE gene primers</td>
<td></td>
</tr>
<tr>
<td>upE-Fwd</td>
<td>GCAACGCGCGATTCCAGTT</td>
</tr>
<tr>
<td>upE-Rev</td>
<td>GCCTCTACACGGGACCCATA</td>
</tr>
<tr>
<td>upE gene probe</td>
<td></td>
</tr>
<tr>
<td>upE-Prb</td>
<td>(FAM)-CTCTTCACATAATCGCCCGAGCTG-(TAMRA)</td>
</tr>
</tbody>
</table>

3.9 Statistical analysis

A 2-way ANOVA test was used to analyze differences in mean RNA values between hDPP4 transduced mice and control mice. All results were expressed as means ± SEM. P-values less than 0.05 were considered statistically significant.
Chapter Four: Results

4.1 ACE2 peptide peptibodies and sACE2 fusion protein

4.1.1 Generation of ACE2 peptide peptibodies

Peptibodies are a fusion of a peptide and the Fc portion of an antibody. They have been previously developed as therapeutics to modulate biological processes. We aim to utilize peptibodies for treatment of disease caused by pathogens. Based on the study done by Han et al. 2006., one peptide consisting of amino acids 22-44 and 351-357 of derived from the viral receptor binding domain of the host cell receptor protein ACE2 (EEQAKTFLDKFNHEAEDLFYQSSGLGKDFR) linked together by glycine was observed to have potent antiviral activity against SARS-CoV. To generate a peptibody containing this peptide, hereafter called ‘ACEP’, the nucleotide sequence was codon optimized for mammalian species codon usage and cloned into the MCS of plasmid vector pFUSE-mlG2a-Fc. This generated vector pFc-ACEP-N which when transfected, codes for the peptide fused to the N terminus of a murine IgG2a Fc domain (Figure 5A). The vector was designed to express a short hinge region (PRGPTIKPCPPCKCPA) between the peptide and amino end of the Fc domain. Mouse IgG has been found to have high levels of Fc-effector functions based on the antibody subclass. Studies have also found zybosides, which are recombinant antibodies that have been generated with multiple antigen combining or recognition sites at both the N and C terminals of the heavy and light chains, have been shown to increase specificity of the antibody. Based on this idea, ACEP was also cloned onto the C terminal of the mlG2a Fc (pFc-ACEP-C) and at both termini (pFc-ACEP-NC) as seen in Figure 5B and 5C. Additionally, we examined whether we could increase the binding capacity of the peptibodies by joining two ACEP peptides (2ACEP) using a
six-glycine amino acid linker. This peptide was cloned into pFUSE-mlgG2a-Fc in a similar manner as described for ACEP, generating plasmid vectors pFc-2ACEP-N and pFc-2ACEP-C (Figures 5D and 5E). Although the hinge region was not added to the peptide when it was cloned into the C terminus, the first peptide repeat of 2ACEP would extend the second repeat away from the Fc domain in a similar manner.

Figure 5. Schematic diagrams of generated peptibodies (A) Fc-ACEP-N, (B) Fc-ACEP-C, (C) Fc-ACEP-NC, (D) Fc-ACE2P-N and (E) Fc-ACE2P-C.
Peptide sequence inserts were confirmed with sequencing using primers upstream of the vector encoded IL-2 secretion sequence and downstream of the Fc open reading frame (ORF). Peptibodies were produced by transfecting 293T cells with each of the five vectors at a concentration of 2 µg per well of a 6 well dish. Cell lysates and supernatants were collected 48 hours later for Western blot analysis using a fluorescing tagged antibody able recognize mouse IgG. From my Western blot, Fc-ACEP-C and Fc-2ACEP-C peptibody expression was detected as seen in Figure 6A. Surprisingly, little to no protein expression was observed in the supernatant and cell lysates in samples transfected with plasmids pFc-ACEP-N, pFc-2ACEP-N and pFc-ACEP-NC (Figure 6B). After careful re-evaluation, it was determined that the original primers used to amplify ACEP into the C terminal domain had inadvertently removed part of the IL-2 secretion signal that is part of the pFUSE-mIgG2a-Fc1 plasmid and ensures that the Fc conjugates produced in the cells are secreted. Subsequently, adjustments were made to the primers and sequencing was used to confirm peptide insertion and the full IL-2 signal sequence. Western blot analysis was repeated following transfection in 293T cells with revised plasmid vectors. Peptibody expression of Fc-ACEP-N, Fc-2ACEP-N and Fc-ACEP-NC was observed in the supernatants and cell lysates (Figure 6C). Analysis of ACEP and 2ACEP peptibodies showed bands at ~43 kDa and 50 kDa, respectively. Wild type pFUSE-mIgG2a-Fc vector was expressed in the same manner and bands were observed at ~34 kDa (Figure 6D). The increase in molecular weight of peptibodies with the ACEP insertion(s) from the wild type vector further confirms the fusion of ACEP and 2ACEP peptides to murine Fc.
Figure 6. Peptibody expression in 293T cell lysates and supernatants collected 48 hours post transfection. Peptibody expression was detected by Western blot using IRDye800 anti-mouse IgG (1:5000). (A) Expression of Fc-ACEP-C and Fc-2ACEP-C peptibodies. (B) Expression of Fc-ACEP-N, Fc-2ACEP-N and Fc-ACEP-NC using the early plasmid vectors. (C) Peptibody expression of Fc-ACEP-N, Fc-2ACEP-N and Fc-ACEP-NC following transfection with redesigned plasmid vectors. (D) As a positive 293T cells were transfected with plasmid pFUSE-mlgG2a-Fc and the supernatant of untransfected cells were as a negative control. (E) Expression of Fc-eGFP peptibody.
4.1.2 Evaluation of transfection efficiency of plasmid pFUSE-mIgG2a-Fc

In order to maximize fusion protein production, transfection efficiency of peptibody vector pFUSE-mIgG2a-Fc needed to be determined. In a similar manner to the ACEP peptibodies, an enhanced GFP (eGFP) gene was cloned at the N terminus of the mouse IgG Fc and analyzed by Western blot (Figure 6E). Fluorescence levels of Fc-eGFP were observed via microscopy for evaluation of transfection efficiency. First, several transfection reagents were evaluated. Lipofectamine 2000, Lipofectamine 3000, TransIT® LT1, CalPhos™ and FuGENE®6 were each used to transfec...
Figure 7. Expression of eGFP in 293T cells 48 hours after transfection in 293T cells using different transfection reagents. Plasmid pFc-eGFP was transfected at a concentration of 2µg per well of a 6 well dish using transfection reagents (A) Lipofectamine 2000, (B) Lipofectamine 3000, (C) TransIT® LT1, (D) CalPhos™ and (E) FuGENE®6. As a negative control, 293T cells were mock transfected with transfection reagent(s) alongside Fc-eGFP and are represented by panel (F).

Next, we wanted to determine the effect of plasmid concentration in transfection efficiency. Moving forward with Lipofectamine 2000, 293T cells were transfected with pFc-eGFP at concentrations of 1 µg, 2 µg, 2.5 µg, 3 µg and 4 µg. These concentrations were chosen based on a range recommended by the manufacturer guidelines. Overall, a slight increase in fluorescing cells was observed with each increment of plasmid DNA concentration after a 48-hour incubation period. The most dramatic difference was exhibited between cells transfected...
with 1 μg of vector DNA versus 2 μg (Figures 8A and 8B), while the variation between 2 μg/2.5 μg (Figures 8B and 8C) and 3 μg/4 μg (Figures 8D and 8E) were negligible. Although these results suggest that using 4 μg of plasmid DNA per 9.5 cm² of cells would yield the highest amount of protein production, higher concentrations of DNA would require larger volumes of transfection reagent as transfection of plasmid DNA occurs at a 3:1 ratio. During production scale up, these parameters could be costly. With that in mind, I decided to move forward with using 2 μg plasmid DNA per 9.5 cm² as the increase of fluorescence at 4 μg did not seem that significant enough for preliminary studies.
Figure 8. Expression of eGFP in 293T cells 48 hours after transfection at different plasmid DNA concentrations. Plasmid pFc-eGFP was transfected at concentrations of (A) 1 μg, (B) 2 μg, (C) 2.5 μg, (D) 3 μg, and (E) 4 μg using transfection reagent Lipofectamine 2000 at a ratio of 3 μl reagent per 1 μg of DNA. (F) As a negative control, 293T cells were mock transfected with Lipofectamine 2000 reagent alongside pFc-eGFP.

4.1.3 ACEP peptibody binding capacity to SARS-CoV S protein

I next evaluated whether the ACEP peptides expressed in the peptibodies were able to bind to viral spike protein. This was determined by immunofluorescence assay. Cells expressing SARS-CoV spike protein were incubated with peptibody supernatants and binding was detected using a fluorescent antibody able to recognize mouse IgG. Despite previously published reports of the potent antiviral activity of the peptide\(^{50}\), binding was not observed among any of the generated ACEP peptibodies as seen in Figure 9.
Figure 9. Peptibody binding to 293T cells expressing SARS spike protein. 293T cells were transfected with 2 μg of SARS spike expressing plasmid and fixed with 10% formalin 48 hours later. Supernatants containing peptibodies Fc-ACEP-N (A), Fc-ACEP-C (B), Fc-ACEP-NC (C), Fc-2ACEP-N (D) and Fc-2ACEP-C (E) were incubated with the fixed cells for 1 hour at 37°C and then stained using an anti-mouse alexafluor488 (1:200). (F) Plain DMEM was used as a negative control. Cells were imaged using an EVOS FL Cell Imagine System.
4.1.4 Generation of sACE2 fusion protein

**Figure 10.** Schematic of the transmembrane domain of ACE2 cloned into pFUSE-mlG2a-Fc (A) and generated fusion protein Fc-sACE2 (B). This clone was confirmed via sequencing and was used to generate peptibody sACE2. The soluble gene segment of ACE2 was PCR amplified with primers encoding unique cut sites EcoRI and EcoRV.

