Neutralizing antibodies are associated with protection in animal models of coronavirus infection and vaccination.

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In Partial fulfillment of the requirement for the
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In the department of Veterinary Microbiology
VIDO-InterVac, University of Saskatchewan
By
SWARALI S. KULKARNI

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2 Abstract:

Prior to the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002, human coronaviruses were primarily thought to cause relatively mild, respiratory disease. The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 marked the introduction of a second highly pathogenic respiratory coronavirus, which continues to cause sporadic cases in Saudi Arabia. Dromedary camels are currently the only known animal reservoir for MERS-CoV and contact with camels is thought to be the source of over half of all primary human cases. Currently, several efforts are being made to develop a vaccine for camels as an intervention strategy to stop transmission from camels into humans. To date, there have been limited studies regarding the immune response from natural infection or vaccination in camels against MERS-CoV. Given the limitations in accessing and working with dromedary camels, an alpaca model which accurately recapitulates MERS-CoV infection in camels was established and subsequently, the neutralizing antibody response to infection and vaccination was characterized. The induction of neutralizing IgG1 and IgG3 isotypes following experimental MERS-CoV infection protected alpacas from subsequent re-infection. Similarly, a S1-based subunit vaccine delivered intramuscularly induced a similar IgG1 and IgG3 neutralizing antibody response, resulting in nearly complete protection following MERS-CoV challenge in alpacas. Moreover, the antibodies induced following infection as well vaccination were able to neutralize currently circulating MERS-CoV isolates from Saudi Arabian dromedary camels, despite of the presence of amino acid changes in the spike protein, the major target for neutralizing antibodies. This data suggests that the neutralizing antibody response may be useful for predicting protection following infection and vaccination.

The recently emerged severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of one of the worst pandemics, continues to circulate in humans. Since the isolation of the first strain of SARS-CoV-2, various variants of concern (VOC) have emerged with increased transmissibility and potential for immune escape. Using a hamster model, the neutralizing and cross-neutralizing activity for the B.1.1.7 and B.1.351 VOCs of SARS-CoV-2 were evaluated. The neutralizing activity varied depending on the VOC used for the challenge, with B.1.351 infection in hamsters inducing overall lower levels of neutralizing antibodies. These findings were consistent with differential neutralizing activity against VOCs induced by two different S1 based subunit vaccines. In the vaccine study, reduced neutralizing activity against
B.1.351 resulted in reduced protection in hamsters. Finally, to demonstrate that neutralizing antibodies alone can provide protection from infection, two monoclonal antibodies; Ab1 and Ab8 targeting spike protein of SARS-CoV-2 were administered prophylactically and therapeutically in a hamster model of SARS-CoV-2 infection. Both monoclonal antibodies reduced SARS-CoV-2 viral titers as well as lung pathology in dose-dependent manner. Interestingly, higher concentrations of Ab8 were found in hamster lungs compared to Ab1 despite of being administered at comparable doses. The ability of monoclonal antibodies to reduce levels of virus and virus-induced pathology provides further supporting evidence that neutralizing antibodies likely play a critical role in protection from infection with respiratory CoV. These findings have major implications to design better vaccine and therapeutic strategies against the coronaviruses to provide a protection against severe disease.
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<td>Middle East Respiratory Syndrome Coronavirus</td>
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<td>SARS-CoV</td>
<td>Severe Acute Respiratory Syndrome coronavirus</td>
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<td>TGEV</td>
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<td>nsp</td>
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<td>ERGIC</td>
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<td>Virus transport medium</td>
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<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
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<td>50% Tissue Culture Infectivity dose</td>
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<tr>
<td>SST</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>Horseradish Peroxidase</td>
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<tr>
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<td>University Animal Care Committee</td>
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<tr>
<td>UpE</td>
<td>Upstream of Envelop gene</td>
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<tr>
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<td>Viral Neutralization</td>
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<tr>
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<td>BLItZ</td>
<td>Biolayer Interferometry Technology</td>
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1 Introduction:

1.1 General features of coronaviruses.

The coronaviruses are a diverse family of viruses that primarily cause respiratory infection in humans. Coronaviruses are named for their crown like spikes protruding from the surface of virus particles. The first coronavirus was identified in 1960s and since then several outbreaks in human as well as animal populations have been reported. Naturally, some coronaviruses are known to spillover from animals to humans and from humans to humans causing global outbreaks.

1.1.1 Classification of coronaviruses.

Coronaviruses are members of the family Coronaviridae, subfamily Coronavirinae in the order Nidovirales. Coronaviruses are further divided into 4 subgenera: alphacoronaviruses, betacoronaviruses, gammacoronaviruses and deltacoronaviruses (Peiris, 2012) (Figure 1.1). The alpha and betacoronaviruses are known to infect humans and animals while gamma and delta coronaviruses are mainly found in birds (Burrell et al., 2017a). Generally, alpha and betacoronaviruses cause infection of the respiratory tract in humans and the gastrointestinal tract in animals. In humans, four of the coronaviruses primarily cause mild “common cold-like” disease (HCoV-NL63, HCoV OC43, HCoV-229E and HKU-1) (Gagneur et al., 2002; Lau et al., 2006a); however, these viruses can cause more severe disease in infants, younger children, as well as frail or elderly patients. In contrast, to these “seasonal” coronaviruses, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in the betacoronavirus genus can cause moderate to severe infections in humans (Drosten et al., 2003; Zaki et al., 2012a; Zhou et al., 2020). Infection with common cold coronaviruses is generally known to cause disease in the upper respiratory tract of humans and seropositivity has been observed in 30-60% of the Asian population (Corman et al., 2019; Lau et al., 2006b; Zeng et al., 2018). In contrast, SARS-CoV and MERS-CoV cause infection in the lower respiratory tract and more often results in severe disease with a case-fatality rate of 10 % and 35%, respectively.

In addition to human coronaviruses, many animal CoV species have also been recovered from domestic animals as well as livestock resulting in serious economic loses.
Figure 1.1: Classification of coronaviruses.

The coronavirus subfamily categorized under *Coronaviridae* family and order *Nidovirales*. The coronaviruses (CoV) are divided into 4 genera Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronaviruses. While the alpha and beta coronaviruses are known to infect mammals, including humans, gamma and delta coronaviruses are only known to infect birds (Burrell et al., 2017b).
For example, bovine coronavirus (BCoV) (Boileau and Kapil, 2010), canine coronavirus (CCoV) (Decaro and Buonavoglia, 2008), and equine coronavirus (ECoV) (Pusterla et al., 2016) that causes gastrointestinal infections. Similarly, porcine coronaviruses cause serious threats to the swine industry. Gastrointestinal disease of a virus etiology in porcine populations was identified in the late 1970s in European countries and named as porcine epidemic diarrhea virus (PEDV) (Wood et al., 1977). Since its emergence, the most severe outbreaks have occurred in Asian swine population resulting in high mortality and morbidity. While the PEDV is lethal in piglets; infections in adult pigs are generally limited to mild to moderate disease. Swine acute diarrhea syndrome coronavirus (SADS-CoV) was identified between 2016 and causing several outbreaks in porcine populations in China (Gong et al., 2017; Zhou et al., 2019, 2018). Similar to PEDV, SADS-CoV was associated with acute diarrhea and vomiting with 90% mortality in piglets. Apart from these common coronaviruses in porcine population, other coronaviruses such as transmissible gastroenteritis virus (TGEV) (Doyle et al., 1946.; Laude et al., 1993), and porcine respiratory coronavirus (PRCV) (Cox et al., 1990; Groschup et al., 1993; Houben et al., 1995) have been known to cause infections and in some cases may be lethal in adult as well as in juvenile animal population.

SARS-CoV, MERS-CoV, HCoV-NL63 and SARS-CoV-2 may have originated in bats while HCoV-OC43 and HKU-1 are suspected to have originated from rodents (Forni et al., 2017; Lau et al., 2006c; Y et al., 2017). The first identified human coronavirus, HCoV-229E, is genetically related to a coronavirus isolated from Hipposidero sp. of bats in Ghana, Africa (Corman et al., 2015; Tao et al., 2017). Recently, it was determined that Chinese horseshoe bats in the Rhinolopus species likely served as an ancestral host for SARS-CoV (Li, 2005). Similarly, the first bat CoV related to HCoV-NL63 was found in the feces of Vespertilionidae bats in Europe and Africa (Huynh et al., 2012). Further phylogenetic analysis and molecular evidence suggest Civet cats and dromedary camels serve as an intermediate host for SARS-CoV and MERS-CoV, respectively (Kan, 2005; Muller et al., 2014; Reusken et al., 2013; Song et al., 2005; Tu et al., 2004). The molecular and serological evidence continue to reveal more coronaviruses in humans as well as domestic animals, in some cases with pandemic potential.
1.1.2 Genome organization of coronaviruses.

The coronaviruses have a large single-stranded positive sense RNA genome, that in the case of SARS-CoV-2 and MERS-CoV is approximately 29kb and 32 kb in size, respectively (Khailany et al., 2020; van Boheemen et al., 2012). The genome organization possesses 5’-leader-UTR-replicase-S-M-N-E-3’ UTR sequence. All coronaviruses have a unique coding strategy; the first two-third of the genome is translated into two polyproteins, ORF-1a and ORF-1b. These two polyproteins are processed by proteolytic cleavage to generate multiple non-structural proteins (nsp1 - nsp16). The cleaved non-structural proteins include papain-like-protease (PLpro) (nsp3), an exonuclease that provides proof-reading activity (nsp14), Helicase (nsp13). Apart from their primary function, in some cases these nsps also act as interferon antagonists; however, the exact mechanisms remain largely unknown for SARS-CoV-2 and MERS-CoV as for the common cold coronaviruses (Lai, 1997; Shah et al., 2020).

Following ORF1a and 1b, the remaining one-third of the genome is transcribed into 12-18 subgenomic mRNAs which encode four structural proteins (Figure 1.2A&B). Spike protein (S), envelope protein (E), nucleocapsid protein (N) and membrane protein (M) make the skeleton of infectious viral particle. The subgenomic mRNAs also encode accessory proteins which are not actively involved in virus replication but modulate the interferon response in the host. The genome ends are flanked by a 5’ cap and a 3’ poly A tail. Other accessory proteins are known to be redundant; however, evidence suggests they have a role in regulation of the host immune response and promoting viral replication (Fehr and Perlman, 2015a; Frieman et al., 2008; Redondo et al., 2021).

The virion of coronavirus measures approximately 125 nm in diameter with club-shaped spikes that protrude from the envelope of the virion. The trimeric S protein is a class I fusion glycoprotein that mediates attachment to the host receptor. In the case of SARS-CoV-2 and MERS-CoV, S is cleaved by furin-like or other proteases expressed by the host cells into two separate polypeptides S1 and S2 (Figure 1.3A&B) (Shang et al., 2020a). S1 includes the receptor binding domain (RBD), while S2 forms the stalk and mediates membrane fusion between host and virus. The M protein is the most abundant protein of coronaviruses. It contains three transmembrane domains and provides the shape of the virion.
Schematic representation of genomic organization of MERS-CoV and SARS-CoV-2. 1.2A) MERS-CoV genome measures around 32kb and 1.2B) SARS-CoV-2 genome size ranges from 29kb. The positive-sense single-stranded RNA comprises of 6-11 open reading frames (ORF) in coronaviruses. The first ORF comprises approximately two third of the genome, encoding 16 nonstructural proteins (nsps). These nsps code for cysteine proteases; papain-like protease(nsp12), chymotrypsin- (3c) like, protease (nsp5). Further, RNA-dependent RNA polymerase (nsp12), helicase (nsp13) making replication-transcription complex. The other one third of genome code for four major structural proteins including spike glycoproteins (S), membrane (M), nucleocapsid (N), envelope (E) and accessory proteins.
1.3A) MERS-CoV spike protein is divided into S1 and S2 subunit. The S1 region contain signal peptide; SP followed by N-terminal domain (NTD). The receptor binding domain is responsible for binding with receptor on host cells (RBD). Following RBD, the S2 is cleaved from S1 after the attachment in viral lifecycle. The S2 encodes two heptad repeats HR1 and HR2 followed by cytoplasmic domain: CP. The MERS-CoV spike protein measured upto 1326bp. 1.3B) Similarly, the spike protein of SARS-CoV-2 contain S1 and S2. The S1 domain is composed of N-terminal domain; NTD, receptor binding domain; RBD. Following attachment between RBD and host receptor, the S1 and S2 domain get cleaved at the furin cleavage site (S1/S2). The S2 domain contain fusion peptide (FP) and two heptad repeats; HR1 and HR2, central helix; CH and connector domain; CD, transmembrane domain; TM and C-terminal domain; CT. The whole spike protein of SARS-CoV-2 measures up to 1273 bp.
The M protein contain highly glycosylated N terminal and C-terminal ectodomain. Further, the E protein is found in small quantities on coronavirus virion. The envelope (E) protein contains ion channel activity. Accumulated evidence suggests that E protein is not required for virus replication but involved in pathogenesis and virulence. Within envelope, the viral genome is encapsulated with nucleoprotein (N) in the nucleocapsid. The N protein of coronaviruses composed of N-terminal domain (NTD) and C-terminal domain (CTD) both of which are involved in binding with viral genome.

1.1.3 Coronavirus replication.

Virus replication is initiated by an interaction between the S protein and its host cell receptor. Specifically, the amino acid sequences in RBD of S1 is responsible for the attachment to the host cell surface receptors. The interaction between S and the host cell receptor are determinants of host specificity and diversity. Both SARS-CoV and SARS-CoV-2 uses angiotensin enzyme -2 (ACE-2) (Hoffmann et al., 2020b) as a cellular receptor while dipeptidyl peptidase-4 (DPP4) (Wang et al., 2013) is responsible for providing entry MERS-CoV. Following attachment, the virus enters the cytosol by either acid-dependent proteolytic cleavage of S protein, TMPRSS2 (SARS-CoV-2) or other proteases followed by fusion of viral particle and host cell membrane (Hoffmann et al., 2020a). The proteolytic cleavage is generally separate S1 from S2 and the fusion peptides present in S2 are exposed (Alsaadi et al., 2019). In the case of MERS-CoV and SARS-CoV-2, fusion generally takes place in endosomes. The exposed fusion peptides in S2 attach to the host membrane. Following attachment of two heptad repeats present on S2 forms an antiparallel six-helical structure. These helical bundles result in fusion of the viral and host membranes resulting in the release of viral genome into the cytoplasm (Belouzard et al., 2012; Li et al., 2005; Shang et al., 2020b). Following attachment and fusion, the viral genome undergoes translation of the replicase gene. The expression of polyproteins pp1a and pp1b translates through a sequence (5’- UUUAAAC-3’) along with an RNA pseudoknot that results in ribosomal frameshift from one reading frame (rep1a) to another (rep1b) (Kames et al., 2020; Plant et al., 2005) (Figure 1.4). Pp1a and pp1b are cleaved by proteases encoded by ORF1 to give rise to nsp-1 to nsp11 and nsp 12 to nsp-16, respectively. These nsps form the RNA replicase and transcriptase complex. This complex localizes to the rough endoplasmic reticulum derived intracellular membrane in the perinuclear region.
SARS-CoV-2 and MERS-CoV enter the cell following binding between RBD of the spike protein to its cognate receptor, ACE-2 and DPP4 respectively. Upon binding, proteolytic cleavage of S1 and S2 takes place in the endosome in a pH dependent manner to release the viral genomic RNA into the cytoplasm. ORF1a and ORF1b are translated to produce pp1a and pp1b polyproteins, which are cleaved by replication-transcription complex (RTC). The RTC further drives the synthesis of full length (-) RNA and is used as a template for full length (+) genome. Transcription takes place to produce several accessory proteins and four structural proteins (S, M, E, N). S, M and E localize to the endoplasmic reticulum and N is encapsulated with genomic RNA. Budding takes place into the lumen of endoplasmic reticulum-Golgi intermediate compartment (ERGIC). The mature virions are then released from the infected cells through exocytosis.
In this region, production of negative sense RNA takes place (Figure 1.4). In the next step, viral RNA synthesis produces genomic RNA and 6-7 subgenomic RNAs. These subgenomic RNAs act as a mRNA template for structural and accessory proteins. The viral structural proteins M, E and S are transported to the endoplasmic reticulum and Golgi intermediate complex (ERGIC) through secretory pathways. In this complex, the viral genomic RNA is encapsulated along with N protein. The ERGIC membrane containing the genome and N protein buds out to form a mature virion (Fehr and Perlman, 2015b; Saraste and Prydz, 2021). Finally, the mature virion is transported to cellular membrane in vesicles and released by exocytosis (Figure 1.4).

1.2 Middle East respiratory syndrome coronavirus (MERS-CoV).

Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in 2012 in Jeddah, Saudi Arabia from a patient with severe pneumonia that subsequently died from multiorgan failure (Zaki et al., 2012b). Since its emergence, MERS-CoV has continued to be introduced into the human population resulting in sporadic cases with frequent nosocomial transmission. To date, the majority of human cases are continuing to be reported in Arabian Peninsula, although as a result of travel MERS-CoV cases have also been reported in 27 countries, resulting in ~2578 cases and 888 deaths. Following an incubation period of 2-14 days, initial flu-like symptoms such as fever and nausea can rapidly progress to pneumonia and can lead to hypoxemic respiratory distress with multiorgan failure which may lead to death. With an absence of approved vaccines and antivirals, current treatment remains limited to supportive care.

1.2.1 Transmission of MERS-CoV.

Similar to other coronaviruses, bats are presumed to be an ancestral reservoir for MERS-CoV and may be involved in transmission as a result of spillover events (Memish et al., 2013). Coronaviruses similar to MERS-CoV have been isolated from bats; however, epidemiological evidence showing a direct role of bats in transmission is still lacking. Until now epidemiological and serological data suggest that camels are the only animal reservoir and may transmit MERS-CoV to humans (Haagmans et al., 2014) (Figure 1.5). Serosurveys of dromedary camels in the Middle East, as well as in certain parts of Africa, routinely find high rates of seropositivity for MERS-
Figure 1.5: Transmission of MERS-CoV.

Bats are presumed to be an ancestral host of MERS-CoV which may have spilled over from bats to camels. Camel-to-camel and camel-to-human transmission takes place as a result of close contact. Infected humans transmit MERS-CoV in nosocomial settings as well as in household and to lesser extent community settings. Zoonotic transmission (indicated in by dotted lines) of MERS-CoV from camels to the community is poorly understood.
CoV antibodies (Hemida et al., 2014; Muller et al., 2014). The current working hypothesis is that MERS-CoV, or an ancestor, likely originated from bats, probably in African bats, a spillover event occurred that introduced this virus to dromedary camels, where it became established with camels becoming their own reservoir for MERS-CoV and then starting in 2012 spillover from camels to humans became more common (Figure 1.5).

Sporadic cases of MERS-CoV in humans continue to occur, which further has resulted in onward human-to-human transmission. In the absence of strict nursing protocols, human-to-human transmission in nosocomial settings remain a major cause of secondary cases in Saudi Arabia. Approximately 50% of cases of MERS-CoV in outbreaks were linked to hospitals in Saudi Arabia (Al-Tawfiq et al., 2014; Assiri et al., 2013; Balkhy et al., 2016). Apart from nosocomial transmission, the human-to-human transmission has also been observed in household settings. The transmission between families of infected individuals were only 13-21% (Arwady et al., 2016; Hemida, 2013). The proposed routes of infection in household setting include sleeping in close proximity to index patients and contact with contaminated surfaces.

While imported cases have occurred in North America, Europe and Asia, outbreaks related to travel have not resulted with one exception. An outbreak of MERS-CoV was reported in South Korea that was track back to a single person (Ki et al., 2015). A 56-year-old male with a travel history to Saudi Arabia returned to South Korea and was subsequently hospitalized with respiratory symptoms. A total of 186 cases in South Korea were eventually linked to transmission in hospitals (Kim et al., 2016a). The reproductive index ($R_0$) of MERS-CoV has varied by outbreak, ranging from 8.1 (South Korea outbreak) to 0.45 in Saudi Arabia. As with SARS-CoV, superspreading events have occurred which result in >5 consecutive cases and have been observed on numerous occasions in Saudi Arabia. Presently there is no approved vaccine or therapy available other than supportive care. The control of hospital-borne outbreaks is typically controlled by hospital closures in Saudi Arabia (Al-Tawfiq et al., 2014; Balkhy et al., 2016).

1.2.2 Dromedary camels as a reservoir of MERS-CoV.

Dromedary (Arabian) camels were domesticated approximately 2500 years ago in Arabian countries and African countries. The first evidence that camels may be involved in MERS-CoV transmission came from a serological study in dromedary camels where MERS-CoV reactive antibodies were detected (Reusken et al., 2013). Shortly thereafter, MERS-CoV genomic material was isolated from one camel in Qatar and showed to be genetically similar to two human cases
who had close contact with camels on the affected farm (Haagmans et al., 2014). Further investigation of the affected farm reported that 11 out of 14 camels were infected with MERS-CoV and anti-MERS-CoV antibodies were present in all of them. Similarly, another study in Saudi Arabia reported infection of a camel farm owner who had direct contact with 4 infected camels up until he was diagnosed with symptoms for MERS-CoV (Azhar et al., 2014).

These findings also indicate the potential risk of transmission of MERS-CoV from camels to humans as a result of close contact. Seroprevalence studies in two slaughterhouses and one live camels farm found 6-19% seropositivity in workers in Abu Dhabi at each sampling round between 2014-2017 (Khudhair et al., 2019). The 1.4% of workers were seroconverted over the study period suggesting high-risk exposure of MERS-CoV between these workers. A study was conducted on 498 individuals out of which 298 had occupational contact with camels and 204 had no contact histories with dromedary camels in Qatar and Europe (Reusken et al., 2015). Serological testing of these individuals showed 7% seropositivity among individuals who had close contact with camels while no seropositivity was observed in persons who had no contact with camels. Furthermore, a nationwide cross-sectional study conducted in Saudi Arabia of over ten thousand individuals showed high seropositivity among individuals with exposure to dromedary camels. Seropositivity was 15 times higher in live farm workers and 20 times higher in slaughterhouse workers compared to the rest of the study population (Müller et al., 2015).

These studies point towards the association of camels with MERS-CoV infection in humans; however, the exact mechanism by which this occurs still needed to be deciphered. An experimental infection of 3 camels with EMC/2012, a first human isolate of MERS-CoV, showed infection in the upper respiratory tract with significant viral loads as well as infectious virus of infected camels. Despite the high viral loads, camels essentially have no evidence of disease, which is consistent with the observation of natural infection in camels (Adney et al., 2014a; Alharbi et al., 2020a). The tissue tropism towards the upper respiratory tract in camels is attributed to the presence of the DPP4 receptor in those areas, in contrast to humans where DPP4 is found primarily in the lower respiratory tract infection (Letko et al., 2018). Natural and experimental infections of dromedary camels have also detected MERS-CoV viral loads in milk, feces, and rectal swabs with a possible role in a transmission of MERS-CoV from camels to humans (Van Doremalen et al., 2014).
Serosurveys against the MERS-CoV spike protein (the major surface antigen) have been observed in up to 90% of dromedary camels assayed in Middle Eastern and African countries, including Oman, Jordan, Sudan, Somalia, Nigeria, Ethiopia, Mali, Tunisia, and Saudi Arabia (Falzarano et al., 2017a; Hemida et al., 2017a; Meyer et al., 2014; Miguel et al., 2017; Muller et al., 2014; Payne et al., 2016; Reusken et al., 2013). Moreover, analysis of banked camel serum dating back 30 years was also reactive against the MERS-CoV spike suggesting that the introduction of the MERS-CoV into dromedary camels is not a recent event (Muller et al., 2014). Based on the epidemiological and surveillance studies, it is suggested that dromedary camels are a reservoir for the MERS-CoV and that spill over to humans occurs as a result of close contact. However, the exact route and mechanism of transmission from dromedaries to humans are not well understood.

1.3 Antibody response in camels to MERS-CoV.

MERS-CoV causes asymptomatic infections in dromedary camels; however, it still causes productive infection and induces seroconversion as evidenced by the IgG response. Several immunoglobulin classes such as IgM, IgA, IgG have been identified in camelids; however, the presence of anti-spike IgG has been observed against MERS-CoV in camels. In addition to conventional IgG1, camelids also contain a non-conventional antibody, which are not found in any other mammalian species (except in sharks) (Hamers-Casterman et al., 1993). The conventional IgG antibody is composed of two heavy chains and two light chains while camelids single chain IgG isotypes are made up of two heavy chains and are devoid of light chains. These camelids IgG isotypes are classified as IgG1 (conventional IgG), IgG2, and IgG3 (heavy chain antibodies). Further, the heavy chain antibodies compose approximately 70% of total serum IgG in camels and 50% in alpacas.

1.3.1 Structural features of conventional IgG1 in camelids.

A conventional IgG antibody is composed of two heavy chains and two light chains joined together by disulfide bonds in a structure that forms a Y-shaped complex (Janeway et al., 2001). Each chain consists of constant regions denoted as CH1, CH2 and CH3 on the heavy chain and CL of the light chain. A distinct part of the antibody, the variable region containing the variable light (VL) and variable heavy (VH) domains, is responsible for antigen recognition. Pairing of the CH1 and VH of heavy chains and the VLs of light chains forms the Fab region (Figure 1.6A). The Fab region is further divided into a variable fragment (Fv) consisting of VH- VL on the heavy and
light chains, respectively. Generally, the variable region is divided into a framework region (FR) and a hypervariable region (HV). The FR consists of beta scaffolds and has a fairly constant amino acid sequence. The HV contains more heterogeneity in amino acid sequences, and it is this region where antigen specificity is based on. The FR region scaffolds project into HV region. Hypervariable driven antigen recognition diversity is achieved by variation in 6 complementary determining regions (CDRs). These 6 CDRs are divided into VH (H1, H2, H3) and VL (L1, L2, and L3) on the heavy and light chains, respectively. The VH-VL forms a non-covalent interface to bring these 6 CDRs into close proximity with each other which forms a paratope or antibody-antigen binding site. Several studies have shown that mutational changes in these CDRs change the structural stoichiometry of the binding site and have a greater effect on antibody affinity. The Fab region further forms the Fc region composed of the CH2 and CH3 domains. While the Fab region is responsible for antigen binding, the Fc region is more involved in effector functions of IgGs. The flexible CH2 domain in the Fc region binds to the C1q molecule with the Fc gamma receptors (FcγRs) and activates the complement mechanism. Similarly, the Fc regions along with FcγR present of immune cells and activate antibody- dependent cell cytotoxicity (ADCC), antibody-dependent neutrophil phagocytosis (ADNP), and antibody- dependent cellular phagocytosis (ADCP) to eliminate the viruses.

1.3.2 Heavy chain antibodies in camelids.

In addition to full-length conventional IgG antibodies (described above), camelids also have two unique sets of IgG isotypes - IgG2 and IgG3 (Hamers-Casterman et al., 1993). Camelid IgG2 and IgG3 are heavy chain only antibodies and devoid of light chain (Figure 1.6B&C). Structurally, the heavy chain antibodies (hcAb) contain only 1 constant region (CH1) in the heavy chain. The CH1 region is eliminated by splicing during mRNA processing guided by a point mutation in the intron/exon boundary of the first C region. In conventional IgG structure, the CH1 domain is connected to the light chain. Since the absence of light chain in IgG2 and IgG3, the antigen binding is navigated by a shorter heavy chain region, called the VHH region, which has a MW of approximately 15kDa. Biochemical analysis indicates that the VHH region incorporates hydrophilic amino acids, at specific locations, such as Phe42, Glu49, Arg50 and Gly52, that confer high solubility and stability to the antigen-binding domain (Chan et al., 2008). In contrast, the heterodimer of conventional antibodies is further stabilized by hydrophobic amino acids, such as at Val47, Gly49, Leu50, and Trp52, on the opposing surfaces of the VL and VH domains. The
presence of hydrophilic amino acids further increases the solubility of heavy chain antibodies compared to conventional IgG isotype.

The absence of light chains in IgG2 and IgG3 is compensated for by an extended complementary determining region 3 (CDR3) of heavy chain antibodies that provides a sufficient antigen-binding surface and demonstrates resemblance to those of conventional IgG1 antibodies in camelids (Transue et al., 1998)(Figure 1.6B&C). The elongated CDR3 allows the heavy chain antibody to fit in a pocket of epitopes, which would be typically inaccessible by full-length IgG antibodies (Bannas et al., 2015). The extended CDR3 of IgG2 and IgG3 can also potentially increase the diversity of paratopes. Interestingly, the CDR1 of old-world camelids contain disulfide bonds formed by cysteine residues in the FR region, which provide increased affinity for the antigen. Furthermore, the germline of VHH contains additional Cys residues along with Cys 22 and Cys 92 which further increase the paratope repertoire (Nguyen et al., 2000).

The VHH of IgG2 and IgG3 recognizes a broad range of epitopes with greater affinity. The hcAb repertoire is diversified by somatic hypermutation (SHM) resulting in novel and unique paratopes that are different from full length IgG isotypes (Ciccarese et al., 2014).
1.6A) Conventional full length IgG1 is composed of two heavy chains and two light chains with a molecular weight of approximately 150kDa. The variable region of the heavy chain (VH) and light chain (VL) contain complementary determining region (CDR) which are responsible for creating the antibody repertoire. The constant region on the heavy chain is called CH1, CH2 and CH3.

1.6B) Heavy chain antibodies contain only a heavy chain and are devoid of light chains. The variable region in heavy chain is called VHH region. IgG2 contains an elongated hinge region compared to full length IgG1 and has a molecular weight of 95kDa.

1.6C) The heavy chain antibody IgG3. Similar to IgG2, the IgG3 is composed of heavy chain only and has a smaller hinge region compared to IgG2. The IgG3 has a molecular weight of approximately 90kDa.

**Figure 1.6: IgG isotype structure in camelids.**

Schematic representation of IgG isotypes found in camelids including dromedary camels and alpacas. 1.6A) Conventional full length IgG1 is composed of two heavy chains and two light chains with a molecular weight of approximately 150kDa. The variable region of the heavy chain (VH) and light chain (VL) contain complementary determining region (CDR) which are responsible for creating the antibody repertoire. The constant region on the heavy chain is called CH1, CH2 and CH3. 1.6B) Heavy chain antibodies contain only a heavy chain and are devoid of light chains. The variable region in heavy chain is called VHH region. IgG2 contains an elongated hinge region compared to full length IgG1 and has a molecular weight of 95kDa. 1.6C) The heavy chain antibody IgG3. Similar to IgG2, the IgG3 is composed of heavy chain only and has a smaller hinge region compared to IgG2. The IgG3 has a molecular weight of approximately 90kDa.
1.3.3 IgG response in camels against MERS-CoV infection and vaccination.

The majority of studies on camel immune response to MERS-CoV have centered around evaluating the presence of MERS-CoV specific antibodies. However, the correlation of the IgG response and its mechanism in virus clearance has not been clearly defined, limiting vaccine development to control MERS-CoV infection and transmission in camels. An IgG response can be induced in camels despite infection typically being asymptomatic. Anti-spike IgG antibodies are generally detected from one-month post-exposure for at least 1 year. Both adult and young camels are more likely to be seropositive compared to calves (Alharbi et al., 2020a; Corman et al., 2014; Hemida et al., 2014; Tolah et al., 2020). The induction of antibodies in young calves may take several months to mount a response as compared to the rapid response in adults (Meyer et al., 2016a). Furthermore, newborn calves acquire maternal IgGs which decline after 5 months making them more vulnerable to infections.