Since results suggested ACEP and 2ACEP peptibodies were unable to bind to SARS-CoV spike protein, I instead cloned the soluble domain of the ACE2 receptor into the MCS of our plasmid vector pFUSE-mlG2a-Fc to generate plasmid pFc-sACE2 (Figure 10A). The soluble ACE2 protein expressed amino acids 1-740, which deleted the transmembrane domain of ACE2 (Genbank accession no. BAB40370.1). This was done as the transmembrane domain may have interfered with secretion of the Fc-conjugated ACE2 protein. As previously described, cloning was confirmed via sequencing and the plasmid was then transfected into 293T cells to produce the fusion protein (Figure 10B). Supernatant was collected for Western blot analysis and
presence of Fc-sACE2 fusion protein was detected using a secondary antibody to mouse IgG (Figure 11A).

To determine if other factors such as low protein concentration or non-specific proteins played a role in the negative results observed in the IFA binding assay, I transfected 293T cells in multiple plates with plasmids pFc-sACE2 (10 x 10 cm²) and pFc-ACEP-N (20 x 10 cm²)to increase protein yield. Plasmid Fc-ACEP-N was chosen as this vector exhibited the strongest protein expression compared to the other vectors following Western blot analysis as previously seen in Figure 6C and good expression was confirmed again (Figure 11C). Fc-ACEP-N supernatants were then purified through a protein G affinity column and eluted in 2 ml fractions.

**Figure 11.** Fc-sACE2 and Fc-ACEP-N peptibody detection in crude and purified supernatants. Plasmids pFc-sACE2 and pFc-ACEP-N were used to transfect 293T cells seeded onto four T875 and T150 flasks, respectively. Peptibody expression was detected by Western blot using IRDye800 anti-mouse IgG (1:5000). **(A)** Expression of Fc-sACE2 in crude supernatant. **(B)** Detection of Fc-sACE2 and Fc-ACEP-N peptibodies in eluted fractions post purification. Elutions were collected in 2 ml aliquots.
4.1.5 Binding capacity of Fc-sACE2

Protein concentration of each eluted fraction was measured using a Qubit fluorometer, which found the highest concentrations of antibody in fraction 4. Binding capacity of purified Fc-sACE2 (26.4 µg/ml) and purified peptibody Fc-ACEP-N (58.9 µg/ml) were evaluated using an immunofluorescence assay. Fusion protein Fc-sACE2 was detected bound to the surface of cells expressing SARS spike protein after the addition of 200 µl from purified stock (Figure 12A), while purified Fc-ACEP-N was not (Figure 12B). This data shows that Fc-sACE was able to efficiently bind to SARS-CoV spike protein. The result also suggests that the inability of Fc-ACEP-N binding to SARS spike was not influenced in the data shown in Figure 9 by low peptibody concentration or potential interfering components in the crude supernatant.

**Figure 12.** (A) Fc-sACE2 and (B) Fc-ACEP-N binding to 293T cells expressing SARS spike protein. 293T cells were transfected with 2 µg of SARS spike expressing plasmid and fixed with 10% formalin 48 hours later. Purified supernatants containing Fc-sACE2 fusion protein and Fc-ACEP-N peptibody were incubated with the fixed cells for 1 hour at 37°C and then stained using an anti-mouse secondary antibody conjugated with alexafluor488 (1:200). (C) Plain DMEM was used as a negative control. Cells were imaged using an EVOS FL Cell Imagine System.
Immunofluorescence results with the peptibodies were further confirmed using ELISA using purified spike protein bound to the ELISA plate for test for interaction with spike. As expected, low absorbance levels were observed with ACEP and 2ACEP fusion proteins (Table 4). High absorbance was observed by diluted Fc-sACE2, indicating either strong binding to SARS spike and/or high concentrations of peptibody.

Table 4. Binding capacity of ACEP peptibodies to purified SARS spike protein. Purified SARS spike protein was coated at a concentration of 1.2 μg per well. Purified Fc-sACE2 (1:30) and peptibody Fc-ACEP-N and crude supernatants of peptibodies Fc-ACEP-C, Fc-ACEP-NC, Fc-2ACEP-N and Fc-2ACEP-C were incubated in each well and bound peptibodies were detected with a secondary antibody to mouse IgG that is conjugated to a fluorescent label. As a positive control, an anti-SARS spike antibody was used at a concentration of 20 μg/ml.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ABSORBANCE</th>
<th>BINDING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified Fc-sACE2 (1:30)</td>
<td>2.416</td>
<td>✓</td>
</tr>
<tr>
<td>Purified Fc-ACEP-N</td>
<td>0.046</td>
<td>X</td>
</tr>
<tr>
<td>Fc-ACEP-C</td>
<td>0.051</td>
<td>X</td>
</tr>
<tr>
<td>Fc-ACEP-NC</td>
<td>0.052</td>
<td>X</td>
</tr>
<tr>
<td>Fc-2ACEP-N</td>
<td>0.046</td>
<td>X</td>
</tr>
<tr>
<td>Fc-2ACEP-C</td>
<td>0.066</td>
<td>X</td>
</tr>
<tr>
<td>Anti-SARS spike antibody</td>
<td>1.001</td>
<td>✓</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.054</td>
<td>X</td>
</tr>
</tbody>
</table>

4.1.6 Neutralization capacity of Fc-sACE2

Next, I wanted to determine whether the sACE2 fusion protein is able to effectively bind and neutralize SARS-CoV and prevent infection of tissue culture cells using a modified TCID$_{50}$ neutralization assay. For this assay I produced a larger quantity of Fc-sACE2 by transfecting 4 x T875 cm$^2$ flasks and collecting the supernatant at two time points (72 and 144 hours post transfection) which were pooled and purified by protein G column chromatography. Eluted
fractions were quantified and assessed for protein purity via automated electrophoresis. The highest concentrations of Fc-sACE2 were found to be eluted in fraction 5 at a concentration of 1.588 µg/µl (Table 5).

Table 5. Protein concentration of purified Fc-ACE2. Concentrations of eluted fractions following purification of Fc-sACE2 supernatant collected at 72 and 144 hours post transfection of 293T cells (4 x T875 cm\(^2\)) and purified by protein G column chromatography. Each fraction was analyzed by electrophoresis using an Agilent 2100 BioAnalyzer.

<table>
<thead>
<tr>
<th>FRACTION OF ELUTED Fc-sACE2</th>
<th>CONCENTRATION (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>165</td>
</tr>
<tr>
<td>5</td>
<td>1588</td>
</tr>
<tr>
<td>6</td>
<td>1343</td>
</tr>
<tr>
<td>7</td>
<td>509</td>
</tr>
<tr>
<td>8</td>
<td>530</td>
</tr>
<tr>
<td>9</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>208</td>
</tr>
<tr>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>12</td>
<td>69</td>
</tr>
</tbody>
</table>

SARS-CoV was generated using reverse genetics in Vero E6 cells and a subsequent viral stock was grown from this rescue. The stock was titred using a TCID\(_{50}\) assay and virus concentration was found to be 2.11 x10\(^6\) TCID\(_{50}\)/ml. A 1:2 dilution series of Fc-sACE2 starting with fraction 5 (1.588 µg/ml) was prepared and 200 µl was incubated with 100 TCID\(_{50}\) units of SARS-CoV for 1 hour at 37°C before adding 100 µl to Vero E6 cells in a 96-well plate. By day 6, CPE was observed in all control wells and the highest dilution of Fc-ACE2 that was able to completely inhibit CPE was recorded (Table 6). Based on triplicate repeats of the assay the
percentage of wells where CPE was not observed was calculated, and the median inhibition concentration (IC\textsubscript{50}) of Fc-sACE2 was calculated to be 1.23 µM (Appendix).

**Table 6.** Inhibition of SARS-CoV infection in Vero E6 cells by Fc-sACE2. Inhibition was measured by percent of wells protected from SARS-CoV virus infection by Fc-sACE2 after 6 days. In a modified TCID\textsubscript{50} neutralization assay, Vero E6 cells were seeded into a 96-well plate. A 1:2 dilution series of Fc-sACE2 was prepared ranging from 1.588 µg/µl to 0.01 µg/µl. From each dilution, 200 µl was incubated with 100 TCID\textsubscript{50} units of SARS-CoV virus for 1 hour at 37°C and subsequently transferred 100 µl onto confluent cells in triplicate columns. Cells were monitored for CPE daily after 48 hours. As a control, virus was incubated with sterile media and subsequently transferred onto cells.

<table>
<thead>
<tr>
<th>Fc-sACE2 CONCENTRATION (µg per well)</th>
<th>% WELLS PROTECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>79.4</td>
<td>100</td>
</tr>
<tr>
<td>39.7</td>
<td>100</td>
</tr>
<tr>
<td>19.9</td>
<td>100</td>
</tr>
<tr>
<td>9.9</td>
<td>100</td>
</tr>
<tr>
<td>5.0</td>
<td>66.6</td>
</tr>
<tr>
<td>2.5</td>
<td>33.3</td>
</tr>
<tr>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>0.6</td>
<td>0</td>
</tr>
</tbody>
</table>
4.2 Generation of a MERS-CoV mouse model

The progression of testing potential therapeutics after in vitro experimentation would naturally be to evaluate in an animal model. For my Fc-sACE2 fusion protein, a SARS susceptible mouse model is available because the virus was adapted to growth and high virulence in mice by serial passaging in mouse lungs\textsuperscript{121}. I have considerable interest in testing the Fc-sACE2 construct in this mouse model of infection. Although I was not able to complete this work as a M. Sc. student in the Biosafety Level 4 Laboratory at the NML, it is a priority for the group and will be completed once a large batch of Fc-sACE2 is prepared for in vivo experimentation.

However, MERS-CoV is currently the viral threat of most interest and so there is considerable interest in therapeutics for treating the infection and I next turned my attention to this virus and to establishing essential tools to work with it. It would be of interest to develop a peptibody or Fc-receptor conjugate for treating MERS-CoV infection, but MERS-CoV naturally infects a very limited number of animals and we do not have an available small animal model for laboratory work. NHPs are able to develop MERS-CoV infection but due to their large size are expensive to work with and are therefore unsuitable for preliminary testing of novel therapeutics\textsuperscript{23}. While the human DPP4 protein is known to be the host receptor for MERS-CoV it has been shown the DPP4 in most other small animals, typically used in the laboratory, does not bind the MERS spike protein with sufficient efficiency to initiate and sustain an infection\textsuperscript{137}. A previous study conducted by Zhao et al. has shown that transduction of hDPP4 using an Ad5 vector into the airways of mice transiently rendered this model susceptible to infection, but unfortunately this model is not commercially available so an in-house model must be generated. While efficient transduction of airway cells was reported with Ad5\textsuperscript{125}, I was also
interested in evaluating other viral vectors that are known to transduce the airway epithelium to determine whether it would be possible to improve this model. Adeno-associated viruses AAV6 and AAV9 have also been shown to efficiently transduce the lungs and other organs (AAV9) of mice\textsuperscript{138,139}. Here, I wanted to evaluate transduction efficiency and tissue tropism of AAV6, AAV9 and Ad5 vectors and whether they could be used to generate a susceptible MERS-CoV mouse model by delivery of the hDPP4 receptor.