Despite high seropositivity in dromedary camels, it is not known if anti-MERS-CoV antibodies provide complete protection from MERS-CoV re-infection. This may be due to insufficient levels of IgGs or involvement of mucosal immunity are required to achieve complete protection. These findings are supported by a study performed in two herds of dromedary camels between Sep 2014 – May 2015 repeatedly sampled animals to detect viral RNA as well as antibody responses. MERS-CoV viral RNA was found in nasal swabs and sequencing was used to determine that all viral isolates within a herd were identical suggesting circulation of a single strain. Camels that were seropositive for MERS-CoV and negative for viral RNA at the initiation of the study were found to have viral RNA (suggestive of re-infection) at later timepoints, suggesting that the presence of antibodies as a result of a presumed prior infection does not provide complete protection against re-infection (Hemida et al., 2017b). Similar experiments on camel herds in Saudi Arabia also suggested that the presence of a pre-existing IgG response may not be protective against re-infection (Alharbi et al., 2020a). These findings require further investigation to characterize the quality of the immune response and determine what sort of immune response prevents or allows re-infection.

Currently, there are no approved vaccines that prevent MERS-CoV infections in either humans or in camels. Therefore, the development of a vaccine to prevent MERS-CoV infection and transmission in camels is a one health approach with the aim of reducing or eliminating transmission and to humans. Currently three vaccine candidates have been evaluated in dromedary
camels so far: 1) a modified Ankara virus (MVA) vector expressing MERS-CoV spike protein; 2) a chimpanzee adenovirus vector expressing spike protein (ChAdOx1); and 3) a DNA vaccine (Hashem et al., 2019; Lam et al., 2016a; Muthumani et al., 2015). The MVA based vaccine was tested in 3 dromedary camels in prime-boost and challenged with MERS-CoV (Lam et al., 2016b). Upon vaccination, high anti-spike antibodies and neutralizing antibodies were detected as compared to unvaccinated camels. Further following MERS-CoV challenge, upper respiratory tract samples showed high titers of MERS-CoV infectious virus in unvaccinated camels while vaccinated camels had reduced viral load. Although further analysis suggested that the MVA vaccine only provided partial protection in camels against MERS-CoV challenge. The ChAdOx1 vaccine was assessed in seronegative as well as seropositive camels, following a single intramuscular dose (Alharbi et al., 2019). Naturally MERS-CoV infected camels were cohoused with vaccinated camels and antibody response as well as, viral loads were monitored. These camels showed increased in neutralizing antibodies following vaccination and subsequently showed reduced levels of virus in nasal swabs. Similarly, vaccination in co-housed individuals (naturally infected) showed infection but lower viral RNA levels in nasal secretions in previously seropositive as well as seronegative camels. Furthermore, vaccination of younger camels was shown to require at least 2 doses of vaccine to obtain a significant immune response as compared to adult camels. A DNA vaccine expressing spike was assessed in three dromedary camels housed in zoo in USA. Following immunization of this DNA vaccine robust neutralizing antibody response was detected up to 11 weeks post-vaccination; however, MERS-CoV challenge studies were not performed in these dromedary camels (Muthumani et al., 2015).

These vaccines were induced strong humoral response in camels and two vaccines provided protection upon MERS-CoV challenge. However, to develop an effective MERS-CoV vaccine for camels many questions still need to be answered, such as what constitutes a protective response, whether the immune response induced from natural infection and whether that prevents subsequent infection and if this is comparable to the immune response induced by specific vaccines. Furthermore, given their unique IgG isotypes repertoire, a better understanding what isotypes are induced and whether these facilitate protection should also be determined.
1.4 Alpaca as an animal model.

Since the emergence of MERS-CoV, various efforts have been made to develop an animal model that can recapitulates what is observed following infection in humans. Experimental infections have been performed in non-human primates (NHP) (de Wit et al., 2017; Haagmans et al., 2015; Vergara-Alert et al., 2017; Yao et al., 2014), rabbits and pigs; however, the receptor specificity has excluded rodents from being used as an animal model against MERS-CoV. To circumvent this, transduction of human DPP4 into mice has demonstrated the possibility of using a mice model to study infection (Agrawal et al., 2015). All of these animal models establish an infection of the lower respiratory tract as observed in humans; however, none of the above animal models recapitulate infection in camels. While dromedary camels would be an ideal animal model to evaluate vaccine efficacy to be used in camel fields, this is impractical due to the lack of availability of animals and difficulties working with camels in CL3. Given these limitations, it would be useful to develop a surrogate model that mimics the diseases that are observed in camels. Alpacas (Vicugna pacos), a close relative of old-world camelids including dromedary camels, are classified as new world camelids, and mainly found in high altitude places. Alpacas are readily available in North America and are readily available from local farms. In addition, their smaller size compared to camels make them more convenient for handling in CL3 facility. To establish a model, female alpacas were infected with MERS-CoV and viral load in respiratory tissues were monitored throughout the study. In the same study, 3 naive alpacas were cohoused with infected alpacas to investigate the transmission events between alpacas (Crameri et al., 2016). Similar to dromedaries, viral replication was observed primarily in upper respiratory tract with absence of clinical disease. Nasal swabs showed detectable levels of viral RNA up to day 15 post-infection. Unlike camels this study did not report any nasal discharge throughout the study from any of the infected alpacas. Furthermore, alpacas were re-infected with MERS-CoV 70 days following initial infection and nasal viral loads as well as the humoral response was studied. A neutralizing antibody response was detected in all alpacas following infection and as well as re-infection. In another study, 3 alpacas were infected with MERS-CoV, and viral loads and neutralizing antibody responses were detected up to 14 days post-infection (Adney et al., 2016). Re-infection of alpacas in both studies showed immune responses developed following initial infection can prevent or protect from re-infection. The infection and re-infection model of alpacas can mimic the infection
and re-infections in camels. However, the differences in viral kinetics and viral shedding between alpaca and camels needed to be addressed.

Apart from experimental infection, alpacas in Qatar, that were located in close proximity to a MERS-CoV positive dromedary camel herd, were found to have antibodies, including neutralizing antibodies, against MERS-CoV (Reusken et al., 2016). Virus was not detected in nasal swabs. This suggests that alpacas are susceptible to MERS-CoV infection through non-experimental routes as well despite being present in small numbers in the Middle East. This accumulated evidence suggests that alpacas can be used as an animal model to recapitulate MERS-CoV infection in camels.

1.5 Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2).

At the end of 2019, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) emerged in Wuhan, China causing an outbreak of pneumonia (Wu et al., 2020; Zhou et al., 2020). SARS-CoV-2 is highly transmissible and has caused a global pandemic with over 276 million cases and over 5 million deaths worldwide. Similar to MERS-CoV and SARS-CoV, the most common symptoms of SARS-CoV-2 are fever, cough, breathlessness, fatigue, nausea and vomiting, specific symptoms like loss of smell or taste and in severe cases, pneumonia which can lead to death. Current data suggests that all age groups of humans are susceptible for SARS-CoV-2; however, there are stark differences in outcome associated with advanced age (Yang et al., 2021). Co-morbidities such as diabetes, immune suppression and obesity can also lead to more severe outcomes (Bajgain et al., 2021; Ng et al., 2021). The mortality rate among critical cases is approximately 49%; while the overall case-fatality rate for SARS-CoV-2 ranges between 2.3 and 5% depending upon region. The onset of disease to recovery is range between 5-10 days in mild cases irrespective of age of an individual.

1.5.1 Transmission of SARS-CoV-2.

Growing evidence indicates that the dominant route of transmission of SARS-CoV-2 between individuals is respiratory. The majority of cases in humans are mainly due to close contact with infected patients, initially assumed from droplets but with the potential to be spread by aerosols as well (Jayaweera et al., 2020) (Figure 1.7). Aerosol generating actions such as talking, coughing and poor ventilation may contribute to the transmission of SARS-CoV-2 in humans (Tang et al., 2020). Secondary transmission among health care workers in proximity to patients infected with SARS-CoV-2 is approximately 3% with or without personal protective equipment.
(Ehrlich et al., 2020; Fernandez et al., 2021). However, the high percentage of infections in health care workers are mainly associated with repeated exposure and high aerosols in poorly ventilated hospitals. Inadequate ventilation systems in household settings, restaurants and/or casinos have also been considered a contributing factor to clusters of cases. Findings also suggest that vertical transmission may occur, as well as possible involvement of domestic and farm animals in human (Day et al., 2020). Several studies have documented SARS-CoV-2 infections in domestic animals such as cats, and ferrets. SARS-CoV-2 can replicate in cats and is transmissible in cats and ferrets (Halfmann et al., 2020; McAloose et al., 2020; Sawatzki et al., 2021). However, there is currently no evidence supporting transmission between cats to humans. Transmission events could also potentially occur at deer farms and with certain captive animals in zoos, such as lions and tigers (Bosco-Lauth et al., 2020; Segalès et al., 2020). Furthermore, minks are also susceptible to SARS-CoV-2 infection and infectious virus has been recovered from farms, where widespread transmission between animals was observed (Fenollar et al., 2021). It is still unclear whether SARS-CoV-2 transmission from mink to humans is possible. One study isolated SARS-CoV-2 like coronavirus RNA from Malayan pangolins confiscated as a result of illegal wildlife trade (Wacharapluesadee et al., 2021). Virus isolated from pangolins had 85.5-92.4% homology with that of human SARS-CoV-2 isolates. However, the role of pangolins in transmission, or as a potential reservoir of SARS-CoV-2 remains to be determined.
Figure 1.7: Transmission of SARS-CoV-2.

Similar to other coronaviruses bats are speculated to be an ancestral host for SARS-CoV-2. Bats may have transmitted SARS-CoV-2 to intermediate wild or domestic animals followed by zoonotic transmission; however, the exact link is unknown. The confirmed route of human-to-human transmission is a result of droplet, aerosols formation or airborne route. The dotted line indicates speculated transmission.
1.6 Antibody response in humans against SARS-CoV-2.

Limited to no pre-existing immunity in humans is assumed to be a primary cause for extraordinary rise in the cases of the SARS-CoV-2 worldwide. Protective immunity induced following infection and vaccination in the SARS-CoV-2 has been studies in rhesus macaques as well as in clinical cases (Addetia et al., 2020; Chandrashekar et al., 2020a; Deng et al., 2020). Experience with the SARS-CoV, suggests the circulating IgG response are detectable for up to 3 years and neutralizing antibodies up to 17 years after onset of disease (Anderson et al., 2020). Further data for the SARS-CoV, suggests that neutralizing antibodies developed following infection prevent viral infection and thus may provide protection in humans and animal models (Post et al., 2020a; Tay et al., 2020; Theel et al., 2020). Early clinical findings from the SARS-CoV-2 in humans suggested that neutralizing antibodies may play a role in prevention of infection and may follow similar path as previously observed in other human coronaviruses.

1.6.1 Antibody response in infection against SARS-CoV-2.

Recovery from the SARS-CoV-2 in humans appears to be mostly rely on antibody response while in some cases T cell response may also play an important role. Most people with symptomatic infection of the SARS-CoV-2 develop specific antibody responses and these antibodies can be detected as early as ≤7 days post onset of disease. Studies of the kinetics of the humoral response following SARS-CoV-2 infection, have found that virus specific IgM responses peak between 1 and 4 weeks, and then rapidly decline and are largely undetectable by 5 weeks. The IgG response peaks between 2 and 7 weeks, remains at the peak for 8-9 weeks and then begin to decline over time (Post et al., 2020b). Follow up studies in humans show IgG persistence for at least 6 months to 1 year following the onset of disease (Goto et al., 2021; Turner et al., 2021). Neutralizing antibodies are crucial for virus clearance by interfering with virus binding to receptors and blocking virus uptake into host cells. Studies in animal models examining the immune response have shown to induce robust neutralizing antibody response alone providing protection against infection (Chandrashekar et al., 2020a; McMahan et al., 2020). In SARS-CoV-2 infection, it has been observed that antibodies are raised against viral proteins with major response directed against epitopes present on the spike protein containing RBD domain (Iyer et al., 2020; Röltgen et al., 2020). Neutralizing antibodies are generally detectable between 7-10 days following disease onset and remain present up to 7-8 months in SARS-CoV-2 infected patients as well as in experimental infections in animal models (Bylicki et al., 2021; Dispensieri et al., 2021). The
estimated half-life of the neutralizing antibody response is around 90 days over the first 8 months after the onset of disease. A recent study found that the majority of mild-to-moderately infected patients developed an antibody response by 21 days post-onset; however only 75% of individuals had titers greater or equal to 1:80 when tested with a validated neutralization assay (Premkumar et al., 2020). Further analysis of the neutralizing antibody response found that the equivalent of 20% of total humoral response is sufficient to provide 50% protection against mild to moderate SARS-CoV-2 cases (Khoury et al., 2021). These findings suggest that lower levels of neutralizing antibodies might be sufficient to block the viral entry and reduce the severity of infection. However, based on limited evidence it is yet to completely determined if the neutralizing antibody response wanes over time and if the low titers are enough to provide complete protection against SARS-CoV-2 infection.

Whether the neutralizing antibody response induced following initial infection protect from re-infection is one of the critical questions in SARS-CoV-2. Studies exploring re-infection to SARS-CoV-2 have been evaluated in animal models (Chandrashekar et al., 2020b). The neutralizing antibody response induced in initial infection with SARS-CoV-2 appears to provide some degree of protection up to day 40 against re-infection (Chandrashekar et al., 2020b). A recent study in a large UK cohort study has shown the presence of anti-spike IgG antibodies induced following infection may protect from re-infection up to 6 months (Lumley et al., 2020). A similar study in Denmark estimated protection of 80% in patients for up to 7 months who have previously tested positive for SARS-CoV-2 (Hansen et al., 2021).

Currently, amino acid changes are detected in S protein of SARS-CoV-2 variants are hotspot for mutations resulting in rise in variants of concern (VOCs). Combining data from antibody responses against SARS-CoV-2 suggests the neutralizing mediated protection may decline substantially against re-infection of VOCs (Garcia-Beltran et al., 2021; Wang et al., 2021; Zhou et al., 2021). The correlates of protection and duration of immunity to wild type strains and newly emerging variants are remain unknown. Further, the binding antibodies that are non-neutralizing may also contribute to the viral clearance of VOCs and reinfection particularly when neutralizing antibodies decline over time (Peiris and Leung, 2020). Defining clear correlates of protection, duration of protective immunity from infection and neutralizing activity against VOCs is crucial to design better vaccine strategies to obtain a protection against SARS-CoV-2.
1.6.2 Antibody response to vaccination against SARS-CoV-2.

The assessment of the immune response induced by SARS-CoV-2 vaccines have largely focused on antibody responses including neutralizing antibodies. Currently, there are several vaccines that have been approved for use to prevent SARS-CoV-2 induced disease which are based on a number of different approaches including 1) inactivated viral vaccine; 2) mRNA based vaccines; 3) adjuvanted subunit vaccines; and 4) viral vector-based vaccines (Kaur and Gupta, 2020). The spike glycoprotein is a component of all these approaches, and assays have really focused on investigating the response to the RBD of S, since the antibody response against RBD most often blocks the interaction with ACE-2 (Krammer, 2020; Martínez-Flores et al., 2021). BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) are lipid nanoparticle-formulated, nucleoside- modified vaccines that encodes a pre-fusion stabilized full length spike protein. Following administration and entry in to host cells, the mRNA translated into the full-length protein, which then localizes to the cell membrane and induces an adaptive immune response (Baden et al., 2021; Polack et al., 2020). Early studies in NHPs with mRNA vaccines found that they induced a spike-specific antibody response in a dose-dependent manner and reduced viral replication following SARS-CoV-2 challenge (Corbett et al., 2020; Vogel et al., 2020). Clinical studies using a mRNA vaccine have been shown to induce high titers of anti-S or anti-RBD binding antibodies that are equivalent or exceed to those observed in naturally infected patients (Havervall et al., 2021; Krammer et al., 2021). This data suggests that protection following a vaccination may rely on neutralizing antibodies or possibly high titers of binding, non-neutralizing antibodies which confer additional protection through other effector functions. The third, now approved vaccine candidate ChAdOx-1 nCoV-19 (AZD1222) has been also evaluated following single and two dose regimens, both pre-clinically as well as clinically. Immunization of NHPs with a single dose of AZD1222 vaccine induced neutralizing antibodies (1:5 -1:40 in prime, 1:160 following boost), which correlated with reduced SARS-CoV-2 mediated lung pathology (van Doremalen et al., 2020). Another replication-incompetent adenovirus vector vaccine (AdV26.CoV2. S) containing a full-length version of the spike protein in which a polybasic cleavage site is absent containing two stabilizing proline sites was developed (Sadoff et al., 2021). Pre-clinical testing of this vaccine resulted in induction of a strong neutralizing antibody response (1:100) on day 2 post-boost, which resulted in reduced viral RNA in the lungs following challenge (Solforosi et al., 2021).
With the current approved SARS-CoV-2 vaccines it is speculated that neutralizing as well as binding antibodies could be important, it is still unknown how long this response provide protection against SARS-CoV-2 in individuals. Also, it is also not known if the duration of vaccine-induced antibodies will differ from that of natural infection. However, initial results from both the BTN162b2 and mRNA-1273 vaccines against laboratory confirmed cases have been shown neutralizing antibodies up to 5-6 months following second dose of vaccination (Naaber et al., 2021; Widge et al., 2021). Studies regarding the kinetics of antibodies following vaccination appear to follow similar trends as observed from actual SARS-CoV-2 infections. Neutralizing antibodies following mRNA-1273 vaccination are estimated to have half-life of 68-202 days, whereas binding antibodies had a half-life of 102 days (Doria-Rose et al., 2021). Although current data suggests that protective immunity is conferred through antibody-mediated responses, further analysis of more specific immune correlates of protection and the establishment of a titer threshold that is required to provide protection is crucial. Currently, the absence of well-defined immune correlates makes it more difficult to predict when the updated vaccination strategies in response to emerging viral variants will be needed. New viral adaptations with amino acid changes in spike protein are continuing to emerge and it is possible that pre-existing immunity conferred as a result of prior infection of from vaccine-induced immunity may cease to be protective. Identification of antibody response as correlates of protection would allow the comparison between vaccine candidates and in predicting the durability of vaccine derived protection against newly emerging viral variants and post-pandemic interventions.

1.6.3 Monoclonal antibodies as a therapy against SARS-CoV-2.

Apart from immunity following infection and vaccination, neutralizing antibodies can be delivered therapeutically against SARS-CoV-2. The majority of monoclonal antibodies that are used therapeutically are full length IgG antibodies (primarily IgG1); however, neutralizing monoclonal antibodies recovered from humanized mice, in addition to synthetically generated with human Fc region are also being evaluated clinically (Wang et al., 2020). Currently, three monoclonal antibodies targeting distinct epitopes on spike protein have been authorized for the treatment of mild to moderate cases of SARS-CoV-2. Bamlanivimab was authorized to treat mild to moderate cases in patients with potentially high risk of developing severe disease (Gottlieb et al., 2021). The bamlanivimab is a human IgG1 monoclonal antibody targeting cognate epitopes in RBD, preventing the attachment of virion to the host cell receptor. In phase II clinical trials in
adults mild to moderately infected patients were received a single infusion at dose of (700, 2800 or 7000mg) showed decreases in viral loads in 2-3 days following first post infusion (Gottlieb et al., 2021). However, single administration of bamlanivimab increased viral variant resistance in this monoclonal antibody population which resulted in revocation of emergency use authorization (Peiffer-Smadja et al., 2021). To reduce the resistance, the combination of bamlanivimab and etesevimab (Fc modified IgG targeting S protein) are infused together against SARS-CoV-2 (Dougan et al., 2021) resulting in a significant reduction in viral loads 3-11 days post-infusion compared to placebo groups. Moreover, the infusion of both monoclonal antibodies was associated with reduction in 70% hospitalizations and deaths following SARS-CoV-2 infection in phase III trials (Dougan et al., 2021). Similarly, combination therapy casirivimab and imdevimab (REGN-CoV2), target distinct overlapping epitopes of RBD was also authorized to be used in mild to moderate cases of SARS-CoV-2 (Weinreich et al., 2021). In extensive neutralizing testing against SARS-CoV-2 spike mutations, the combination of these antibodies retained its neutralizing ability \textit{in vitro}. Therapeutic administration of REGN-CoV2 therapy in rhesus macaques and hamster model not only reduced viral loads but also protected from pneumonia compared to the placebo group (Baum et al., 2020). The ongoing phase I/II/III clinical trial investigating a single infusion of this combination at 2400mg or 8000mg dose for symptomatic adults not only reduced SARS-CoV-2 viral loads but also resulted in lower rates of hospitalization compared to the placebo group (Gupta et al., 2021; Weinreich et al., 2021). Further, sotrovimab, which was initially developed against the SARS-CoV spike protein was administered intravenously in mild to moderate cases of SARS-CoV-2. The ongoing phase III trial of sotrovimab reduced viral loads and disease progression in 85% of the cases compared to the placebo group (Gupta et al., 2021). Monoclonal neutralizing antibodies in combination with other medications are an attractive treatment strategy against SARS-CoV-2 infection. However, early clinical trials suggest further investigation on optimal dosing and timing remain to be determined. Similarly, further studies to determine which at-risk population would benefit from prophylactic administration, the duration of protection and its impact on vaccinations will be crucial.

1.7 Animal models for SARS-CoV-2.

To date, the origin and whether there are any intermediate animal reservoirs for SARS-CoV-2 has not yet been established. Several early reports suggested the possibility of pangolins as a potential intermediate, but it is expected that its ultimate origin is in bats (Wacharapluesadee et
Several domestic, as well as commonly used laboratory animals, were tested for their susceptibility to SARS-CoV-2, and ACE-2 tissue tropism. Initial bioinformatics analysis on the binding of spike to ACE-2 showed cats, hamsters, ferrets and NHPs were potentially susceptible to infection with SARS-CoV-2 (Table 1.1).

1.7.1 Mouse model:

ACE-2 from *Mus musculus* (mice) does not facilitate binding with SARS-CoV-2 spike protein to facilitate entry into host cells. Several strategies have been made to mitigate this limitation and develop a mice model which is susceptible to SARS-CoV-2 infection. The spike protein of SARS-CoV-2 can be modified to interact with mouse ACE-2 by adapting the virus which is achieved by performing sequential passaging in mouse (Gu et al., 2020). These mice models can be used to study pathogenesis of SARS-CoV-2, which replicates some of the disease processes that are observed in humans. However, adaptations may also increase the severity of disease in those mice models with more severe disease than is observed in humans. The transient expression of human ACE-2 in mice can be facilitated using an adenovirus viral vector that expresses human ACE-2 (Rathnasinghe et al., 2020a). This mouse model closely recapitulates what observed in humans in terms of viral replication in lungs, histopathological changes, and bilateral pneumonia. These mice also experience weight loss, induction of strong neutralizing antibodies as well as T cell response. Furthermore, it can be also suitable to evaluate the vaccine and therapeutics against SARS-CoV-2 to be used in humans (Rathnasinghe et al., 2020b). However, the transduction of human ACE-2 alters the tissue tropism and includes organs, which are generally not susceptible for SARS-CoV-2. Another approach is to develop a transgenic mouse model by genetic modifications such that the mice express human ACE-2 on tissues instead of mouse ACE-2. Three different human ACE-2 transgenic mice have been developed that makes use of varying promoters such as the Krt18 promoter for epithelial cells (Zheng et al., 2020), a universal promoter such as combination of a cytomegalovirus enhancer and the β-actin promoter 137 (Muzumdar et al., 2007; Xu et al., 2001) or using the endogenous expression of mice ACE-2 promoter (Bao, 2020). Although all these transgenic mice are susceptible to SARS-CoV-2, there are notable differences in the pathogenicity and disease severity ranging from mild to severe in these mice models. These transgenic mice models are suitable to study pathogenesis and disease in addition to being suitable for the evaluation of vaccines, antivirals, and therapeutics antibodies against SARS-CoV-2.
1.7.2 Non–human primates:

Unlike the mouse model, some non-human primates (NHP) are naturally susceptible to SARS-CoV-2. Three non-human primate models have been developed for SARS-CoV-2 including rhesus macaques (Macaca mulatta), African green monkey (Chlorocebus aethiops) and Cynomolgus macaques (Macaca fascicularis) (Chandrashekar et al., 2020; Finch et al., 2020; Munster et al., 2020; Rockx et al., 2020). Following infection, viral RNA can be detected up to 14 days post-infection in the respiratory tract. Histopathological changes include viral pneumonia and mild clinical signs of disease. These NHP models have been used to evaluate SARS-CoV-2 infection and its correlation with age (Rockx et al., 2020; Yu et al., 2017). Consistent with data from humans, aged macaques were found to shed infectious virus for a longer duration as compared to younger adult macaques. In another study, the Cynomolgus macaques from the immune response induced in initial infection were protected against subsequent re-infection with SARS-CoV-2. These models have also been used to study the efficacy of vaccine candidates against SARS-CoV-2 (Chandrashekar et al., 2020a). A recent study with Cynomolgus macaques showed reduced viral loads and strong adaptive immune responses against SARS-CoV-2 challenge following the prime boost regimen of vaccination (Corbett et al., 2020; van Doremalen et al., 2020; Vogel et al., 2020). Taken together, NHPs can be used to study disease pathogenesis and its correlation with risk factors, immune response against SARS-CoV-2. However, specialized CL3 facilities and handling makes less convenient model for SARS-CoV-2.

1.7.3 Ferrets:

Ferrets (Mustela putoris furo) are also susceptible for SARS-CoV-2 infection and develop mild disease with no clinical changes (Gortázar et al., 2021; Kim et al., 2020; Sawatzki et al., 2021). Following infection via the intranasal route, virus replication is restricted to the upper respiratory tract, where viral RNA can be recovered for up to 14 days post-infection. The lack of disease following infection with SARS-CoV-2 makes ferret unsuitable for pathogenesis and of limited utility for vaccine efficacy studies. However, the transmission studies have demonstrated efficient transmission from infected to naïve animals kept in close proximity in experimental settings (Kim et al., 2020; Richard et al., 2020). The induction of neutralizing antibodies has also been reported in ferrets; however, the levels are low in initial weeks of infection.
1.7.4 Golden Syrian hamster model.

Initial in silico analysis predicted a strong interaction between Syrian golden hamster ACE-2 and the SARS-CoV-2 spike protein suggesting hamsters might be susceptible to SARS-CoV-2. Intranasal inoculation of hamsters with SARS-CoV-2 resulted in mild to moderate disease progression with significant weight loss (Imai et al.;2020). High virus titers are observed in lung lobes and nasal turbinates with histological changes that include immune cell infiltration and, in some cases, alveolar hemorrhage. Immunohistochemistry analysis revealed nucleocapsid protein in lung lobes as early as day 2 with resolution by day 14 post-infection. Viral RNA is also be detected in other tissues such as blood, tissues from gastrointestinal tract and kidney. By two weeks following infection hamsters recover from infection in studies reported by different groups (Imai et al., 2020). Furthermore, since age dependent severity observed in humans, infection of young and aged hamsters was performed to assess the outcome upon infection with SARS-CoV-2 (Osterrieder, 2020). High titers of viral load and significant weight loss was observed in aged hamsters compared to young hamsters. Histopathological changes were age dependent with young hamsters showing earlier immune infiltration compared to aged hamsters, which had a delayed influx of inflammatory cells and accompanying delayed recovery. Similarly, when compared based on sex, male hamsters had more severe disease outcomes compared to female hamsters (Yuan et al., 2021). These age- and sex- dependent differences in hamsters may provide valuable insights to evaluate correlation with risk-factors and vaccine efficacy against SARS-CoV-2. Hamsters are currently widely used to study innate immune response upon infection with SARS-CoV-2. Upon infection, the expression of cytokines and chemokines in the lungs of hamsters peaks at day 5 post-infection and then gradually declines between days 7 and 8 post-infection (Francis et al., 2021). SARS-CoV-2 induced lung pathology was reduced in STAT-2 knock- out hamsters, while viral loads was increased (Boudewijns et al., 2020). Furthermore, various neutralizing antibodies that are approved or currently in clinical stages were evaluated in hamster models against SARS-CoV-2 infection (Kam et al., 2007a; Li et al., 2020). Upon administration of therapeutic antibodies, the viral load and lung pathology was markedly reduced against SARS-CoV-2 infection. Similarly, passive transfer of convalescent serum significantly reduced viral loads in the respiratory tract of hamsters (Imai et al., 2020; Takamatsu et al., 2021).

In addition, various vaccine candidates have been evaluated in the SARS-CoV-2 hamster model. Inactivated rabies- vectored vaccine carrying the SARS-CoV-2 spike protein was given in
conjugation with MPLA-AddaVax, a TLR-4 agonist in the hamster model (Kurup et al., 2020). Upon challenge with SARS-CoV-2, a significant reduction of in viral loads was noted as early as 3 days in vaccinated hamsters compared to unvaccinated hamsters. Vaccination induced strong neutralizing as well as binding antibody response and a strong Th-1 biased cellular response. Similarly, the Ad26.CoV2 vaccine candidate showed a reduction in viral loads and a dose-dependent induction of neutralizing antibodies (Tostanoski et al., 2020; van der Lubbe et al., 2021).

A caveat of the hamster model is absence of immunological reagents and research tools, which are critical to study the immune response to SARS-CoV-2. Overall, the knowledge from preclinical data and the efficacy testing of vaccines in hamster model can be used to design better vaccine and therapeutic strategies to be used in humans.
Table 1.1: Animal models of SARS-CoV-2.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose/Route</th>
<th>Sites of Virus Replication</th>
<th>Clinical signs and Histological alterations</th>
<th>Immune response</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c mice</td>
<td>10^5 TCID₅₀ /IN</td>
<td>lung, trachea, turbinate</td>
<td>weight loss, severe interstitial pneumonia</td>
<td>innate immune response</td>
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<tr>
<td>K18-hACE-2 mice</td>
<td>2.5x10⁴ pfu /IN</td>
<td>lung, heart, spleen, kidney, brain</td>
<td>innate immune response and lymphopenia</td>
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<tr>
<td>Adv-hACE-2 mice</td>
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<td>respiratory tract, brain</td>
<td>weight loss and pneumonia</td>
<td>N/A</td>
</tr>
<tr>
<td>hACE-2 mice</td>
<td>4x10⁵ pfu /IN</td>
<td>nasal turbinate, trachea</td>
<td>weight loss (aged hamsters), interstitial pneumonia</td>
<td>specific IgG antibodies at 7-8 dpi.</td>
</tr>
<tr>
<td>Ferrets</td>
<td>10⁵ TCID₅₀ /IN</td>
<td>nasal turbinate, trachea</td>
<td>mild weight loss, neutralizing antibody response</td>
<td></td>
</tr>
<tr>
<td>Rhesus Macaques</td>
<td>2.6x10⁶ TCID₅₀m/IN, IT/OC</td>
<td>nose, throat, lungs, intestinal tract</td>
<td>reduced appetite, neutralizing antibodies</td>
<td></td>
</tr>
<tr>
<td>African green monkeys</td>
<td>4.6 x10⁵ pfu/ IT IN</td>
<td>respiratory tract, brain, eyes, lymphoid tissues</td>
<td>weight loss, typical interstitial pneumonia</td>
<td>neutralizing antibodies response</td>
</tr>
<tr>
<td>Cynomolgus macaques</td>
<td>10⁶ TCID₅₀ /IT/IN</td>
<td>RNA in nasal, respiratory tract, lymphedema</td>
<td>virus specific antibodies</td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td>$5 \times 10^5$ TCID$_{50}$ /IN</td>
<td>lungs, nasal turbinate</td>
<td>weight loss, pneumonia, inflammatory cells infiltration</td>
<td>neutralizing antibody response</td>
</tr>
</tbody>
</table>
2 Rationale to study the antibody response against MERS-CoV and SARS-CoV-2 infection and vaccination:

Antibodies can serve as a critical barrier to virus infection. For many viruses, one of the key approaches to control virus infection is to block virus entry with neutralizing antibodies. Understanding the role of neutralizing antibodies in protection for interventions such as vaccines.