### 4.2.1 Generation of an AAV6-hDPP4 viral vector

To generate an AAV6 virus for the purpose of expressing human DPP4 in mouse lungs, hDPP4 sequence (GenBank no. NM\_001935.3) was cloned into plasmid pACAGGFP to produce plasmid pACAGhDPP4 (Figure 13A).

![Figure 13. Schematics of plasmids pACAGhDPP4 (A) and pDGM6 (B) used to produce AAV6 virus expressing hDPP4. Human DPP4 was PCR amplified from plasmid pCAG-hDPP4 (National Microbiology Laboratory) using primers containing SacI and HindIII restriction enzyme cut sites to permit cloning using the InFusion kit.](image)
method into pACAGGFP to generate pACAGhDPP4. Plasmids pACAGGFP and pDGM6 were provided by Dr. Wootton of the University of Guelph.

Following confirmation of the gene insert via sequencing, human DPP4 expression from plasmid pACAGhDPP4 was evaluated using an immunofluorescence assay. Receptor expression was detected on the surface of the cells transfected with plasmid pACAGhDPP4 as seen in Figure 14A. Subsequently, AAV6-hDPP4 virus was generated by co-transfecting 293T cells with plasmids pACAGhDPP4 and pDGM6, a plasmid that provides the AAV6 capsid proteins, AAV replicase function and Adenovirus 5 helper functions, (Figure 14B) at a DNA concentration ratio of 1:2 and harvesting supernatants 72 hours later. Supernatants were purified using heparin HP columns and viral titres were determined via RT-PCR by Dr. Wootton at the University of Guelph (Table 7).

Figure 14. Expression of human DPP4 from plasmid pACAGhDPP4 (A) and a positive control plasmid expressing hDPP4 (B). Evaluation of hDPP4 expression was carried out by transfecting 293GT cells with 2 µg plasmid pACAGhDPP4 per 6 well dish. Cells were fixed after 48 hours with 10% formalin and DPP4 expression was detected using a rabbit polyclonal anti-CD26 primary antibody (Abcam) at a concentration of 1:1000 followed by an anti-rabbit secondary antibody conjugated with alexafluor488 (1:200). (C) As a negative control, 293T cells were transfected with transfection reagent and sterile media.
Infection, daily monitoring and sacrificing of mice was carried out by Anders Leung (NML), Darwyn Kobasa (NML), Mable Hagan (NML) and Kevin Tierney (NML).

Table 7. Stock titres of viral vectors AAV6-hDPP4, Ad5-hDPP4 and Ad5-ctrl.

<table>
<thead>
<tr>
<th>VIRAL VECTOR</th>
<th>TITRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV6-hDPP4</td>
<td>2.5x10^{13} vg/ml</td>
</tr>
<tr>
<td>Ad5-hDPP4</td>
<td>7.5x10^6 TCID_{50}/ml</td>
</tr>
<tr>
<td>Ad5-ctrl</td>
<td>7.5x10^6 TCID_{50}/ml</td>
</tr>
</tbody>
</table>

4.2.2 Transduction of the mouse airway with AAV6, AAV9 and Ad5 vectors

To determine if transduction of mouse airways with AAV6-hDPP4 is able to generate a MERS-CoV susceptible mouse model, mice (n=18) were infected via the intranasal route with 2.5 x 10^{12} viral genome (VG) copies of AAV6-hDPP4 virus. They were then challenged with 10^5 PFU of MERS-CoV EMC 2012 (Genbank JX869059) per mouse at one, two and three weeks post transduction (n=6) via the intranasal route. Untreated control mice (n=2) were also challenged with virus in parallel at one, two and three weeks. Mice were monitored daily for clinical signs of disease and weight loss and sacrificed at 4 and 7 days (n=2/day) post virus challenge for viral RNA detection in the lungs, liver, kidneys, heart and spleen*. While viral RNA was not detected in the liver, kidneys heart or spleen at either time point (data not shown), MERS-CoV RNA was found in the lungs (Figure 15). Differences in MERS-CoV viral RNA levels were not significant between control mice and transduced mice and viral genomes did not exceed 10^5 copies per gram of tissue. There was also a slight decrease in viral RNA copies in the lungs of mice sacrificed at 7 days compared to 4 days.

*Infection, daily monitoring and sacrificing of mice was carried out by Anders Leung (NML), Darwyn Kobasa (NML), Mable Hagan (NML) and Kevin Tierney (NML).
Figure 15. MERS-CoV RNA levels in mouse lungs following transduction of hDPP4 with AAV6-hDPP4 virus. BALB/c mice (n=18) were infected intranasally with $2.5 \times 10^{12}$ vg of AAV6-hDPP4 virus and then challenged with $10^5$ PFU of MERS-CoV EMC 2012 (JX869059) per mouse at one, two and three weeks post transduction (n=6) via the intranasal route. Untreated control mice (n=2) were also challenged with virus in parallel at one, two and three weeks. Mice were sacrificed at 4 and 7 days (n=2) post virus challenge for viral RNA detection. Statistical significance was determined using 2-way ANOVA test (p-value = 0.8296) comparing mice sacrificed at days 4 and 7 to controls.

We next evaluated AAV9 and Ad5 vectors. Ad5 has been previously demonstrated to successfully transduce the lungs of mice with hDPP4 and produce a rodent model that is susceptible to infection\textsuperscript{125}. AAV serotype 9 was evaluated as it has been shown to not only efficiently transduce airway epithelial cells, but other tissue types as well\textsuperscript{139}. Ad5 and AAV9 expressing human DPP4 and control vectors were commercially purchased from Abm Inc. rather than cloned in the laboratory. Ad5-hDPP4 and Ad5-ctrl viral stocks were grown up by infecting 293GT cells with seed stock received from Abm in a T150 cm\textsuperscript{2} flask per vector. I then harvested after ~8-9 days post infection as described in section 3.2.2 and stock titres (Table 7)
were determined by end point dilution assay as described in section 3.6.2. Based on the reported successful transduction by Ad5 by others, the viral vectors were immediately evaluated in mice.

Mice were transduced with AAV9-hDPP4 and AAV9-ctrl (n=3) by tail vein injection of 1x10^{10} genome copies (GC) of viral vector in DMEM-BSA. Mice were then challenged with MERS-CoV EMC 2012 at 10^5 PFU per mouse 14 days post transduction and sacrificed 2, 4 and 6 days later (n=1/day). For Ad5, mice were infected intranasally (n=6) with of 7.5x10^6 TCID_{50} units of Ad5-hDPP4 and Ad5-ctrl and challenged through the intranasal route with MERS-CoV EMC 2012 (10^5 PFU per mouse) 4 days post transduction. Ad vectors rapidly produce high levels of viral and transgene expression and as a consequence induce a strong immune response and are cleared relatively quickly in mice. AAV viral vectors on the other hand often show a significant delay in transgene expression but are also able to sustain protein production for weeks. Additionally, Zhao et al. had shown that hDPP4 expression in the lungs of mice following transduction was sufficient for a MERS-CoV infection and clearance of the viral vector did not result in a loss of receptor expression due to maintenance of hDPP4 in cells for a longer period. These observations suggest that mice transduced with hDPP4 using an Ad vector should be susceptible to MERS-CoV infection within 1 or 2 days after vector deliver while mice that are transduced using an AAV vector might not be susceptible to MERS-CoV infection for a week or more. Mice were then sacrificed at 2, 4 and 6 days post MERS challenge (n=2/time point). Similar to the AAV6 experiment, mice transduced with Ad5 and AAV9 viral vectors were monitored daily post infection for clinical signs of disease and weight loss and organs (lungs, liver, kidney, heart and spleen were harvested) for viral RNA detection.
4.2.3 *In vitro* analysis of AAV9 and Ad5 reporter genes

**Figure 16.** Fluorescence detection of mice transduced with Ad5-hDPP4, Ad5-ctrl, AAV9-hDPP4 and AAV9-ctrl. *(A)* A representative image of GFP fluorescence in BALB/c mice at 0, 1, 2, 3 and 4 days post infection with viral vectors Ad5-hDPP4 and Ad5-ctrl. *(B)* A representative image of luciferase-induced luminescence following injection of furimazine in BALB/c mice at 0, 4, 7, 12 and 14 days post infection with AAV9-hDPP4 and AAV9-ctrl vectors.

Additionally, the Ad5 and AAV9 viral vectors encode GFP or luciferase reporter genes, respectively. Each group of Ad5-hDPP4, Ad5-ctrl, AAV9-hDPP4 and AAV9-ctrl infected mice were imaged periodically following transduction using an UVP iBox® Scientia™ small animal imaging system. AAV9 infected mice required an additional tail vein injection with NanoLuc substrate for fluorescence. As seen in Figure 16, fluorescence was not observed in any of the Ad5 or AAV9 transduced mice at any time point.
**Figure 17.** MERS-CoV RNA levels in mouse lungs following transduction with hDPP4 using AAV9 and Ad5 viral vectors. (A) BALB/c mice (n=3) were infected with 1x10^{10} GC AAV9-hDPP4 and AAV9-ctrl via tail vein injection and then challenged through the intranasal route with MERS-CoV EMC 2012 (10^5 PFU per mouse) 14 days post transduction. Mice were sacrificed at 2, 4 and 6 days post MERS challenge (n=1) and MERS titers in the hDPP4 transduced animals compared to the titers in animals that received the control vector. (B) BALB/c mice (n=6) infected intranasally with 7.5x10^{6} TCID_{50} units of Ad5-hDPP4 and Ad5-ctrl and then challenged through the intranasal route with MERS-CoV EMC 2012 (10^5 PFU per mouse) 4 days post transduction. Mice were sacrificed at 2, 4 and 6 days post MERS challenge (n=2) and MERS titers in the hDPP4 transduced animals compared to the titers in animals that received the control vector (p-value = 0.0119 for all hDPP4 transduced animals compared to controls). (C) TCID_{50} concentration of MERS-CoV from 2, 4 and 6 dpi lung homogenates of Ad5-hDPP4 transduced mice. Statistical significance was determined using 2-way ANOVA test.

MERS-CoV RNA levels in the airways of both the control group and human DPP4 expressing AAV9 transduced mice were similar to the results observed in AAV6 treated mice.
where no significant differences were observed (Figure 17A). In contrast, RNA levels detected in the lungs of Ad5-hDPP4 transduced mice showed a significant increase when compared to control group infected with Ad5-ctrl (Figure 17B). We hypothesized that RNA levels detected in mouse lungs may have resulted from residual virus from initial inoculation. To evaluate whether there was live virus replication taking place, we cultured lung homogenate in a TCID$_{50}$ assay. While no CPE was observed in cells incubated with lung homogenates obtained from AAV6 and AAV9 infected mice (data not shown), lung homogenates from Ad5-hDPP4 treated mice yielded live replicating virus from 2 and 4 dpi, but not at 6 dpi (Figure 17C).

**Figure 18.** GFP gene expression *in vitro* by Adenovirus serotype 5 viral vectors. Magnification is indicated by the measurement in each image (400 μm). 293GT cells were infected with Ad5-hDPP4 **(A)** and Ad5-ctrl **(B)** in a 6-well dish. GFP reporter gene expression from Ad5-hDPP4 and Ad5-ctrl were read at 488 nm along with a negative control **(C)** 48 hours post infection.
The Ad5 and AAV9 vectors carrying hDPP4 and the reporter genes (GFP and luciferase, respectively) are commercially prepared so I did not initially evaluate them for either expression of the hDPP4 or reporter constructs prior to the mouse experiments. Since no fluorescence/luminescence was observed in mice following transduction, viral vectors were evaluated in vitro for GFP and luciferase expression. For GFP expression in Ad5 vectors, cells were infected with both Ad5 vectors and analyzed at 488 nm 48 hours later for fluorescence. As seen in Figure 18A-C, strong GFP fluorescence was observed in both Ad5-hDPP4 and Ad5-ctrl infected cells compared to mock infected cells. In a similar manner, cells were infected with both AAV9 vectors and supernatants were collected 7 days later. NanoLuc is a novel form of luciferase that is secreted from cells. Supernatants were then incubated with furimazine, the substrate for NanoLuc, for 20 minutes and read for luminescence, however, none was detected (data not shown).
Figure 19. Reporter gene and human DPP4 expression in vitro by immunofluorescence assay. Magnification is indicated by the measurement in each image (400 μm). 293GT cells were infected with Ad5-hDPP4 (A), Ad5-ctrl (C), AAV9-hDPP4 (B) and AAV9-ctrl (D) at MOI's ranging from 0.1-100 per well in a 6 well plate. Human DPP4 expression of Ad5-hDPP4 (A) and Ad5-ctrl (C) and AAV9-hDPP4 (B) and AAV9-ctrl (D) infected cells were evaluated after fixing with cold methanol 4 and 7 days post infection, respectively. As a positive control, 293GT cells were transfected with 2 µg of a hDPP4 expressing plasmid (E), while mock infected cells were used as a negative control (F) and fixed 48 hours post transfection. DPP4 expression was detected using a rabbit polyclonal anti-CD26 primary antibody (Abcam) at a concentration of 1:1000 followed by an anti-rabbit secondary antibody with alexafluor488 (1:200).

Endogenous hDPP4 was also evaluated in vitro for both Ad5 and AAV9 vectors at 4 and 7 days post infection using immunofluorescence assay, respectively. Expectedly, human DPP4
was observed on cells infected with Ad5-hDPP4 and not in Ad5-ctrl (Figure 19A and 19C).

Although alexafluor488 secondary antibodies excitation and emission are at the same wavelengths as the GFP gene, fluorescence of GFP was no longer observed by day 4 when the IFA was carried out for hDPP4. AAV9-hDPP4 did not appear to express any DPP4 and immunodetection for hDPP4 gave the same outcome as with the AAV9-ctrl which does not contain hDPP4 (Figure 19B and 19D).
Chapter Five: Discussion

5.1 ACEP peptibody targeting SARS-CoV

The main goal of my thesis was to generate and evaluate a potential therapeutic against SARS-CoV, as a proof-of-concept model for later development of a similar therapeutic to MERS-CoV, using a fusion between the Fc portion of an antibody and a biologically active peptide called a peptibody. Peptibodies have been previously used to modulate biological processes such as chronic immune thrombocytopenia and diabetes mellitus type 2\textsuperscript{140}, but their application in treating viral infections has not been studied. The principle of this fusion protein is based on the growing array of therapeutic peptides that have been described. Peptides show a lot of promise as a therapeutic against infectious and metabolic diseases as well as cancer due to their small size, specificity and ease of production. However, there are downfalls to these biopharmaceutical agents, the most significant issues being poor stability and short biological half-lives\textsuperscript{140,141}. Conjugation of peptides to the Fc domain of IgG antibodies not only increases the stability of the peptide, but also allows these fusion proteins to take advantage of the neonatal Fc receptor salvage pathway responsible for prevention of IgG antibody degradation\textsuperscript{142}. In this particular study, we chose to fuse an ACE2 derived peptide (ACEP) previously shown to have potent antiviral activity against SARS-CoV, due to its direct high affinity interaction with the viral spike protein, to the Fc domain of a mouse IgG2a antibody. While our hopes were to increase the stability of the small peptide for therapeutic use, there was potential to exploit other properties of the Fc domain as well such as stimulation of antibody dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) effector mechanisms, to enhance clearance of virus. Typically in humans,
IgG1 and IgG3 trigger these mechanisms more readily, but IgG2a is only able to induce a subtle response\textsuperscript{143}. However, in mice, IgG2a is the most efficient at fixing complement and exhibits the most CDC of all the IgG subclasses. Previous studies have shown effective CDC response by murine IgG2a fusion proteins \textit{in vitro} and \textit{in vivo} against opportunistic pathogen \textit{Pneumocystis jirovecii}\textsuperscript{144}.

Initially, five fusion proteins were generated by fusing one or two ACEP peptides at N, C and both termini of the Fc domain of a murine IgG2a antibody. The ACEP peptide was derived from two amino acid sections from the RBD of ACE in a study carried out by Han \textit{et al.} that combined amino acids 22 to 44 with 351 to 357 with a small linker between them to generate a peptide that interacts with the receptor binding domain of the viral spike protein. Current peptibodies undergoing clinical trials for the treatment of other diseases such as Romiplostim and AMG 386 were manufactured in \textit{E. coli} bacterium\textsuperscript{145,146}. These peptibodies were produced in \textit{E. coli} as single chained polypeptides expressed in inclusion bodies. These insoluble protein products require subsequent refolding and purification that is carried out via multiple rounds of differential precipitation and chromatography. While this process for manufacturing peptibodies has been found to be more efficient than mammalian cell culture\textsuperscript{140}, we opted to move forward with production of our peptibodies in mammalian 293T cells. Downstream processing would have been laborious for the small amounts of protein initially being produced. Additionally, peptibodies produced in mammalian cell culture undergo glycosylation unlike their \textit{E. coli} manufactured counterparts, which supports antibody stability and ADCC/CDC activity\textsuperscript{147}. Results from Western blot analysis showed all five peptibodies were secreted in the supernatants from 293T cells, however expression seemed to be stronger in peptibodies with
ACEP fused at the N terminus (Figure 6). As expected, the Fc fused with the peptides had a slight increase in molecular weight compared to that of mouse IgG Fc, which was used as a positive control, further confirming ACEP expression in my peptibodies.

To evaluate transfection efficiency, I generated an enhanced-GFP -mlG2a fusion protein to be used a transfection control during peptibody production and to optimize transfection conditions. Fluorescence observed via microscopy was compared between five transfection reagents: Lipofectamine 2000, Lipofectamine 3000, TransIT® LT1, CalPhos™ and Fugene®6. Of the six reagents evaluated, transfections with Lipofectamine 2000 and TransIT® LT1 exhibited the most fluorescence (Figure 7). I decided to proceed with Lipofectamine 2000 due to the negligible difference in fluorescence between the two best reagents and shorter transfection time. Additionally, this reagent has been widely utilized in previous generations of fusion peptides\(^{144,148}\). Using the same Fc-eGFP fusion protein I tested whether an increase of DNA concentration would correspondingly increase transfection rates. Increased DNA concentration was observed to slightly increase fluorescence (Figure 8). However, higher concentrations of DNA require higher volumes of transfection reagent, which could get expensive and for this reason I decided to proceed with 2 \(\mu\)g DNA per 9.5 cm\(^2\) of cells.

Before evaluating the therapeutic potential of the ACE2 derived peptibodies in vivo, I needed to determine whether they were are able to efficiently bind SARS-CoV spike protein and neutralize virus infectivity in vitro. Based on previous studies, small peptides generated from key fragments of ACE2 were able to interfere with viral entry and subsequent infection by inhibiting interaction between the spike protein and host ACE2 receptor\(^{50,76,149}\). These interactions have been studied in great detail and three regions within ACE2 have been
identified to be important for binding viral S glycoprotein. Structural studies based on a small
fragment of SARS spike containing the RBD found ACE2 residues Q24, T27, K31, H34, E37, D38,
Y41, Q42, L45, L79, M82, Y83, N90, Q325, E329, N330, K353 and G354 made direct contact with
the viral protein. Similarly, analysis by alanine-scanning mutagenesis using the full intact S
protein further identified additional amino acid residues E22, E23, K26, D30, K31, H34, E35, E56
and E57. It is of interest to note K26 and D30 were not found to interact with spike RBD but
appeared to still be critical in virus/host receptor interaction. Additionally, ACE2 amino acids
K353, D355 and R357 are important for binding the S1 domain of spike protein, more
specifically, K353 and G354 make direct contact with the receptor-binding motif (RBM) of S1.