The development of a MERS-CoV vaccine for dromedary camels is a One health approach that should also prevent transmission of MERS-CoV from camels to human, and thus prevent human cases. As vaccine efficacy studies in camels will be difficult, we need to identify correlates of protection that can be used to predict immunity and to do so, we need to understand the IgG isotype response against natural MERS-CoV infection in camels. To date, the majority of serological studies in camels in regard to MERS-CoV infection have focused simply on seroprevalence; the kinetics and the role of individual IgG isotypes have not been clearly defined in camels. Heavy chain antibodies are known to recognize a broader repertoire of antigen, which may have different kinetics against viral infections compared to full length IgG1 isotype antibodies. As alpacas are close relatives of camels, characterizing the antibody response in this animal model may provide insights into similar response in camels.

Similarly, the importance of the antibody response against SARS-CoV-2 is not completely understood, and it is not known if neutralizing antibodies are an absolute requirement for protection. The role of the neutralizing antibody response induced by infection or vaccinations and how these are affected by new SARS-CoV-2 variants that continue to arise are critical questions. Although, evidence of a robust neutralizing antibody response results in protection from moderate to severe disease, very low levels of neutralizing antibodies may also facilitate protection. Additionally, all currently approved SARS-CoV-2 vaccines are based on the spike sequence from the ancestral virus isolate. With currently rising variants of SARS-CoV-2 it is not known if reduced neutralizing activity as has been observed against many of the VOCs.
Hypothesis:
Characterization of the neutralizing antibody response following either infection with or vaccination against emerging respiratory coronaviruses, such as MERS-CoV or SARS-CoV-2, can be used as a correlate to predict protection from infection.

Objectives:
1. Characterize the IgG isotype response that is induced in alpacas following MERS-CoV infection and vaccination, and to determine the relative contribution that each isotype makes towards neutralization.
2. Develop reagents to allow the assessment of the IgG response and neutralizing activity induced following MERS-CoV infection and vaccination in camels.
3. Determine the effect of amino acid changes in spike protein of variants of concerns (VOCs) on neutralizing antibodies against homologous and heterologous virus isolates induced following infection and vaccination in the hamster model of SARS-CoV-2.
4. Identify whether full length Ab1 and engineered single domain human Ab8 therapeutic monoclonal antibodies can prevent the infection with SARS-CoV-2 in a hamster model.
3 Materials and methods:

3.1 Maintenance of cell lines.

Vero76 (African green monkey kidney cells) (VERO C1008. CRL- 1586, ATCC, USA) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (D5796- 500ml, Thermo Fisher Scientific, USA) with 4.5g/l glucose, sodium pyruvate, 2mM L-glutamine, supplemented with heat inactivated (56°C for 30 mins) 10% fetal bovine serum (FBS) (Cat. 10091148 Qualified New Zealand, Thermo Fisher Scientific, USA), 100 U/ml Penicillin and 100 U/ml Streptomycin (Penn/Strep) (Cat. 15140122, Thermo Fisher Scientific, USA). Cell lines were maintained in 5% CO₂ at 37 °C under humidified condition.

3.2 MERS-CoV stock preparation and storage.

MERS-CoV (EMC/2012 strain) was originally isolated from a patient in Saudi Arabia and was subsequently obtained from Rocky Mountain Laboratories, NIAID, (Hamilton, MT USA). 8 µl of virus suspension was propagated on Vero76 cells in DMEM with 2% FBS, 100U/ml (1X) Penn/Strep. Supernatant from infected cells was harvested on day 3 of infection. The stock purified was designated as p.6/RMLp.2/VIDOp.2 (named as P2 stock). The titer of stock virus was calculated by 50% Tissue culture infectivity assay (TCID₅₀/ml) using Reed- Muench method (Reed and Muench, 1938). This virus isolate is known to contain point mutation at position T1015N, which has previously been characterized as cell culture adaptation (Scobey et al., 2013). We found our EMC/2012 stock contained this mutation at rate of 9:1 (Cell culture adaptation: wild type). The virus stock was stored at -80°C.

Ten MERS-CoV RT-qPCR positive nasal swabs in virus transport medium (VTM) from dromedary camels were obtained from Saudi Arabia (kindly supplied by Drs. Taseen Desin and Naif Alharbi, KAIMRC, Riyadh, Saudi Arabia). Following centrifugation at 5000xg for 5 min. VTM was diluted in DMEM with 2% FBS and absorbed on Vero76 cells in 6 wells cell culture plate. Cytopathic effect (CPE) was monitored up to day 3. Typical MERS-CoV CPE with cell rounding and syncytia formation was noted from 7 of these isolates. Blind passaging the three isolates with CPE did not result in CPE formation on subsequent passages, of note these samples had the lowest level of MERS-CoV viral RNA. On the day when 80 – 90 % CPE was observed, supernatant from Vero76 cells were collected separately. The 100 µl of supernatant was then used to infect Vero76 cells in T175 tissue culture flask for each virus isolate. On day when 80 – 90 %
CPE was observed, supernatant from Vero76 cells were collected separately in 50 ml Falcon tubes (Cat. 05-539-13, Fisher brand, Ottawa, Canada) and clarified by centrifugation (4500xg, 10 mins). Clarified supernatant was aliquoted in 2.0 ml Starstedt tubes (Cat. 72.694.406, Fisher Scientific, Ottawa, Canada) and stored at -80 °C until further use.

3.3 Viral titer determination by TCID$_{50}$ Assay.

Confluent Vero76 cells were split 1:3 the day prior to starting the assay to obtain approximately 90% confluent cells on the assay day. Virus stocks were thawed from -80°C and serially diluted at 1:10 in DMEM supplemented with 2%FBS and 1X Penn/Strep. Media was removed from Vero76 cells and 50 μl of virus dilutions were added in triplicate to the 96 well plate. Virus was allowed to absorb on Vero76 cells for 1 hr at 37°C in 5% CO$_2$. The virus suspension was removed, and cells were overlaid with DMEM supplemented with 2% FBS and 1X Penn/Strep. Cells were incubated for 5 days, and CPE was monitored on days 1, 3 and 5. On Day 5, the number of wells showing CPE was recorded and the virus titer determined using the Reed and Muench method (Reed and Muench, 1938).

3.4 Alpaca animal study for MERS-CoV infection.

3.4.1 Animal study outline.

All animal work with SARS-CoV-2 was approved by the University Animal Care Committee (UACC) at University of Saskatchewan. Ten female alpacas aged 3-14 years were obtained from a local farm and subsequently housed in the APLA3 facility at VIDO. One day prior to challenge, all alpacas were microchipped with temperature probes, three groups of alpacas were inoculated by either the intranasal (IN) route (n=3), intratracheal (IT) route (n=3) or by a combination of IT and IN routes (n=4) with 5ml IN (MAD Nasal™ Intranasal Mucosal Atomization Device, Cat. MAD300) and/or 10ml IT containing a total of 1x10$^6$ TCID$_{50}$/ml of EMC/2012. Nasal and oral swabs were collected daily for 21 days post-infection (dpi) to detect viral RNA levels and viral titers by virus titration assay (TCID$_{50}$/ml). At 7 dpi, 1 alpaca from the IN/IT group was euthanized and Nasal turbinates, lung lobes, larynx, pharynx, lymph nodes and kidney tissues were collected (Figure 3.1).
Figure 3.1: Alpaca study outline for MERS-CoV infection and re-infection.

3 groups of female alpacas were infected with $1 \times 10^6$ TCID$_{50}$/ml intranasally (IN) (n=3), intratracheally (IT) (n=3) or by combined routes (IN/IT) (n=4). Nasal and oral swabs were collected from 1 to 21 dpi from all three groups. Serum samples were collected weekly. On day 7, one alpaca infected via the combined route was euthanized and tissues were collected for histology and viral titrations. Alpacas infected via the IT and IN alone were euthanized on day 28 and tissue samples were collected. On 61 dpi, alpacas infected via the combined route were re-infected with $1 \times 10^6$ TCID$_{50}$/ml of MERS-CoV and nasal and oral swabs were collected daily up to day 14 post-reinfection (74 dpi). Serum samples were collected every week up to day 21 post-reinfection (81dpi). On day 21 all alpacas were euthanized, and tissues were collected. The red arrow indicates euthanasia timepoints.
The alpacas infected via the IN and IT route alone were euthanized on 28 dpi and tissues collected. Blood samples were collected in serum separator tubes (SST) every 7 days following infection. To separate serum, blood samples centrifugated at 300xg for 10 mins, serum removed and subsequently stored at -80 °C. Further, 61 days following infection the remaining 3 alpacas in the IN/IT group were re-infected with 1x10^6 TCID_{50}/ml of EMC/2012 by the Intranasal route (IN). Following re-infection all alpacas were sampled for nasal and oral swabs daily up to day 14 post-reinfection (74dpi). At day 21 post-reinfection (81dpi) all remaining alpacas were euthanized, and tissues collected as previously described. Similar to the primary infection, serum samples were collected at every 7 days and stored at -80°C.

3.4.2 MERS-CoV viral RNA purification and RT-qPCR in nasal and oral swabs and tissues.

To collect the nasal swabs, alpacas were restrained, and a sterile flocked swab (Cat. 220526, BD biosciences, USA) was inserted in each nostril. The swab was rotated several times against the nasal wall in both nostrils and transferred to 500 μl of DMEM supplemented with 100U/ml of Penn/Strep for each alpaca. Similarly, the oral swabs were collected by placing sterile flocked swabs at the end of buccal surface of oral cavity. The swabs were then transferred to 600 μl of DMEM containing 100U/ml Penn/Strep. All samples were kept on ice packs until processed. Viral RNA was isolated from nasal and oral swabs using the QIAamp Viral RNA Mini Kit (Cat. 52906, Qiagen, Ontario, Canada). Briefly, 140 μl of nasal swab liquid was transferred to 560 μl of viral lysis buffer (AVL) and stored at -80 °C until further processed. The oral swabs were briefly mixed by vertexing for 10s and 140 μl of oral swab was transferred to 560 μl of buffer AVL. On the day of viral RNA purification, samples in AVL were thawed at 37°C and viral RNA was precipitated in 560 μl of 100% ethanol. Inactivated samples were brought out of CL3 as per approved SOPs. The precipitated samples were transferred to containment level 2 lab and processed according to the manufacturer’s instructions. Viral RNA was eluted in 50 μl of AVE buffer provided with kit and stored at -80°C.

RNA from alpaca tissues was isolated using the RNeasy tissue viral RNA kit (Cat. 74104, Qiagen, Ontario, Canada). At necropsy, approximately 30 mg of individual tissues were placed in 1ml of RNAprotect (Cat. 76154, Qiagen, Ontario, Canada) and stored at 4°C for 24-48 hours. RNAprotect was subsequently removed, and the tissues were transferred to -80°C until further processing. For RNA isolation, tissues were weighed, 600 μl of tissue lysis buffer (RLT)
containing 0.1% of β-mercaptoethanol was added along with a 2.38mm metal beads (Cat. 13117-500, Qiagen, Ontario, Canada). The tissues were homogenized for 6 min at 30Hz to completely disrupt all tissues using a TissueLyserII (Cat. 85300, Qiagen, Ontario, Canada). Following homogenization, the homogenate was centrifuged at 5000xg for 5min. on a benchtop centrifuge. The tissue lysate was diluted with RLT to achieve a final tissue concentration of 30 mg of tissue in 600 μl and incubated for 10min at room temperature. The tissue lysate was then transferred to a new tube containing 600 μl of 70% ETOH. Following precipitation, samples were removed from CL3 according to approved SOPs and RNA was purified according to manufacturer’s protocol. RNA from tissues were eluted in 60 μl of RNase free water (Cat No. 129112, Qiagen, USA) and stored at -80°C. All samples were then processed for RT-PCR.

A primer/probe set targeting upstream of E gene (upE gene) of MERS-CoV was detected by RT-qPCR using isolated RNA from nasal and oral swabs and tissues. A total reaction volume of 25 μl was used including 5 μl of RNA, 12.5 μl of 2X reaction buffer in Quantifast probe RT-PCR kit (Cat. 204356, Qiagen, USA). The following program was used 55°C for 3 min, followed by 45 cycles of 95°C for 15s and 58°C for 30s (Table 3.1) (Corman et al., 2012) on StepOnePlus™ Real-time PCR system (Cat. 4376600, Thermofischer Scientific, Canada).

### 3.4.3 Quantification of infectious virus in nasal and oral swabs and tissue samples.

Infectious virus was determined in nasal and oral swabs as described in section 3.3. Briefly, 10-fold serial dilutions were prepared with an initial dilution of 1:10. One day prior to assay, Vero76 were seeded to obtain ~90% confluency. On the day of assay, media was removed from cells and dilution of nasal washes were added in triplicates in 96 well plates. Following 1 hr incubation at 37°C in 5% CO₂, virus suspension was replaced with DMEM with 2% FBS in was added and kept for 5 days. The CPE was recorded on day 5 and infectious titer was calculated by Reed and Muench method as mentioned in section 3.3.

To determine the quantity of infectious virus in tissues, protocol was modified from section 3.3. on the day of assay tissues were weighted, 1000 μl of DMEM and 2.38mm metal beads were added. The tissues were then homogenized in TissueLyserII as mentioned in section 3.4.2. Following homogenization and centrifugation at 5000xg for 5 mins, tissue homogenate was serially diluted 1:10 and absorbed on ~90% confluent Vero76 cells. Infectious tissue titer was calculated and noted as TCID₅₀/mg of tissue. As mentioned in section 3.3.
Table 3.1: Primer and probe sequence for MERS-CoV UpE gene.

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Position/gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer upE</td>
<td>27458-27474</td>
<td>GCAACGCGCGATTCAGTT</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse primer upE</td>
<td>27459-27530</td>
<td>GCCTCTACACGGGACCCATA</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>27477-27502</td>
<td>(6-carboxyfluorescein [FAM]-CTCTTCACATAATCGCCCAGCTCG-6-carboxy-N, N, N,N'-tetramethylrhodamine [TAMRA]</td>
</tr>
</tbody>
</table>
3.5 Virus microneutralization assay.

Vero76 cells were seeded 1:3 in 96 well plates (Cat. 167008, Corning, USA) a day prior to the assay in DMEM supplemented with 10%FBS and 1X Penn/Strep to obtain ~90% confluency. Serum collected from alpacas was heat inactivated at 56°C for 30 mins. Inactivated serum samples were serially diluted 1:2 in DMEM supplemented with 2% FBS and 1X Penn/Strep in a 96 well round bottom plate (Cat.3799, Corning, USA). Virus (EMC/2012 or as indicated) was diluted to obtain a final concentration of 100 TCID\textsubscript{50}/50 μl (2000 TCID\textsubscript{50}/ml) in DMEM with 1X Penn/Strep. Virus was added to the diluted serum and incubated for 1 hour at 37°C in a humidified incubator with 5% CO\textsubscript{2}. The serum/virus suspension was added to Vero76 cells and incubated for 5 days at 37°C in 5% CO\textsubscript{2} incubator under humidified condition. On day 5, CPE was noted in each well and the neutralization titer was determined.