Despite previously described antiviral activity and abundance of critical residues of
peptide ACEP, none of the initial five peptibodies were able to bind to SARS spike protein
expressed on Vero E6 cells in our immunofluorescence assay (Figure 9). The free ACEP peptide
is apparently conformationally free to adopt a structure that optimally interacts with the SARS-
spike protein with sufficient affinity to interfere with binding of the virus to the ACE2 protein on
cells. However, it is unclear whether the ACEP is similarly free to efficiently interact with spike
when it has been conjugated to Fc. The inability of the ACEP-Fc peptibodies to recognize cell-
expressed spike protein indicates that binding is not able to occur and there are at least two
possible reasons that could explain this outcome. First, it is possible that fusion of the ACEP is
forcing it to adopt a conformation that is suboptimal for efficient interaction with spike.
Residues 22-44 form an α-helix that amphipathic, exhibiting a surface that is highly hydrophilic
and another surface that is highly hydrophobic. The side of the helix that interacts and binds
to viral S protein is highly polar with a multitude of charged amino acids while the opposite side
that would normally face the interior of ACE2 is hydrophobic. Based on this, the α-helical conformation would be less stable in the aqueous environment the peptibody would be exposed to. Additionally, residues 351-357 are originally part of a secondary β-turn-β structure in intact ACE2, however the peptibodies are lacking residues that support formation of this structure. SARS spike RBD contains multiple epitopes that elicit strong neutralizing antibody responses, but they are all conformation dependent. The antiviral activity of the free peptide suggests that conformation likely resembles how the peptidyl sections bind to spike glycoprotein in the ACE2 crystal structure.

On the other hand, it is possible that in the fusion of ACEP to the Fc antibody domain that the Fc portion may sterically hinder the interaction of ACEP and spike even if ACEP can adopt a conformation that is suitable for interaction. It is also conceivable that binding was not observed due to insufficient peptibody concentrations resulting in undetectable fluorescence or inhibiting factors present in the tissue culture media.

5.2 Fc-sACE2 fusion protein

To address whether size and conformation was the main issue, we fused the soluble domain of the ACE2 ectodomain (amino acids 1-740) to mouse IgG2a Fc to generate Fc-ACE2. The full human ACE2 receptor is normally comprised of 805 amino acids, but this protein was truncated to remove the transmembrane and cytoplasmic domains. Soluble forms of ACE2 have previously been described to inhibit association between Vero E6 cells and a S protein derived immunoglobulin (amino acids 12-672 of spike conjugated to an antibody Fc). Subsequent immunoprecipitation identified the specific interaction between ACE2 and the SARS-spike proteins. The soluble ectodomain of ACE2 was then later shown to have potent
antiviral activity; pseudoviruses bearing SARS-CoV spike protein were not able to infect 293T cells when pre-incubated with supernatants containing soluble ACE2 providing further evidence that the removal of the transmembrane and cytoplasmic domains does not affect ACE/SARS-spike interaction\textsuperscript{151}.

Similar to the ACEP peptibodies, I produced Fc-ACE2 in mammalian 293T cells and expression was confirmed by western blot analysis (Figure 11). Molecular weights estimated from the bands were consistent with the observed sizes of a free ACE2 protein and Fc produced by pFUSE-mlG2a-Fc which are approximately 100 kDa\textsuperscript{151} and 34 kDa, respectively. To address any concerns about having a high enough protein concentration and purity, I made larger protein preparations of both Fc-ACE2 as well as Fc-ACEP-N and purified the protein from cell supernatants using protein G affinity chromatography. Concentration of purified Fc-sACE2 and Fc-ACEP-N were determined to be 26.4 µg/ml and 58.9 µg/ml, respectively.

We anticipated the ectodomain of ACE2 would be large enough to allow for proper structural conformation without interference from mouse IgG Fc and there is a short linker contained in the Fc cloning vector that allows for independent folding of the Fc domain and fused protein. Results from immunofluorescence assays provided evidence that the fusion of sACE2 to Fc did not inhibit the ability of sACE2 to bind spike and there was efficient interaction between Fc-sACE2 to SARS spike protein (Figure 12). This potentially indicates that Fc-sACE2 was able to fold into a tertiary structure that allowed interaction with high affinity residues within the first \( \alpha \)-helix and \( \beta \)-sheet\textsuperscript{57}. However, using crystal structure analysis, further structural studies would need to be carried out for confirmation. With that being said, Fc-sACE2 appeared to be binding at rather low levels since only a few individual cells were bound by the
fusion protein. This could be a result of low binding affinity and/or low transfection rates of the
spike receptor on the cell surface. Because the cells that are labelled are highly labelled this
suggests that the latter possibility is true. Only a few cells were successfully transfected with
the construct to express the SARS-Spike protein but any cell that was successfully transfected
expressed high levels of spike that was recognized efficiently Fc-sACE2. If immunofluorescence
was being limited by low affinity of Fc-sACE2 we would expect to see more cells that show weak
labelling and this is not observed, all cells that are labelled show strong fluorescence. This could
also explain why Fc-ACEP-N was not observed bound to the surface, despite higher protein
expression and purification of Fc-ACEP-N.

To further confirm binding of Fc-sACE2 to SARS spike, an additional assay was carried
out. Using purified SARS spike protein in an ELISA based assay, Fc-sACE2 at an 1/30 dilution
from the purified stock, was observed to bind to the glycoprotein providing a high ELISA signal,
whereas the signal for detection of undiluted Fc-sACE2 was out of range (data not shown).
Meanwhile, purified Fc-ACEP-N along with the crude supernatants of the other ACEP
peptibodies exhibited zero interaction with SARS spike. These results suggest that inability of
peptibody Fc-ACEP-N to bind to spike is due to other reasons, whether it be peptide
conformation, steric hinderance or otherwise, rather than low protein concentrations or
inhibitors present in the media.

Unlike ACE2, segments of human DPP4 have yet to be evaluated for antiviral potential.
Rather, many studies have focused on using the RBD that interacts with hDPP4 to elicit
neutralizing antibodies for isolation or potential vaccination strategies\textsuperscript{152,153}. Results of our
binding assays with Fc-sACE2 suggest it may be possible to use the ectodomain of hDPP4,
similar to sACE2, in peptibody development. It has also been shown that using monoclonals that targeted both RBD specific and non-RBD specific areas, it was possible to protect from lethal dosage of virus and delay the emergence of escape mutants\textsuperscript{154}.

Next, I assessed whether Fc-sACE2 was able to exhibit antiviral activity as was previously seen with the ACE2 ectodomain. When pre-incubated with 25 TCID\textsubscript{50} units SARS-CoV virus, Fc-sACE2 was able to inhibit infection of Vero E6 cells at an IC\textsubscript{50} (the half maximal inhibitory concentration) of 1.23 µM. Compared to other small compounds that target various proteins of SARS-CoV, these results provide evidence of the promising potential of Fc-sACE2 as a therapeutic. A small molecule targeting non-structural protein 3, an essential component of SARS virus replication, was able to considerably inhibit \textit{in vitro} replication of the virus with an EC\textsubscript{50} of less than 1 µM. Other compound targeting the helicase protein also exhibited anti-viral activity with EC\textsubscript{50} values ~10 µM\textsuperscript{155}. Some compounds such as chloroquine, chlorpromazine, loperamide, and lopinavir were shown to inhibit MERS-CoV replication \textit{in vitro}. The EC\textsubscript{50} values of these compounds were in the range of 3-8 µM\textsuperscript{26}.

As a fusion protein, the Fc segment lends some advantage over using the soluble ACE2 ectodomain on its own to inhibit viral replication. Ricks \textit{et al.} investigated the effects of different IgG subtypes fused to Dectin-1 in reducing \textit{Pneumocystis} organisms in the lungs of mice. They observed a reduction in viability of \textit{P. murina} by a Dectin-1:mlG2a Fc fusion protein through a complement dependent process \textit{in vitro} where as Dectin-1:mlG1 Fc did not mediate killing. These results are consistent with previous observations of mouse IgG2a exhibits the most CDC function and is the most efficient isotype at fixing the complement system\textsuperscript{156}. Interestingly, IgG1 isotype showed higher protein expression and was more effective in
reducing *Pneumocystis* asci (cyst forms) burden, while Dectin-1:mlgG2a was observed to only slightly reduce overall organism burden in the lungs of immunodeficient mice. They concluded that overall protein levels may have been the main contributing factor to lack of efficacy *in vivo*, suggesting complement-dependent killing induced by Dectin-1:mlgG2a likely requires higher protein concentrations. It is important to note that mice in this study were treated with an expression plasmid construct expressing the fusion protein and Dectin-1:mlgG was produced within the animals\(^{144}\). This would be less of a concern for Fc-sACE2 as the protein would be produced and purified *in vitro* rather than injection with the plasmid construct. Injection of the vector into the host to produce the proteins *in situ* is advantageous where the peptibody concentration levels will build within the host and purification is not necessary. However, to treat an acute viral infection, the time required to generate the protein may not be ideal.

In response to a disease state, such as that caused by infection, the expression levels of many type I and type II transmembrane proteins may be reduced at the cell surface either by decreased transcription and translation, protein internalization or ectodomain shedding. Ectodomain shedding, where the extracellular domain of a transmembrane protein is proteolytically removed from the cell surface, has been observed among several biologically active proteins and in each case, has a unique effect on function\(^ {157}\). For example, the shedding of heparin-binding-EGF-like growth factors results in self-activation and the release of these proteins bind to EGF receptors that stimulate cell proliferation. In contrast, other proteins such as membrane-bound forms of Kit ligand and ephrins have little to no biological activity following their release from the cell surface\(^ {158}\). ACE2 has been found to undergo ectodomain
shedding from human epithelial cells mediated by tumor necrosis factor-alpha convertase (ADAM17) \(^{159}\). In addition to retaining its role as a SARS-CoV receptor, soluble ACE2 obtained from primary human airway epithelia and Calu-3 cells was still able to convert ANG II to ANG (1-7) \(^{160}\). As previously mentioned, ACE2 plays an important role in the protection from severe ALI and it has been found that ARDS progression in wild-type and ace2 knockout mice can be attenuated by treatment with a catalytically active recombinant ACE2 protein. Although the intention of evaluating Fc-sACE2 is as a proof-of-concept model for generation of a SARS-CoV therapeutic, our peptibody could potentially be used in tandem to protect against ALI.