3.6 MERS-CoV S1 Enzyme Linked Immunosorbent Assay (ELISA).

ELISA assays to assess antibody binding to MERS-CoV S1 were performed on serum samples collected at the indicated timepoints. Clear flat bottom MaxiSorp nonsterile 96 ELISA plates (Cat. N442404, Thermo Fisher Scientific, USA) were coated with 1 μg/ml of MERS-CoV S1 in PBS (pH 7.4) at 4°C overnight. S1 contained a HIS-tag and was produced in a mammalian cell culture expression system (Cat.40069-V08B1 Sino Biologicals, China). The following day, ELISA plates were blocked with blocking buffer (5% non-fat skim milk, 0.05% Tween20) in PBS (pH 7.4)) and incubated at room temperature for 1 hour. Plates were washed with wash buffer (PBS, pH7.4 with 0.05% Tween 20) for three times. Following washing, wells were incubated with serial dilutions (1:2) of serum diluted in blocking buffer at 37°C for 1 hour. Subsequently, all wells were washed with 200 μl of wash buffer 3 times at room temperature. To detect alpaca total IgG, rabbit anti-llama IgG antibody with horseradish peroxide (HRP) (Cat. AS101517, Agrisera, Sweden) was diluted in blocking buffer (1:100) added to the plate and incubated at 37°C for 1 hour in a humidified incubator. Following incubation, a washing step was performed as described previously. For detection, an o-phenylenediamine (OPD) containing 10mg/tablet (Cat. PI-34006, Thermo Fisher\textsuperscript{TM} Pierce\textsuperscript{TM}, USA) was dissolved in 9 ml of distilled water and 1 ml of substrate buffer (Cat. PI- 34062, Thermo Fisher\textsuperscript{TM} Pierce\textsuperscript{TM}, USA). The OPD substrate was added to wells and incubated at room temperature for 30 mins. The optical density (OD) was measured at 490nm on an xMark microplate spectrophotometer (Cat. 1681150, Bio-rad, Canada). The end-point titer was determined from optical density of known positive and negative controls were run parallely.
in the assay. In the case where positive control was not available, the titer cut off was calculated from optical density of pre-immune sera (negative control). To calculate the titers, the forecast function in an intercept program built in Excel was used to generate the intercept values.

3.7 IgG isotype response.

3.7.1 Purification of IgG isotypes.

The purification protocol of IgG isotypes from alpacas were adapted from a published methods for purification of IgG isotypes in camelids (Daley et al., 2005). Serum samples collected at day 21 post-infection and 21 post-reinfection from alpacas inoculated via the combined IT/IN route were diluted 1:5 in 20mM phosphate buffer saline (pH 7.0) (Appendix). The diluted serum sample was then passed through 0.025μm filter to remove contaminants (Cat. VSWP04700, MF-Milipore, MA, USA). Purification was performed on the fast protein liquid chromatography (FPLC), AKTA (Cat. SKU:8149-30-0006, GE healthcare, USA). The AKTA FPLC was primed with 5 column volumes of 20mM phosphate buffer saline (loading buffer/priming buffer) and a pre-packed 5ml protein G affinity chromatography columns were used (Cat. GE17-0405-01, GE healthcare, USA). The column was washed with 3-5 column volume (CV) of loading buffer at a flow rate of 1ml/min. Diluted serum was added at the rate of 0.5ml/min. A chart plotter was connected to the system and the column run trace was recorded. Following a wash of 10 CV, the fraction connector was started. IgG3 was eluted with 0.15 M NaCl, 0.058% acetic acid (pH 3.5) at the rate of 0.5ml/min and 0.5ml fractions were collected. Once the chart reached a flat line, 5 column volumes of priming buffer was injected into the system. IgG1 was eluted with 3 CV of 0.1M glycine-HCl (pH 2.7) at a rate of 0.5ml/min and 0.5ml of fractions were collected. The unbound fraction from the protein G column was again absorbed two times on protein G column and all remaining IgG3 and IgG1 were eluted as mentioned above. The flow through unbound Ig from the protein G column was collected in a separate vial and filtered through a 0.025μm filter.

The 5ml protein A column (Cat. GE-17-0403-01, GE healthcare, USA) was then attached to system and washed with priming buffer for 3-5 CV. The unbound fraction from the protein G column was then absorbed onto the protein A column at 0.5ml/min. Following absorption, IgG2 was eluted with 0.15M NaCl, 0.058% acetic acid (pH 4.5) at a rate of 0.5ml/min and 0.5ml fractions were collected. The flow through from the protein A column was again absorbed on protein A column again and the remaining IgG2 was eluted. Both the columns were filled with 0.1M HCl for long term storage. All eluted IgG isotypes were concentrated using Amicon Ultra-
15 Centrifugal Filter Unit with Ultracel-30 membrane (Cat. UFC903024, Sigma, USA) to obtain 1ml of IgG1 and IgG3 and 0.5ml of IgG2 fractions. All IgG isotypes then neutralized with 0.1M Tris- HCl (pH 9.0) and stored at 4°C for short term storage or -20°C for long term storage.

3.7.2 Protein concentration estimation.

The concentration of each IgG isotype was measured by DC™ protein assay (Cat. 5000111, Bio-rad, USA). To determine the protein concentration, a known concentration of bovine gamma globulin supplied with the protein assay kit was serially diluted (1:2) in 20mM phosphate, pH 7.0 buffer to obtain protein concentrations between 1.5mg/ml and 0.2 mg/ml. Similarly, purified IgG isotype fractions were diluted 1:10 and 1:100 in 20 mM sodium phosphate buffer. For the assay, 5 μl of each dilution of standard and IgG isotype were added separately in each well. Next, 25 μl of reagent A was added to each well followed by 200 μl of reagent B. The plate was incubated for 15 mins and absorbance was measured at 750nm in a xMark plate reader. The protein concentration was extrapolated from the bovine immunoglobulin standard curve in mg/ml using GraphPad Prism9.2.

3.7.3 SDS-PAGE and western blot analysis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the purification of camelid IgG isotypes (Cat.165-8000, Bio-rad, USA). For detection of IgG isotype in alpacas, 12% polyacrylamide gel (Appendix) was used. The purified IgG isotypes were diluted 1:4 in SDS sample buffer with or without 5% β-mercaptoethanol for reducing and non-reducing conditions, respectively (Cat. M3148, Sigma Aldrich, USA) (Appendix 1) and heated at 100°C for 10 mins in heating block. An SDS-PAGE gel containing was loaded with 30μl of each purified IgG isotype in sample buffer. Similarly, 7 μl of Precision Plus protein dual color standard (Cat. 1610374, Bio-Rad, USA) was also loaded in one of the well. SDS-PAGE gels were run at 90-110 volts in 1X SDS buffer (Appendix). SDS-PAGE gels were either stained with Coomassie Brilliant Blue stain (Cat. 1610400, Bio-Rad, USA) or subjected to western blot analysis.

For western blot analysis, Amersham™Hybond™ 0.45μm polyvinylidene difluoride (PVDF) (Cat. 45-004-110, GE healthcare, USA) was activated in methanol for 5 mins. Transfer sponges were soaked in either anode or cathode buffer (Appendix 1) for 5 minutes. The transfer sponges soaked in anode buffer was set on anode side of Trans-Blot® SD Semi-Dry Electrophoretic Transfer cell (Cat.1703940 Bio-Rad, Canada). The methanol soaked PVDF
membrane was placed on anode sponge and air bubbles were rolled out. The gel was briefly placed in anode buffer and then placed on top of the membrane. The filter pad in the cathode buffer was added on top and a roller was used to remove any air bubbles. Finally, the top of the apparatus was put in place. The proteins were transferred to the membrane at 0.8 A/cm² (60 mA per minigel) for 90 minutes. Following transfer, the membrane was incubated in blocking solution (5% skim milk in PBS with 0.05% Tween20) for 1 hour at room temperature. The primary antibody was diluted in blocking solution and incubated with the membrane for 1 hour at room temperature with constant rocking. Subsequently, the membrane was washed three times in PBS with 0.05% Tween for 10 minutes each. The secondary antibody was diluted in blocking solution and incubated with the membrane for 1 hour at room temperature. The membrane was washed three times in PBS with 0.05% Tween20 for 10 minutes each and then washed once more in PBS.

To detect IgG1, IgG2 and IgG3, the membrane was incubated with mouse monoclonal antibodies that recognize IgG1 (27E11), IgG2 (19D8) or IgG3 (8E11) at a 1:100 final concentration (kindly provided by Judith Appleton, Cornell University, NY, USA)(Daley et al., 2005). The secondary antibody anti-mouse IgG was diluted 1:15000 (IRDye ®800 CW) (Cat.Ab216772, Abcam, Cambridge, United Kingdom).

3.7.4 Alpaca IgG isotype ELISA.

To determine the relative quantity of the different IgG isotypes in serum samples, 1μg/ml of S1 protein was coated on ELISA plates as mentioned in section 3.6. Following blocking, serially diluted (1:2) serum samples or purified IgG isotypes were added to blocked plates and incubated for 1 hour at 37°C. Further, washing steps were performed as mentioned in section 2.6. For detection of individual IgG isotype, 1:100 diluted anti-IgG1 (27E11), anti-IgG2 (19D8) and anti-IgG3 (8E11) antibodies mentioned in section 3.7.3. were added separately and incubated for 37°C for 1 hour. Following washing steps, goat anti-mouse IgG HRP antibody (Cat. 62-6520, ThermoFisher scientific, Canada) was added at a 1:10000 dilution and incubated for 37°C for 1 hour. Finally, the IgG isotypes were detected with OPD substrate as mentioned in section 3.6.

3.7.5 Microneutralization assay on IgG isotypes.

A microneutralization assay for IgG isotypes was adapted from section 3.5. The purified IgG isotypes were diluted 2-fold serially from starting concentration 2 μg in DMEM supplemented with 100 U/ml Penn/Strep and without FBS. Similar to section 3.5, 100TCID₅₀/50 μl of EMC/2012 was incubated with diluted IgG isotypes and incubated for 1 hour at 37°C. Upon incubation, IgG
isotype and virus mixture was added on Vero76 cells and incubated for 5 days. Neutralization titer was estimated as EC$_{50}$ using GraphPad Prism 9.0.

3.7.6 Surface plasmon resonance (SPR) analysis.

Surface plasmon resonance (SPR) analysis was performed on a XPR36 ProteOn instrument (Cat.1760100, Bio-Rad, Canada) to determine the affinity of purified alpaca IgG1, IgG2 and IgG3 antibodies against MERS-CoV S1 antigen (Cat.40069-V08B1 Sino Biologicals, China). For SPR analysis, all reagents were prepared in degassed Milli-Q water. The ProteOn GLC sensor chip (Cat. 1765011, Bio-Rad, Canada) was sensitized with the amine coupling kit (Cat. 1762410, Bio-Rad, Canada) containing N-ethyl-(3-diethylamino-propyl) carbamide (EDC) and N-hydroxysuccinimide. Immobilization buffer was prepared using 1M acetate with a pH ranging from 5.5 to 4.5. All experiments were performed with continuous flow at 30µl/min of filtered and degassed priming buffer phosphate buffer (pH 7.4) supplemented with 0.5% bovine serum albumin (BSA) and 0.05%Tween 20. S1 antigen was immobilized at pH 4.5, 5.0 and 5.5 onto a GLC chip on channel and 1 channel was used as PBS control without any protein immobilization. Among all pH conditions, pH 5.0 had maximum immobilization of S1 on the GLC chip and thus used for the assay. Further assay conditions were optimized in separate run and saturation levels were obtained at 500 nmole/ml to 100nmole/ml.

Purified IgG isotypes were separately 2fold serially diluted in priming buffer at concentration of 100 nmole/ml. Diluted IgG1 was injected over immobilized S1 at the rate of 30µl/min and resonance unit (RU) was noted in real time. Following the IgG1 interaction, GLC chip was regenerated with 0.1M glycine-HCl (pH 2.7). Similar steps were performed for diluted IgG2 and IgG3 separately followed by regeneration steps in between each IgG isotype. The resulting RU and binding kinetics were analyzed using Langmuir 1:1 method and global fitting model and recorded as association contact (ka), dissociation (kd) and dissociation constant (KD in Bio-Rad ProteOn software (Cat. 1760200, Bio-Rad, Canada).

3.8 Immunization of alpacas with an S1 subunit vaccine against MERS-CoV.

Male alpacas were purchased from a local farm and housed in VIDO farm. All alpacas were microchipped with temperature probe. For immunization, MERS-CoV S1 protein containing a 6X HIS tag was produced at the VIDO protein expression facility using a 293T mammalian expression system. The expressed 25 µg of MERS-CoV S1 protein was formulated with Tri-Adj, a combination adjuvant developed by VIDO, containing 250 µg IDR-1002 peptide, 500 µg
polyphosphazene and 250 μg poly (I:C) (Garg et al., 2017a). Alpacas were divided into 3 groups and inoculated intramuscularly (IM) or intranasally (IN) or with PBS (IM) as a control. Next, immunized alpacas were re-immunized with same dose of vaccine on day 28 post-vaccination. Following 2 doses of vaccine, at day 57 all alpacas were challenged with 1x10^6 TCID_{50} of EMC/2012 intranasally (IN) using a syringe driven atomizer, as described previously (section 3.4.1). Serum samples were collected on day 0, 28 and 57 post-vaccinations, as well as on day 64 and 85 (days 7 and 28, respectively post-challenge, dpc). Serum samples were analyzed to determine the binding titer of individual IgG isotypes to MERS-CoV S1 using the isotype-specific ELISA described in section 3.7.4. In addition, each IgG isotype was purified using protein A and protein G chromatography as described in section 3.7.1 from the serum samples (day 56 post vaccination and 28dpc) collected from alpacas vaccinated by IM route. Following purification, the neutralizing activity of each IgG isotype was determined by EC_{50} assay as previously described in section 3.7.5.

3.9 Purification of camel IgG isotype.

Dromedary camel serum was purchased from Cedarlane (Cat. ABX800078 Cedarlane, Canada). Previously established protocols for the purification of alpaca IgG isotypes, described in section 3.7.1, were modified for dromedary camel IgG isotype purification. Crude camel serum was filtered through 0.025 μM filter and diluted 1:5 in 20mM phosphate buffer (pH 7.4). Similar to section 3.7.1 the protein G column was primed with 20mM phosphate buffer (pH 7.4) and diluted camel serum was absorbed on protein G column at a rate of 0.5ml/min. The flow through was collected in fraction collector in 1ml fractions. IgG1 was eluted as previously described in section 3.7.1 followed by purification of IgG3. This process was repeated 3 times to remove all IgG1 and IgG3 isotypes from the flow through. At each step of elution, 0.5ml fractions of IgG1 and IgG3 were collected. Next, a protein A column was primed with 20mM phosphate buffer (pH 7.4) as previously described in section 3.7.1 and flow through collected from the final protein G column run was absorbed onto the protein A column. The IgG2 isotype of camels was eluted using 0.15M NaCl, 0.58% acetic acid (pH 4.5) and repeated 2 times to elute all traces of IgG2. Upon purification, all eluted fractions of IgG1, IgG2 and IgG3 were concentrated and neutralized with 0.1M Tris HCl (pH 9.0).

The purity of each IgG isotype was estimated by SDS-PAGE assay under non-reducing conditions and/or reducing conditions. Similar to section 3.7.2, the concentration of each IgG
isotype was measured the BioRad DC protein assay using a known concentration of bovine immunoglobulin as a standard as mentioned in section 3.7.2.

3.9.1 Development of anti-IgG3 monoclonal antibody.

A monoclonal antibody against dromedary camel IgG3 was developed and subsequently scaled up by Genscript, USA. Briefly, Balb-C (n=5) and C57BL/6 (n=5) were immunized subcutaneously with 50 μg of purified IgG3 along with Keyhole limpet hemocyanin (KLH) adjuvant. On day 21, blood samples were collected, and a second immunization as previously described, was performed. On day 7 post-second immunization, all mice were test bleed to estimate the antibody titer in serum. Following the test bleed, all mice were immunized with a final (third) dose, as previously described. All serum samples collected following immunization were delivered to VIDO for assessment in a camel IgG isotype-specific ELISA to determine the specificity to IgG3 as well as the cross-reactivity to IgG1 and IgG2. Based on these results, Balb-C mice were eliminated from the study as they had low antibody response to IgG3.

Following euthanasia, blood samples and spleens were collected from C57BL/6 (n=5) mice. To develop hybridoma cells, a single cell suspension of spleenocytes was prepared and spleenocytes were fused with myeloma cell by electrofusion. The average fusion efficiency was around 2x10⁴/ml. Fused cells were plated in 96 well plates at a concentration of 1 fusion per well with up to 15 plates being generated. The fused cells were maintained in conditioned media developed according to Genscript protocols. The supernatant from hybridomas were collected on day 7 post fusion and ELISAs were performed using purified IgG1, IgG2 and IgG3 from camels as the coating antigen. Hybridomas that had high cross-reactivity to IgG1 and IgG2 were eliminated. Subsequently, 10 hybridoma supernatants with high specificity for camel IgG3 were selected and expanded for scale-up production. Of these, only 5 hybridoma cell lines were stable out of which from 1 cell lines anti- IgG3 monoclonal antibody was affinity purified and delivered to VIDO-InterVac. In our lab, anti-IgG3 monoclonal antibody was screened by isotype ELISA.

3.9.2 Camel IgG isotype-specific ELISA.

Camel IgG isotype specific ELISAs were established based on methods described in section 3.7.4. Briefly, purified IgG1, IgG2 and IgG3 as well as serum from camels (1:1000 dilution) were coated onto a MaxiSorp plate as described in section 3.7.4 at concentration of 1μg/ml. Next, the IgG isotypes were detected using the anti-camel IgG3 mouse monoclonal antibody developed in section 3.9.2 and anti-camel IgG1 mouse antibody (Cat. ABIN1981267,
Antibodies.online, PA, USA). Following incubation of primary antibodies and washing steps, detection was provided by goat anti-mouse IgG tagged with HRP followed by OPD substrate as previously described in section 3.7.4.

3.10 isolation of SARS-CoV-2 from clinical isolate.

SARS-CoV-2/Canada/ON/VIDO-01/2020 was isolated from a human patient nasopharyngeal swab collected on January 23\textsuperscript{rd}, 2020, in Ontario, Canada with travel history to Wuhan, China (EPI_ISL_425177). Vero76 cells were plated in 24 well tissue culture plate in DMEM supplemented with 10 % FBS and 100U/ml Penn/Strep. On the next day, ~45 µl of nasopharyngeal swabs collected in virus transport medium (VTM) were diluted in 150 µl of DMEM supplemented with 2% FBS, 100U/ml Penn/Strep and 16 µg/ml of TPCK trypsin and absorbed on ~90% confluent Vero76 cells. The plate was then centrifuged for 1 hour at 25°C, 800xg followed by incubation at 37°C in 5% CO\textsubscript{2} for 30 mins. Following incubation, the viral inoculum was removed and replaced with DMEM supplemented with 2% FBS, 100U/ml Penn/Strep and 16 µg/ml of TPCK trypsin. The infected cells were monitored for 72 hours for typical SARS-CoV-2 CPE (detached rounded cells).

On day 3 post-infection, the supernatant from infected cells was absorbed on fresh Vero76 cells in a single 6-well and incubated at 37°C with 5% CO\textsubscript{2} for 1 hour. Following incubation, the virus inoculum was replaced by DMEM supplemented with 2% FBS, 100U/ml Penn/Strep and 1 µg/ml of TPCK trypsin. The infected cells were monitored for 72 hours for typical SARS-CoV-2 CPE (detached rounded cells).

Similarly, a nasopharyngeal swab from a patient infected with B.1.351 (also known as beta VOC) lineage was obtained from the Roy Romanow Provincial Lab (Regina, SK). The virus was isolated as previously described; however, the amount of trypsin decreased to 1 µg/ml for all steps. The virus stocks (p.2) were prepared in T175 flasks and stored at -80°C until further use.

3.10.1 SARS-CoV-2 virus stock preparation from stock virus.

The B.1.1.7 lineage isolated from nasopharyngeal swab of infected patient collected at Marion County, Oregon, USA on February 6\textsuperscript{th}, 2021 (EPI_ISL_1055380) (Originally named as hCoV-19/USA/OR-OHSU-PHL00037/2021 also known as Alpha variant). A vial of original stock was shipped by BEI resources, USA in DMEM to VIDO-InterVac. At VIDO-InterVac, the virus stock was absorbed on Vero76 cells in T175 flasks in DMEM supplemented with 2% FBS, 1µg/ml of Trypsin treated with TPCK and 100 U/ml Penn/Strep. The viral supernatant was collected at
day 3 to prepare a stock and stored at -80°C. Similarly, the P1 of SARS-CoV-2/Canada/ON/VIDO-001/2020 and B.1.351 from section 3.10 were scaled up to make stock virus and labelled as P2 stock. The stock virus was aliquoted in 2.0 ml Startedts tube and stored at -80°C. The titer of each isolate was obtained from virus titration with TCID$_{50}$/ml using Reed-Muench method as mentioned in section 3.4.3.

3.11 Hamster model infection with B.1.1.7 and B.1.351 of SARS-CoV-2.

Male golden Syrian hamsters aged 5-6 weeks were obtained from Charles River Laboratory (Strain code:049, Charles River Laboratory, Montreal, Canada) and housed at the APLA3 facility at VIDO for at least 1 week prior to the start of the study (Figure 3.2). Following acclimatization, all hamsters were microchipped with a temperature probe. In separate studies, hamsters were infected with either B.1.1.7 (n=8) or B.1.351 (n=8) intranasally with 50 μl/nare containing a total of 1x10$^5$ TCID$_{50}$/hamster. Body weights and body temperature were measured daily. Nasal washes and oral swabs were collected under isoflurane anesthesia on day 1,3,5,7, and day 10 post-infection (Figure 3.2). Oral swabs were collected on individual hamsters with Starplex swabs and subsequently placed in 600 μl of DMEM supplemented with 100U/ml Penn/Strep. Nasal washes were collected from restrained hamsters positioned such that one nare was over the sample collection tube and a teat cannula was inserted into the other nare. 500 μl of PBS was gently injected through the cannula and approximately 350 μl of fluid was recovered.

On 5 dpi (n=4) and 10 dpi (n=4), hamsters from both studies were euthanized. Terminal blood samples were collected in serum separator tubes to collect serum samples.
Figure 3.2: Schematic for the experimental outline for SARS-CoV-2 hamster infection.

6–8-week-old male hamsters were infected with B.1.1.7 or B.1.351 intranasally. Following infection, nasal washes, oral swabs were collected at 1, 3, 5, 7, and day 10 and serum at day 5 and 10 post-infection. On day 5 and day 10, 4 hamsters were euthanized to collect respiratory tissues and serum samples. Each blue arrow showing day of sampling.
At necropsy, the left lung was perfused with 10% neutral buffered formalin solution for histopathology and immunohistochemistry studies. Tissues from the right lung lobes, nasal turbinate and trachea were collected to detect viral load and viral RNA post-infection.

3.11.1 SARS-CoV-2 viral RNA detection in nasal washes and tissues.

The previously established protocol from section 3.4.2 was modified for detection of SARS-CoV-2 RNA levels in nasal, oral and tissue samples in hamsters. SARS-CoV-2 viral RNA was isolated from nasal and oral swabs using the QIAamp Viral RNA Mini Kit (Cat. 52904, Qiagen, USA) and from tissue samples using RNeasy tissue viral RNA kit (Cat. 74104, Qiagen, USA) as described in section 3.4.2. The upstream of E gene (UpE) primers and probe for SARS-CoV-2 was used to RT-qPCR. A total of 25 μl reaction using 5 μl of viral RNA was set up with Quantifast RT-PCR kit (Cat. 210212, Qiagen, USA). The primers and probe were designed, and cycle conditions were adapted against SARS-CoV-2 (Corman et al., 2020) (Table 3.2).

3.11.2 SARS-CoV-2 infectious virus detection in hamster samples.

The quantity of infectious virus from nasal washes, oral swabs and indicated tissues were determined by TCID$_{50}$ assay. An established protocol (section 3.4.3) was modified to detect SARS-CoV-2 infectious virus on timepoints post-infection in hamsters. Infectious virus titers were calculated using the Reed and Muench method and reported as TCID$_{50}$/ml for nasal and oral samples and as TCID$_{50}$/mg for tissues.

3.11.3 Histology and immunohistochemistry of SARS-CoV-2 infected tissues.

At necropsy the 5 lung lobes or only left lobe were fixed with 10% neutral buffered formalin and placed in cassettes immediately. The fixative volume was 5 greater than the approximate tissue volume. Tissue cassettes were stored at 4°C for 1 week in fixative. After 1 week, fixative was changed with fresh 10% neutral buffered formalin and tissue cassettes were transferred to CL2 facility. Further, tissues were embedded as per established protocols at Prairie Diagnostic Services (PDS, Saskatoon, SK). Briefly, each section of tissue was incubated in a sequential gradient of 70%, 80% and 95% ethanol twice to denature cellular proteins. Tissue cassettes were then submerged in xylene for approximately 1 hour and then embedded in paraffin wax for 2 hours. The paraffin was trimmed and cut in 5 μm sections of tissues on a microtome. All tissue blocks were then subjected to deparaffinize and hematoxylin and
Table 3.2: primer and probe sequence for SARS-CoV-2.

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>UpE gene</th>
<th>ACAGGTACGTTAGTAAATAGCGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>(246 E_Sarbeco_F1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td>UpE gene</td>
<td>ATATTGCAGCAGTACGCACA</td>
</tr>
<tr>
<td>(247 E_Sarbeco_R2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>UpE gene</td>
<td>ACACTAGCC Zen\textsuperscript{TM}</td>
</tr>
<tr>
<td>(248 E_Sarbeco_P1)</td>
<td></td>
<td>ATCCTTACTGCCTTCG</td>
</tr>
</tbody>
</table>
eosin staining. The histopathological changes in lung lobes were scored from 0-4 based on inflammation, pneumonia, and signs of lesions. Lung tissue sections were also prepared for immunohistochemical staining, which was conducted at PDS (Saskatoon, SK) using an automated slide Stainer (Autostainer Plus, Agilent Technologies Canada Inc., Mississauga, ON). Epitope retrieval was performed in a Tris/EDTA pH 9 buffer at 97°C for 20 minutes. The primary antibody was a rabbit polyclonal antibody against nucleocapsid protein of SARS-CoV-2 (SARS2-N). The SARS2-N antibody was produced in-house by VIDO (Animal Study number AS20-012). The SARS2-N antibody was diluted 1:800 in PBS and incubated with the slides for 30 min at room temperature. After washing, the bound SARS2-N antibody was then detected using an HRP-labelled polymer detection reagent (EnVision+ System - HRP Labelled Polymer, Agilent Technologies Canada Inc., Mississauga, ON). Immunostaining was categorized as no staining, weak staining intensity or strong staining intensity.

3.12 SARS-CoV-2 viral neutralization assay.

Viral microneutralization assays against different variants of concern (VOC) of SARS-CoV-2 virus were performed on serum samples collected at day 5 and day 10 post-infection as described previously in section 3.5. Briefly, serum samples collected from SARS-CoV-2 infected hamsters were inactivated for 30 mins at 56°C and then diluted 2-fold in DMEM supplemented with 2% FBS and 100U/ml Penn/Strep. The neutralization assay was performed in 3 technical replicates. VIDO-01, B.1.1.7 (BEI, resources) or/and B.1.351 (isolated in Regina, SK) were diluted separately to 25 TCID50/50 μl (500 TCID50/ml) in DMEM without any supplement. The 60 μl of diluted virus was mixed with 60 μl of the serum dilutions in a 96 well round bottom plate at 37°C for 1 hour. Following incubation further steps were performed as described in section 3.5 and the serum dilution factor for the wells with no CPE at 5 dpi was defined as the serum neutralization titer.

3.13 SARS-CoV-2 S1 and RBD ELISA.

SARS-CoV-2 S1 and RBD ELISA were performed as described previous (section 3.6). ELISA plates (Cat. N442404, Thermo Fisher Scientific, USA) were coated with either purified S1 or RBD protein from VIDO-01 (similar to Wuhan isolate) B.1.1.7 (Cat. 40591-V08H12 and 40592-V02H1, Sino Biologicals, China) and B.1.351 (Cat. 40591-V08H15 and V0592-V08H185B, Sino Biologicals, China) at concentration of 1 μg/ml. The coated plates were incubated at 4°C overnight. On following day, blocking was performed as described in section 3.6.
Two-fold dilutions of serum collected from hamsters infected with either B.1.1.7 or B.1.351 were added to plate and incubated at 37°C for 1 hour followed by washing steps. Goat anti-hamster IgG HRP (Cat. PA1-29626, ThermoFisher Scientific, Canada) were diluted in blocking buffer to obtain a working concentration of 1:7000 and incubated at 37°C for 1 hour. Following washing steps as per mentioned in section 3.6, the reaction was developed with OPD substrate and absorbance was measured at 450nm after 30 mins at room temperature. The cut off value was determined by taking the average of the pre-infection sera plus 3 standard deviations. To calculate the titers, the forecast function in an intercept program built in Excel was used to generate the intercept values.

3.14 SARS-CoV-2 S1 vaccine formulation.

COVAC-1

The S1 protein of SARS-CoV-2 was manufactured by Biodextris (400 μg/ml, PBS pH=7.3 containing 277 mM NaCl; Lot#: C2003-VID-DSP-E-002). The S1 protein has a 12-histidine tag at its C-terminus and was expressed in HEK293T cells. TriAdj is composed of of 10 μg PCEP (EP3), 10 μg poly I:C (Product A) and 20 μg peptide IDR-1002 (Product B). The TriAdj components were manufactured by Dalton; Product A (Dalton, Lot# 373-2943 DOM 11/03/2020); Product B (Dalton, Lot# 374-2943 DOM 11/06/2020) and mixed by manufacturer. On the day of vaccination, 25 μg S1 and desired adjuvant were mixed at VIDO facility.

COVAC-2

The S1 antigen was manufactured by Biodextris (400 μg/mL, PBS pH=7.3 containing 277 mM NaCl; Lot#: C2003-VID-DSP-E-002). SWE was used for adjuvant. SWE was provided by the Vaccine Formulation Institute (CH, Chemin des Aulx 14, 1228 Plan-les-Ouates Switzerland). SWE Lot# is ESSAI O/W 1849101 Batch: L00820. SWE was mixed 1:1 with S1 protein.

3.14.1 Hamster immunization.

This was a randomized control trial (RCT), and technicians were blinded. 5-6 weeks old male hamsters were obtained from Charles River Laboratory (Strain code – 049) and housed in the animal care facility at VIDO for approximately 7-10 days. Hamsters were randomly assigned to groups, cages, euthanasia days and chipped accordingly. There were 3 groups of hamsters; Group 1: COVAC-1, group 2: COVAC-2 and group 3 PBS control. All hamsters were monitored for weight change, temperature throughout study. The group 1 was immunized with TriAdj and 25μg/per dose of S1 protein. Similarly, SWE+25 μg of S1 was injected intramuscularly in both thighs. Vaccines were administered as a prime and a boost at a 4-week interval, on day 0 and day
28, respectively. The vaccines were given via the intramuscular (IM) route with an injection volume of 70 μl/side and injected at both sides of the thighs (Figure 3.3).