Likewise, a soluble form of DPP4 has been found in in the body. It is most commonly detected in bodily fluids such as plasma, seminal fluid, but low levels have also been detected in cerebral spinal fluid \(^{161}\). It is well established that DPP4 functions as a regulator of diverse biological functions and dysregulation of its enzymatic properties could lead to many issues. In a variety of neurological and inflammatory diseases as well as cancer, the abundance of both membrane-bound and circulating DPP4 appears to be altered. Previous studies have identified sDPP4 as a novel adipokine (cytokine that is secreted by adipose tissue), which is known to be involved in regulation of glucose homeostasis through the incretin system \(^{162}\). Transmembrane DPP4 cleaves a variety of substrates such as growth factors, hormones, neuropeptides and chemokines. Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are two incretin hormones that are substrates of DPP4. GLP-1 and GIP are released from the intestinal mucosa and contribute to insulin secretion, the effects of which are progressively amplified as the concentration of glucose in the plasma rises \(^{163}\). As soluble DPP4 is still catalytically active, high levels of sDPP4 in serum have been correlated with insulin resistance, metabolic syndrome
and increased expression and secretion of pro-inflammatory cytokines\textsuperscript{164,165}. In comparison with sACE2, retention of enzymatic activity of a sDPP4 fusion protein may be detrimental rather than beneficial to the host and should be carefully examined \textit{in vivo}.

Reportedly, Hong Peng Jia et al. identified a single mutation, L584A, lead to the prevention of sACE2 shedding. This particular residue was speculated to be within a sheddase "recognition motif" that typically reside in the distal portions of the ectodomain of released proteins. It was thought that replacement of a normally hydrophobic leucine by alanine would alter the tertiary structure of ACE2 by interfering with interactions with neighboring hydrophobic residues and thereby interrupt virus binding with the mutation located near the catalytic and receptor binding domains of ACE2. However, no differences in susceptibility were observed in cells expressing wild-type or L584A mutant ACE2 receptors and the ectodomain of the receptor was not shed\textsuperscript{160}. It also appears that soluble DPP4 is generated as a result of proteolytic cleavage rather than secreted like other adipokines. A fraction of sDPP4 levels in serum likely originates from immune cells, however the main source of circulating DPP4 and its regulation has yet to be determined\textsuperscript{162,166}. It may also be a good idea to insert the L584A mutation in our Fc-sACE2 peptibody as the fusion protein could potentially be cleaved during \textit{in vivo} studies and this should also be considered during design of a sDPP4 fusion protein.

5.3 Transduction of mouse airways

5.3.1 AAV6

The understanding of viral pathogenesis comes mostly from studying animals that simulate human disease. Animal models also play a crucial role in the evaluation of potential treatments and vaccines. Non-human primates (NHPs) are favoured as models as they often
mimic disease and immune response more closely to humans than any other animal. However, the use of NHPs for pre-clinical studies presents significant ethical and economic challenges. Furthermore, they may not be available to all institutions. Smaller animal models are a great alternative as they are inexpensive and larger and more frequent studies can be carried out. Despite mild presentation of illness, some small animal models were found to naturally support SARS-CoV infection. Subsequent serial adaptation of SARS virus in mice and engineering of a transgenic mouse model expressing human ACE2 receptor generated models that displayed overt clinical disease. However, species specific restriction of susceptibility to infection proved to be more of an obstacle when evaluating small animals for MERS-CoV as rodents and most other small animals are not naturally permissive to infection and this is thought to be due to inability of the MERS-CoV spike protein to bind to the DPP4 in those animal species.

Initial studies demonstrated that incorporation of residues 246-505 of the human DPP4, which contains the Spike (S) protein RBD, into ferret DPP4 allowed MERS-CoV to infect ferret cells. Further studies were then carried out to characterize amino acid residues in the region of DPP4 that interacts with the S protein RBD. Six differences were identified between human and mouse DPP4 in this region, 5 of which were predicted to be the cause of the lack of binding affinity\textsuperscript{113}. Since the mouse DPP4 was also unable to bind the MERS-CoV virus, one solution to make mice susceptible to infection would be to supply human DPP4 to cells of the mouse respiratory tract so that it is expressed in those cells. In this study we investigated whether transduction of the hDPP4 gene via viral vectors AAV serotype 6, AAV serotype 9 and Adenovirus serotype 5 vectors into mice was able to generate a suitable rodent model.
Viral vectors for gene delivery have been utilized for years for therapeutic intervention, with AAV emerging as a prime candidate for gene therapy and vaccines. Over 100 naturally occurring (isolated from human and NHPs) or synthesized variants of AAV capsid sequences have been researched. One of the most extensively studied is AAV serotype 2 for gene delivery and treatment of diseases. AAVs are unable to replicate without the assistance of helper viruses and wild type infections have not been associated with human pathogenicity. Transduction by these vectors confers persistent long-term transgene expression. Unfortunately, due to the host humoral response and pre-existing neutralizing antibodies reported to occur in 35-80% of the human population, the potential of this serotype as a gene delivery system may be limited\textsuperscript{167}. More importantly for this study, despite being able to transduce multiple cell types, AAV2 was shown to only be able to transduce lungs at low to moderate rates. Research into other serotypes indicated that transduction efficiency is primarily determined by the source of the Cap proteins. The cap gene produces three viral capsid proteins (VP1, VP2 and VP3) that arrange into icosahedral structure to form the AAV capsid. Competition assays showed that the capsid of different AAV serotypes bind different receptors. AAV6 pseudo-typed vectors were found to transduce lungs more effectively and with lower immunogenicity than AAV2 when delivered by nasal aspiration\textsuperscript{168-170}.

In my studies to develop a mouse that is sensitive to MERS-CoV, the first group of BALB/c mice were infected with an hDPP4 expressing AAV6 viral vector via the intranasal route. It has been shown that distribution and efficiency of the virus is dependent on method of administration, volume and level of anesthesia. Based on previous studies, 100 \( \mu \)l virus at \( 10^{11} \) genome copies/ml was administered using a modified intranasal technique to maximize
transduction efficiency\textsuperscript{138}. Infections were allowed to progress for the duration of 1, 2 and 3 weeks before MERS-CoV virus challenge. AAV6 vectors have a long duration period for protein expression and transduced animals are typically sacrificed 3-4 weeks post vector administration\textsuperscript{138,169} for analysis of transgene expression. Although other organs were also harvested, viral RNA was only detected in the lungs. The RNA levels were observed to be quite low and no significant difference was noted between AAV6-hDPP4 infected and uninfected control mice after intranasal infection with MERS-CoV. Additionally, no clinical signs of disease or weight loss were observed in the mice, although this was anticipated given a similar lack of clinical signs of disease in mice that had been transduced with hDPP4 using an Adenovirus 5 viral vector\textsuperscript{125}. Similar results were observed when mouse adapted MERS-CoV strains were used to infect immune-deficient mice that express only the murine DPP4. In this study, genomic viral RNA was detected by RT-PCR but not messenger RNA\textsuperscript{171} suggesting persistence of the viral inoculum in the tissue but failure of the virus to infect and replicate to any detectable levels. Altogether, these results suggested that either hDPP4 transduction by AAV6 did not occur or receptor levels were too low to allow a robust MERS-CoV infection. Given that previous studies have shown success in using AAV6 to transduce the airways of mice, some modifications could be applied to future studies using this vector. Histological analysis could be carried out to determine optimal time points to challenge transduced mice with MERS-CoV. Additionally, mice could be administered a second dosage with an AAV-hDPP4 of another serotype to boost receptor expression. Halbert et al. found that re-administration with the same serotype is blocked, likely due to neutralizing antibodies generated in the initial vector exposure.
5.3.2 AAV9 & Ad5

AAV9 is another serotype that was observed to efficiently transduce mouse alveolar epithelium when administered by intratracheal instillation. Nineteen human α-1-antitrypsin (AAT) expressing AAV vectors, including well established serotypes AAV1, 2, 5, 6, 7, 8, and 9 were generated by Limberis et al., and hAAT secretion in serum was measured following transduction of mouse lungs. The highest protein concentrations found in serum were conferred by AAV1, 6 and 9. Additionally, it was found that serotype 9 preferentially transduces alveolar epithelium as opposed to AAV6 that has been shown to target mouse conducting airways\textsuperscript{172}. Further support for using other AAV serotypes come from transgene expression and biodistribution studies carried out by Zincarelli et al. comparing serotypes 1-9 over a 9-month period after administration via tail vein injection. This study revealed that AAV9 had some of the highest expression levels, stability, viral genome distribution as well as rapid onset of transgene expression. The highest levels of protein expression by AAV9 were found in the liver, heart and hindlimb skeletal muscles\textsuperscript{139,173}. Although MERS-CoV causes a respiratory infection, hDPP4 is also expressed in the livers of humans. Coronaviruses have also been found in the liver of deceased animals that suffered respiratory distress and acute liver failure\textsuperscript{9,30}.

Many adenovirus vectors previously used in cancer and vaccine therapy are mostly genetically modified versions of serotype 5. When compared to AAV vectors, transgene expression tapers off quite rapidly with almost a 90% loss after 4 weeks. This is likely a result of the strong immune response elicited by Ad vectors. In mice, induction of the innate immune response system directed at capsid proteins begins immediately after intravenous injection and is maximal after 6 hours. Many pro-inflammatory proteins are activated and can be lethal if
doses of $10^{10}$ viral particles are administered to these small animals\textsuperscript{174,175}. For the purposes of this study however, extended expression times are not of high priority. Adenoviruses are advantageous because large exogenous genes can be inserted into a recombinant vector and Ad5 exhibits tropism for respiratory epithelium when introduced intratracheally\textsuperscript{176}. Similar to some serotypes of AAV, Ad5 has also been found to target the liver following delivery by intravenous injection\textsuperscript{177,178}.

In this study, AAV9 was administered via tail vein injection to determine whether a more robust mouse model exhibiting morbidity could be generated, while Ad5 was delivered by the intranasal route. Although direct comparison of intranasal inoculation efficiency to tail vein injection has not been carried out for AAV9, high-level transduction with this serotype has been found in the lungs of mice in individual studies. Tail vein injection has also been found to transduce different tissues at high levels, which is of interest as the DPP4 receptor in humans is expressed in the kidneys, small intestine and liver as well as the lungs. Even in cases of intra-arterial administration where the lungs would be the last organ exposed to AAV9, transduction rates did not appear to be reduced\textsuperscript{179}. Although animal models have been inconsistent in regards to whether MERS-CoV causes systemic infection, in severe human cases renal failure has been a well-known complication\textsuperscript{180}. Moreover, Prescott et al. observed MERS-CoV dissemination in immunosuppressed Rhesus macaques which is interesting as immunosuppression is a comorbidity of clinically severe MERS cases of disease in humans\textsuperscript{181}.

MERS-CoV RNA levels in the lungs of AAV9 infected mice were similar to those observed in AAV6 transduced mouse airways. RNA levels in the lungs appeared to decrease following MERS-CoV infection, likely indicating clearance of the initial inoculated virus from the lungs,
without productive replication of the virus. Viral RNA levels in BALB/c mice whose airways were transduced with Ad5-hDPP4 exhibited a significant difference between Ad5 control, AAV6 and AAV9 mice with over $10^8$ copies/ml detected in the lungs compared to $10^6$ in other mice. When lung homogenates from Ad5-hDPP4 infected mice were cultured in vitro, live replicating virus was detected as shown by cytopathic effect on Vero cells 3 days after passage of tissue lysates. However, mice still did not exhibit signs of clinical disease or weight loss and viral RNA levels decreased over time in the airways following infection. While Ad5 was successful in terms of allowing MERS virus to infect the respiratory tract, human disease was not recapitulated and clearance of virus was rapid. Despite these disadvantages, Zhao et al. was able to examine a few key components in the immune response to MERS-CoV, as well as successfully evaluate a Spike protein derived vaccine and serum transfer treatment using Ad5-hDPP4 transduced immunocompromised mice. They also observed weight loss in aged mice and slowed viral clearance in both immunodeficient and aged mice. This is especially of interest to note as both advanced age and impaired immune function are comorbidities of more severe MERS infections\textsuperscript{23}.

To test the capacity for the virus vectors to efficiently deliver a transgene, both AAV9 and Ad5 vectors were designed to contain reporter genes to observe viral protein expression in vivo. The Ad5 vectors had a GFP gene cloned into both the control and hDPP4. However, at the time the viral transduction studies were conducted in the CL4 lab, the imaging system was not properly configured for GFP detection and we did not have the reagents to repeat the experiment. Subsequent in vitro evaluation of the GFP reporter gene in cells infected with the Ad5 vectors exhibited fluorescence after 48 hours but it remains undetermined whether
fluorescence would have been observed in vivo. Both AAV9 vectors encoded the nanoluciferase transgene but luminescence was not observed in vivo following substrate injection, nor was it seen in subsequent in vitro experiments. When hDPP4 expression was evaluated in vitro, hDPP4 was observed to be expressed at relatively high levels by Ad5-hDPP4 infected cells but not in AAV9-hDPP4 infected cells. These results were consistent with the RNA levels detected in the lungs of infected mice and suggest that hDPP4 was likely not expressed in AAV9 infected mice. For future studies, rather than relying on reporter genes cloned into the vector to determine protein transduction efficiency in vivo, histological examination of the organs could be carried out instead. However, the downside of using this method over looking for reporter gene expression is that the study would require more mice and considerably more of the viral vector preparations.

In terms of developing a model susceptible to a more robust infection, Agrawal et al. was able to produce a lineage of mice globally expressing hDPP4. Severe respiratory and brain infection was established followed by death within days of intranasal inoculation of virus. High loads of infectious virus (~$10^{7.8}$ TCID$_{50}$/g) were detected in the lungs 2 days post infection, approximately 3 logs higher than the live virus found in our Ad5-transduced lung homogenate and 1 log higher than the titres detected by Zhao et al. In addition to the high viral titres, infected mice developed severe bronchointerstitial pneumonia and experienced rapid weight loss prior to death. Another group was able to use this model to evaluate a candidate vaccine and therapeutic for MERS-CoV.

Viral RNA was also detected in the heart, spleen and intestines of the transgenic mice suggesting systemic dissemination following initial inoculation of the respiratory tract, but
infectious virus were not recovered from these organs. While evaluation of dissemination was not carried out in Zhao’s study, the heart, spleen, liver, kidneys and spleen were tested for viral RNA in our Ad5 model, but no RNA was detected (data not shown). Interestingly, viral RNA was not detected in the liver in Agrawal’s transgenic model despite prominent expression of hDPP4 in the liver. Renal failure has been observed in several MERS patients as well as hepatitis in some SARS cases. However, the latter has been thought to be driven by collateral damage mediated by the response of the pulmonary infection rather than direct immune response in the liver and therefore is likely a result of the renal failure seen in SARS infections\textsuperscript{182}.

Li et al. generated a similar hDPP4 transgenic mouse model using different promoters to control receptor expression. A human surfactant protein C (SPC) promoter was used for isolated expression in pulmonary epithelia, while a K18 promoter was utilized for direct transgene expression in the epithelial cells of the airway, liver, kidney, gastrointestinal tract and some cells of the nervous system. K18-hDPP4 mice shared many similar results with the transgenic model reported by Agrawal et al. Intranasally infected K18-DPP4 mice exhibited weight loss and death 6-7 days post infection. Highest viral titres were found in the lungs and brain (6 \( \times \) 10\(^7\) pfu/g and 10\(^8\) pfu/g, respectively). In contrast, SPC-hDPP4 lineage mice were similar to the Ad5 model, where no dissemination of virus outside of the respiratory tract occurred and with even lower viral loads detected in the lungs and no mortality. From these studies, it is perhaps restriction of infection to the lungs that allows mouse innate immunity to quickly diminish virulence factors and subsequently limit damage due to inflammation\textsuperscript{183}.

Over-expression transgenic models, while effective in generating a deadly infection, may complicate results when investigating respiratory pathogenesis. The rapid rate of progression to
death may be an issue for studies that require longer duration, but subsequent studies determined a much lower infectious dose of MERS-CoV (10 TCID$_{50}$) prolonged the duration of disease until death at 6-12 days post infection but still resulted in a 100% mortality rate$^{184}$. This model recapitulated infection seen in humans better than the Ad5 model as dissemination may occur during MERS-CoV infections of humans. Virus has been detected in the blood and urine of previous patients and autopsies of MERS-related patients that died noted a high frequency of dissemination into the CNS. Although some argue whether brain infection in mice is relevant to human disease, it has been hypothesized that encephalopathy may have been missed in some cases of severe infection$^{183}$. In a previous report, severe cases of CNS disease were associated with 3 cases of MERS infections and suggest that other associative cases of CNS disease may be missed due to the use of sedation and CNS blockades$^{185}$. There could be alternative explanations of neurological symptoms, and the limited postmortem data of MERS patients indicates further research is needed to understand the association, if any.

More recent studies have aimed to generate a model that promotes functional MERS-CoV/receptor interactions. CRISPR/cas9 genome editing technology was used to insert human codons into the mouse DPP4 gene at positions 288 and 330, simultaneously allowing efficient MERS infection and preservation of innate species-specific DPP4 expression, signaling activity and tissue distribution. Human (HCoV-EMC/2012), camel (Dromedary/Al-Hasa-KFU-HKU13/2013) and recombinant strains of MERS-CoV (icMERS/MERS-0) were able to replicate to high titres in the lungs (10$^{6-7}$ pfu/ml/g), however, none of the mice exhibited severe clinical signs of disease. Serial passaging a nonpathogenic variant of MERS with a high replication phenotype (MERS-0) in the lungs over fifteen rounds in 288$^+/330^+$ mice yielded strain MERS-15,
which when used in subsequent infection studies resulted in increased replication, weight loss and a ~70% mortality rate\textsuperscript{110}.

Later on, Li et al. followed a similar path where they replaced mouse DPP4 exons 10-12 with the human exon counterparts using a plasmid-mediated gene targeting strategy. Virus replication occurred following intranasal inoculation of the knock in (KI) mice, but like the CRISPR/cas9 model, viral titres were moderate (\~$10^{5-6}$ pfu/g) and no weight loss or mortality was observed. MERS-CoV (EMC/2012) was passaged 30 times to increase lethality and subsequent infection with $10^4$ PFU of the new strain showed progressive weight loss and 80% mortality rate\textsuperscript{127}. Based on the similarities in viral titres and clinical symptoms, this humanized DPP4 mouse model was only advantageous over the CRISPR/cas9 model in regard to a much lower viral load required to achieve the same results ($10^4$ vs $5\times10^6$ PFU). However, this perhaps could be remedied in the CRISPR/cas9 model with further passaging of their MERS-15 strain in mice to achieve better adaptation.