On day 49 (3 weeks following the boost), animals were challenged with 50 μl/nare containing a total of $1 \times 10^5$ TCID$_{50}$ of SARS-CoV-2 VOC B.1.1.7 or B.1.351 via the intranasal (IN) route. SST blood samples were collected prior to each immunization (day 0 and day 28), prior to challenge (day 48) and at euthanasia day 5 or day 10 post-challenge (dpc) (day 54 or day 59 post-vaccination). Serum samples were assayed for IgG reactivity to SARS-CoV-2 S1 antigen or against RBD antigen (Wuhan Hu-1, B.1.1.7 and B.1.351), and for viral neutralization antibody titers (described in section 3.12 and 3.13). Nasal washes and oral swabs were collected a day before challenge and every two days post-challenge for examination of viral loads. Half of the animals (4/group) were euthanized at day 54 (5 dpc), and the remaining animals were euthanized at day 59 (10 dpc). At necropsy, lung tissues and trachea were collected for histopathology (H&E) as described in section 3.11.3. Lung tissues were examined for viral load by RT-qPCR and for levels of infectious virus as previously described. Similarly, nasal turbinates and trachea were also collected for viral load by RT-qPCR and for levels of infectious virus by cell culture as mentioned in section 3.11.1 and 3.11.2.
Figure 3.3: Vaccination with COVAC-1 and COVAC-2 following challenge with B.1.1.7 or B.1.351 in hamsters

6–8-week-old hamsters were immunized with a prime boost regimen, 28 days apart of either COVAC-1 or COVAC-2 via the intramuscular route. Control animals received PBS. All hamsters were challenged on day 48 post-vaccination and nasal and oral swabs were collected on day 1, 3, 5, 7 and day 10 post-infection. Half of the hamsters were euthanized on day 5 and the remaining on day 10 post-infection. At euthanasia, lung lobes, trachea and nasal turbinate were collected for determination of levels of virus, in addition to histological analysis. Serum samples are indicated with yellow tube were collected on day 0, 28 and 48 post-vaccination and day 5 and day post-infection.
3.15  Monoclonal antibody prophylaxis and treatment.

3.15.1 Isolation and characterization of Ab1.

The purification and characterization of IgG1 Ab1 (termed as Ab1), a human IgG1 monoclonal antibody, was carried out at University of Pittsburgh, (Pittsburgh, PA, USA). SARS-CoV-2 RBD-HIS and RBD-Fc, S1-Fc, ACE2-Fc and IgG1 were subcloned into pcDNA3.1. Proteins were expressed with the Expi293 expression system and purified with protein A resin or by Ni-NTA resin. The recombinant RBD proteins was used to pan a naïve human antibody phage display library, which was generated from the antibody cDNA of 490 healthy donors’ PBMCs and splenocytes. These libraries contain bacteriophage (size for each ~1011) and are highly diverse in antibody clones. Biopanning was based on the pull-down method by using streptavidin-M280 Dynabeads (Cat 11205D, ThermoFisher Scientific, USA). After panning, positive binders were selected by phage ELISA. Their binding was subsequently measured by RBD binding ELISA, hACE2 competition ELISA, and the binding kinetics were measured by the biolayer interferometry technology (BLItz). The leading candidates were converted to the full-length anti-spine Ab1 for treatment (Li et al., 2020).

3.15.2 Prophylaxis and therapeutic treatment with Ab1 in the SARS-CoV-2 hamster model.

Male hamsters (9-week-old) were obtained from Charles River Laboratories (Montreal, QC). For evaluation of prophylactic efficacy, all hamsters (n=5) were injected intraperitoneally with 10 mg/kg of Ab1 24 hours prior to intranasal challenge with 50 µl/nare containing a total of 1×10⁵ 50% tissue culture infectious doses (TCID₅₀) of SARS-CoV-2 (SARS-CoV-2/Canada/ON/VIDO-001/2020) (Figure 3.4). For the therapeutic group, hamsters (n=5) were infected as above and treated intraperitoneally with 10 mg/kg of Ab1 at 6 hours post-challenge. Untreated hamsters were kept as a control (Figure 3.4). Nasal washes and oral swabs were collected at 1, 3 and 5 (dpi). Hamsters were bled at 1 and 5 dpi. All hamsters were euthanized on 5 dpi. At euthanasia, lung lobes were collected for virus titration and RNA isolation. For viral RNA and titer determination, nasal washes were determined as mentioned in section 3.11.1 and 3.11.2 respectively. To determine virus titers from tissues, lungs were homogenized with DMEM and then processed as section 3.11.1 and 3.11.2. Cytopathic effect was scored on 3 and 5dpi.

3.15.2 Isolation, prophylaxis, and treatment with Ab8 in hamsters.

VₜAb8-Fc (termed as Ab8) antibody was developed and characterized at the University at Pittsburgh (Pittsburgh, PA, USA). Ab8 antibody was identified by panning of a phage library
as described in section 3.15.1. Ab8 was constructed by fusing a V\textsubscript{H} domain from IgG1 to a human Fc with the native IgG1 hinge. Male hamsters (9-week-old) were obtained from Charles River Laboratories (Montreal, QC). For evaluation of prophylactic efficacy, hamsters (n = 7) were injected intraperitoneally with 10 mg/kg of Ab8 24 hours prior to intranasal challenge as described above (3.15.2) (Figure 3.4). For the therapeutic group, hamsters were infected with (SARS-CoV-2/Canada/ON/VIDO-001/2020) as above and treated intraperitoneally with 10 mg/kg (n = 3) or 3 mg/kg (n = 4) of Ab8 at 6 hours post-infection. Untreated hamsters but infected with same dose of SARS-CoV-2 were kept as a control. Nasal washes and oral swabs were collected at day 1, 3 and 5 post infection (dpi) (Figure 3.4). Hamsters were bled at 1 and 5 dpi. All hamsters were euthanized on 5 dpi. At euthanasia, lung lobes were collected for virus titration and RNA isolation as mentioned in section 3.15.2. Viral detection and histopathology were performed as mentioned in section 3.11.3 for hamster in prophylaxis as well as treatment groups.
3.4A)

![Prophylaxis and treatment with Ab1 and Ab8 in hamster model of SARS-CoV-2](image)

3.4B)

![Prophylaxis and treatment with Ab1 and Ab8 in hamster model of SARS-CoV-2](image)

**Figure 3.4:** Prophylaxis and treatment with Ab1 and Ab8 in hamster model of SARS-CoV-2.

Study design of prophylaxis and treatments with Ab1 and Ab8 in hamster model of SARS-CoV-2. 3.4A) 6-8 weeks old hamsters were treated with 10mg/kg Ab1 or Ab8 intraperitoneally in separate studies and challenged with SARS-CoV-2 VIDO-01 isolate in dose 1x10⁵/hamster intranasally. Nasal and oral swabs were collected on 1, 3 and 5 dpi. Serum samples were collected as indicated by the serum tubes on 1 and day 5 dpi. Lungs, nasal turbinate, and trachea were collected on 5 dpi. 3.4B) Hamsters were infected with 1x10⁵ TCID₅₀/hamster intranasally and treated 6 hours post-infection with either 3mg/kg or 10mg/kg of Ab1 or Ab8 intraperitoneally. Following treatment all hamsters were sampled for nasal and oral swabs at 1, 3 and 5 dpi. Serum samples were collected on 5 and 10 dpi.
3.15.3 Detection of human Ab1 and Ab8 in serum and tissues of hamsters.

To determine the concentration of monoclonal antibodies that remain in circulation as well as the amount of antibody in the lung, the concentration of human Ab1 or Ab8 was determined from hamsters treated at concentration 10mg/kg. A modified SARS-CoV-2 S-1 ELISA was used. S1 protein was coated at 1 μg/ml overnight at 4°C in PBS onto MaxiSorp plates as mentioned in section 3.6. The following day plates were blocked with 5% skim milk and 0.05%Tween20. Serum collected on day 1 and day 5 post-challenge was diluted 1:100 and absorbed for 1 hour at 37°C. Plates were washed and goat anti-human IgG-HRP was added (1:15000). Plates were washed and subsequently developed with OPD (o-phenylenediamine dihydrochloride) substrate. Optical density was measured at 450 nm after 30 mins of incubation. For lung tissues, after blocking, lung homogenates were prepared as mentioned in section 3.4.3 and diluted 1:10. Lung homogenate dilution were incubated overnight at 4°C followed by washing detection with anti-human IgG-HRP and substrate as stated above. To determine the concentration, dilutions of purified Ab1 or Ab8 were used to extrapolate concentration of Ab1 and Ab8 in homogenized tissues using GraphPad Prism 9.0. The Ab1 and Ab8 concentration was reported as µg/mg. The control hamster lung homogenate was used for background correction.

3.16 Statistical analysis.

All statistical analyses were performed using GraphPad Prism version 9.0. Prior to statistical analysis, variables with non-Gaussian distributions were log transformed. When data were collected from individual animals more than once over the duration of the study, they were analyzed using repeated measures Anova (two-way, mixed effects model), followed by Tukey’s multiple comparisons test, where indicated, to compare the groups on specific days. When data were collected on a single day, they were analyzed by one-way Anova followed by Tukey’s multiple comparisons. The residuals from each Anova were examined for evidence of deviation from the assumptions of the Anova model. In all cases, the unit of analysis was the individual animal. Individual and median values of data with log-normal distributions, were plotted against the log 10 scale.
4 Results:

4.1 Characterization of immune correlates of protection in a MERS-CoV alpaca model.

Serosurveys throughout the Arabian Peninsula and numerous regions of Africa consistently find that dromedary camels have a high prevalence of MERS-CoV-specific antibodies (Deem et al., 2015; Falzarano et al., 2017b; Hemida, 2013; Muller et al., 2014). Experimental challenge of dromedary camels with MERS-CoV results in virus replication in the upper respiratory tract with moderate to high levels of virus shedding from nasal secretions (Adney et al., 2014a). Currently, it is not understood if seropositivity correlates with prevention of subsequent infection and shedding with MERS-CoV and clear correlates of protection have not been established yet. In dromedary camels, it is also not known whether systemic immune responses, such as serum IgG levels and neutralizing antibodies, play a role in protection from infection or rather that local, mucosal immune responses may be necessary to confer protection from MERS-CoV infection and shedding. The majority of current data has focused on serum total IgG responses; however, the role of unique heavy chain antibodies (IgG2 and IgG3) as compared to conventional IgG1 antibodies has not yet been established in regard to MERS-CoV infection and vaccination. Given the challenges of obtaining and maintaining dromedary camels in a containment level 3 facility, initial studies characterized the IgG response generated following infection with MERS-CoV in an alpaca animal model.

4.1.1 MERS-CoV virus shedding and IgG isotype response following experimental infection in alpaca model.

Experimental infections in dromedary camels as well as alpacas have resulted in at most mild signs of disease, such as nasal discharge, but more typically no clinical signs are observed (Adney et al., 2016). Here alpacas infected via the IT, IN or combined IT/IN routes showed no change in body temperature following MERS-CoV infection. Notably, no significant nasal discharge was observed in any alpacas following MERS-CoV infection. To detect virus secretion at mucosal surfaces, nasal and oral swabs were collected following infection up to 21 dpi. Regardless of route of infection, all alpacas showed increasing levels of MERS-CoV viral RNA in nasal swabs staring on day 2-3 post-infection (Figure 4.1A). Alpacas inoculated via the IN route demonstrated a faster increase in viral loads earlier and reached at peak levels on day 3 post-infection, in contrast, alpacas infected via the IT route showed a multiple day delay in viral RNA peak; however, similar levels of viral RNA were reached.
Figure 4.1: Quantification of viral RNA and infectious virus in nasal and oral swabs from alpacas infected with MERS-CoV.

Viral RNA and infectious titer were calculated in nasal and oral swabs from alpacas infected with MERS-CoV. 4.1A) Viral RNA in nasal swabs were calculated following MERS-CoV infection in alpacas by RT-PCR. 4.1B) Infectious virus following MERS-CoV infection in nasal swabs of alpacas was detected by TCID<sub>50</sub>/ml assay. 4.1C) Viral RNA in oral swabs were calculated following MERS-CoV infection in alpacas by RT-PCR. Each dot representing a single alpaca.
The levels of viral RNA in all alpacas were maintained until approximately 7 dpi when a gradual decline was noted, with all animals being negative for viral RNA by 14-16 dpi in IN and IN/IT inoculated groups, while two alpacas were positive for viral RNA up to 21 dpi when inoculated via IT route (Figure 4.1A). Viral RNA clearance was slightly delayed in the IT group. Infectious virus titers correlated with peak viral loads with infectious virus being recovered between 2 and 7 dpi. While infectious virus remained low in IT infected alpacas, IN infected alpacas showed detectable levels of infectious virus titer up to 12 dpi. The infectious virus titer in nasal washes in all alpacas ranged between $10^2$-$10^4$ TCID$_{50}$/ml and negative for virus titer by 10-12 dpi regardless of route of infection (Figure 4.1B). Oral swabs were also positive for viral RNA starting on day 2, tending to correlate with peak viral loads in nasal swabs; however, infectious virus was not isolated from oral swabs at any time point. All alpacas were negative for oral viral RNA by 8 dpi (Figure 4.1C).

To assess the distribution of virus in various respiratory tract tissues one alpaca infected via the IT/IN route was euthanized on 7 dpi and the indicated tissues were collected. Nasal turbinates, olfactory epithelium and trachea showed approximately $10^4$-$10^5$ TCID$_{50}$ eq./mg viral RNA compared to negligible levels, below $10^1$ TCID$_{50}$ eq./mg in lung lobes. Moderate levels of viral RNA were detected in larynx and pharynx (Figure 4.2A). A lack of gross pathology in the lungs was consistent with other studies in alpacas as well as dromedary camels (Figure 4.2C) (Adney et al., 2016, 2014b; Crameri et al., 2016). Histological analysis of 10% formalin fixed tissues by immunohistochemistry (IHC) staining was performed on nasal turbinates, trachea, and lungs. Out of all tissues tested, only nasal turbinates (collected in separate three section representing anterior, medial, and posterior regions) had consistent findings of IHC positive cells. Generally, few epithelial cells, with multifocal distribution were observed (Figure 4.2B). Similarly, the alpacas inoculated via the IN and IT routes were euthanized on 27 dpi and IHC staining was performed. All animals were negative for IHC staining against MERS-CoV nucleocapsid in all tissues and otherwise had normal findings by H&E staining (negative data not shown). This suggests that all alpacas in IN and IT were resolved by 27 dpi.
Viral RNA and lung pathology was determined in respiratory tissues (trachea and lung lobes) collected from alpacas at 7 dpi. 4.2A) Viral RNA levels in tissues collected from alpaca inoculated via the IT/IN route with MERS-CoV on 7dpi 4.2B) Immunohistochemistry against MERS-CoV nucleocapsid(N) in nasal turbinate collected at 7dpi from alpaca infected via IT/IN route indicated by black arrow. 4.2C). Gross pathology of a whole lung at day 7 post-infection.
To determine if infection induced an antibody response, the serum IgG response against MERS-CoV S1 as well as neutralization activity was assessed weekly. Prior to infection, no reactivity to MERS-CoV S1 nor neutralization activity was noted, supporting that all alpacas were, as expected, naïve for prior exposure to MERS-CoV. Seroconversion as indicated by S1-specific IgG was noted at day 14 dpi and peaked at 27 dpi. Differences in total IgG levels between the 3 routes of infection (Figure 4.3A) were not noted. To determine whether neutralizing antibodies were present, serum was also subjected to a microneutralization assay. On 7 dpi, 1 alpaca from the IT group and 1 alpaca from the IN group had a low titer neutralizing antibody response, while all the alpacas in the combined route did not have neutralizing antibodies. By day 14 and day 21, all alpacas showed comparable levels of neutralization antibodies (titer) (ranging between 1:32 to 1:256), reaching a peak on 27 dpi. The titers of neutralizing antibodies remained constant from 14 to 27 dpi in IT/IN and IT infected alpacas (Figure 4.3B).

4.1.2 Characterization of the IgG isotype response following experimental infection with MERS-CoV.

To determine, the MERS-CoV S1-specific IgG isotype response in alpacas infected via the combined IT/IN route, serum samples were analyzed using an IgG isotype-specific ELISA. IgG1 was detected as early as 7 dpi, increasing at day 14 and reaching a peak at 27 dpi. IgG3 (a heavy chain only antibody) was detected as early as 14 dpi and peaked at 21 dpi. The quantity of IgG3 binding as compared to IgG1 appeared to be comparably lower. Interestingly, the IgG2 isotype was below the level of detection in all alpacas up to 27 dpi (Figure 4.6A). To determine the IgG isotypes that are responsible for neutralizing activity against MERS-CoV, IgG1, IgG2 and IgG3 were purified from alpacas (n=3) infected via the combined IT/IN route. Affinity chromatography, using protein A and protein G columns was carried out on serum samples collected at 21 dpi to assess IgG responses. IgG1 and IgG3 bind to protein G and can be eluted