Each murine model that has been developed so far has come with advantages and disadvantages. Transgenic mice that show severe disease progression provide a more rigorous assessment when it comes to determining vaccine and therapeutic efficiency. However, the use of viral vectors to transduce cells with the MERS-CoV host cell receptor may be more favorable due to rapid development, for example, if a new pathogenic human coronavirus that does not naturally replicate in mice emerges. Once a receptor is discovered, a receptor-transduced model could be developed within weeks compared to the months it would take for development of other models such as genetically modified animals. The transduction model is useful for rapid screening of potential therapeutics or vaccines, but there are limitations due to...
the lack of clinical signs and symptoms. Ideally in cases of public health emergencies, screening a wide range of animal species would be a fast fire way to discover a susceptible model, however like the various mouse models generated this too has limitations. For instance, studies have shown sequence homology between the DPP4 receptor of rabbits and humans and upon further investigation, rabbits were shown to be susceptible to MERS-CoV infection\(^{121,186,187}\). While clinical signs and symptoms were not overtly obvious this model could still be potentially used to test intervention strategies. Similarly, as the anatomy of domestic pigs (\textit{Sus domesticus}) resembles that of humans, this animal was also evaluated as a potential model. Although MERS virus was found to replicate and shed in the respiratory tract of pigs, the limited levels observed and lack of clinical signs of disease again indicates that this animal would not be the most ideal model but could conceivably be used for quick screens of therapeutics\(^{188}\). Overall, the animal model that naturally most closely recapitulates human infection of MERS-CoV is the NHP model\(^{171}\). As previously mentioned, common marmosets support MERS virus replication, exhibit severe disease progression and pathology has revealed viral RNA in the lungs, blood, kidney, liver and heart indicating viral dissemination. While it would be an effective model for screening therapeutics, the cumbersome and labor-intensive nature of using such large animals, as well as ethical concerns when using primates for preliminary screening of medical countermeasures, has prompted this interest in developing a smaller model.
Chapter Six: Concluding remarks and future directions

This study has identified a new fusion protein consisting of the soluble domain of ACE2 and mouse IgG Fc that was able to inhibit SARS-CoV infection \textit{in vitro}. Generation and evaluation of these fusion proteins has proven to be quick, cost effective and highly specific, which could overcome limitations of monoclonal antibody production. Interactions between virus and target receptors are generally localized to small specific domains that make up the surfaces involved in the protein-protein interactions. My study suggests fusion of the Fc portion of an antibody of a minimal peptide representing a linear binding domain in ACE2 that is known to bind efficiently to the SARS-spike protein hinders conformation and/or interaction with viral spike protein. This may be due to the size of the Fc domain, which could sterically interfere with the interaction between the peptide and the spike protein. However, fusion of the ectodomain of the ACE2 receptor to mouse IgG-FC still allowed successful binding of the Fc-sACE2 fusion protein to SARS-CoV spike protein. Subsequent evaluation showed inhibition by Fc-sACE2 of infection by SARS-CoV in Vero E6 cells. Based on these results, we have shown the potential value for developing a synthetic biological therapeutic product to specifically target coronaviruses. Ideally, the next step would be testing pharmacokinetics, safety, and efficacy of this potential treatment in appropriate animal models and clinical trials. If potent antiviral activity is exhibited \textit{in vivo}, a similar fusion protein can be generated against MERS-CoV using the host target receptor DPP4. Additionally, this approach could be useful for development of similar fusion proteins for therapeutics for other viruses for which the cellular receptor is known.
As most small animals are not naturally permissive to MERS-CoV infection and a commercial model is not available, targeting SARS-CoV was used as a proof-of-concept model in the development of a coronavirus treatment. However, this barrier can be overcome by transduction of the mouse airway with the human DPP4 receptor as seen in this and other studies, using a suitable viral vector to deliver the hDPP4 gene to airway cells. While clinical symptoms were not observed in our Ad5-transduced model, the presence of replicating virus in vector administered mice, compared to control mice that are mock transduced with and empty vector, can allow for quick screening of drugs and vaccine validation. Tweaks in dosage and in administration methods of the viral vector or transduction in immunocompromised mice may allow for more substantial viral replication and clinical signs of disease as seen in other studies. Other approaches may generate a more robust model but use of an Ad5 vector for transduction of mouse airways allows rapid generation and reproducibility. As seen in the cases of both SARS- and MERS-CoV, speed plays a critical role in the development of therapeutics against emerging viruses and can be limited when a susceptible animal model is not immediately available.


58. Yang Z-Y, Huang Y, Ganesh L, et al. pH-Dependent Entry of Severe Acute Respiratory Syndrome Coronavirus Is Mediated by the Spike Glycoprotein and Enhanced by Dendritic Cell Transfer


86. Klimpel GR. Immune Defenses. University of Texas Medical Branch at Galveston; 1996.


123


141. Cavaco M, Castanho MARB, Neves V. Peptibodies: An elegant solution for a long-standing


168. Halbert CL, Allen JM, Miller AD. Efficient mouse airway transduction following recombination


Chapter Eight: Appendix

4X SDS Gel Loading Buffer recipe:
20 ml 1M Tris-HCl pH 7.5
20 ml 20% Sodium Dodecyl Sulfate (SDS)
35 ml Glycerol
0.5 g Bromophenol blue
20 ml β-mercaptoethanol (BME)

Transfer Buffer for Western blot recipe:
6.05 g Tris Base (BP152-1, Fisher Scientific)
28.5 g Glycine (BP381-500, Fisher Scientific)
400 ml Methanol
1600 ml dH₂O

2% SeaPlaque agarose:
20 g SeaPlaque low melting point agar (Lonza)
1 L Tissue culture grade water (HyClone)

IC₅₀ = 140 μg/ml
(140 μg/ml) / (114.084 μg/µmol) = 1.23 µM
Chapter Nine: Copyright Permissions

Figure 1. No authorization needed as stated by Journal policies.

Figure 2. Copyright clearance is attached

Figure 3. Copyright clearance is attached
This Agreement between Yvonne Pho ("You") and Springer Nature ("Springer Nature") consists of your license
details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number 4466610604445
License date Nov 12, 2018
Licensed Content Publisher Springer Nature
Licensed Content Publication Nature Reviews Microbiology
Licensed Content Title SARS and MERS: recent insights into emerging coronaviruses
Licensed Content Author Emmie de Wit, Neeltje van Doremalen, Darryl Falzarano, Vincent J. Munster
Licensed Content Date Jun 27, 2016
Licensed Content Volume 14
Licensed Content Issue 8
Type of Use Thesis/Dissertation
Requestor type academic/university or research institute
Format print and electronic
Portion figures/tables/illustrations
Number of figures/tables/illustrations 2
High-res required no
Will you be translating? no
Circulation/distribution <501
Author of this Springer Nature content no
Title Developing a novel therapeutic against coronaviruses
Institution name University of Manitoba
Expected presentation date Jan 2019
Portions Figure 1 and Figure 2
Requestor Location
Billing Type Invoice
Billing Address
Springer Nature Terms and Conditions for RightsLink Permissions

Springer Nature Customer Service Centre GmbH (the Licensor) hereby grants you a non-exclusive, world-wide licence to reproduce the material and for the purpose and requirements specified in the attached copy of your order form, and for no other use, subject to the conditions below:

1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

   If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Where print only permission has been granted for a fee, separate permission must be obtained for any additional electronic re-use.

3. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

4. A licence for ‘post on a website’ is valid for 12 months from the licence date. This licence does not cover use of full text articles on websites.

5. Where 'reuse in a dissertation/thesis' has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

6. Permission granted for books and journals is granted for the lifetime of the first edition and does not apply to second and subsequent editions (except where the first edition permission was granted free of charge or for signatories to the STM Permissions Guidelines http://www.stm-assoc.org/copyright-legal-affairs/permissions/permissions-guidelines/), and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence.

7. Rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

8. The Licensor's permission must be acknowledged next to the licensed material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

9. Use of the material for incidental promotional use, minor editing privileges (this does not include cropping, adapting, omitting material or any other changes that affect the meaning, intention or moral rights of the author) and copies for the disabled are permitted under this licence.

10. Minor adaptations of single figures (changes of format, colour and style) do not require the Licensor's
approval. However, the adaptation should be credited as shown in Appendix below.

Appendix — Acknowledgements:

For Journal Content:
Reprinted by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION] (Article name, Author(s) Name), [COPYRIGHT] (year of publication)

For Advance Online Publication papers:
Reprinted by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION] (Article name, Author(s) Name), [COPYRIGHT] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION] (Article name, Author(s) Name), [COPYRIGHT] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:
Reprinted/adapted/translated by permission from [the Licensor]: on behalf of Cancer Research UK: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION] (Article name, Author(s) Name), [COPYRIGHT] (year of publication)

For Advance Online Publication papers:
Reprinted by permission from The [the Licensor]: on behalf of Cancer Research UK: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION] (Article name, Author(s) Name), [COPYRIGHT] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Book content:
Reprinted/adapted by permission from [the Licensor]: [Book Publisher (e.g. Palgrave Macmillan, Springer etc) [Book Title] by [Book author(s)] [COPYRIGHT] (year of publication)

Other Conditions:

Version 1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.
This Agreement between Yvonne Pho ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number: 4474990500128
License date: Nov 23, 2018
Licensed Content Publisher: Springer Nature
Licensed Content Publication: Springer eBook
Licensed Content Title: Coronavirus Genome Replication
Licensed Content Author: Stanley G. Sawicki
Licensed Content Date: Jan 1, 2009
Type of Use: Thesis/Dissertation
Requestor type: academic/university or research institute
Format: print and electronic
Portion: figures/tables/illustrations
Number of figures/tables/illustrations: 1
Will you be translating? no
Circulation/distribution: <501
Author of this Springer Nature content: no
Title: Developing a novel therapeutic against coronaviruses
Institution name: University of Manitoba
Expected presentation date: Jan 2019
Portions: Fig. 2.2 Coronavirus genome replication Image on page 27
Requestor Location: [redacted]
Billing Type: Invoice
Billing Address: [redacted]
Total: 0.00 CAD
Terms and Conditions

Springer Nature Terms and Conditions for RightsLink Permissions

Springer Nature Customer Service Centre GmbH (the Licensor) hereby grants you a non-exclusive, world-wide licence to reproduce the material and for the purpose and requirements specified in the attached copy of your order form, and for no other use, subject to the conditions below:

1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

   If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Where print only permission has been granted for a fee, separate permission must be obtained for any additional electronic re-use.

3. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

4. A licence for ‘post on a website’ is valid for 12 months from the licence date. This licence does not cover use of full text articles on websites.

5. Where ‘reuse in a dissertation/thesis’ has been selected the following terms apply: Print rights of the final author’s accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

6. Permission granted for books and journals is granted for the lifetime of the first edition and does not apply to second and subsequent editions (except where the first edition permission was granted free of charge or for signatories to the STM Permissions Guidelines http://www.stm-assoc.org/copyright-legal-affairs/permissions/permissions-guidelines/), and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence.

7. Rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

8. The Licensor’s permission must be acknowledged next to the licensed material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book’s homepage. Our required acknowledgement format is in the Appendix below.

9. Use of the material for incidental promotional use, minor editing privileges (this does not include cropping, adapting, omitting material or any other changes that affect the meaning, intention or moral rights of the author) and copies for the disabled are permitted under this licence.

10. Minor adaptations of single figures (changes of format, colour and style) do not require the Licensor’s approval. However, the adaptation should be credited as shown in Appendix below.

Appendix — Acknowledgements:

For Journal Content: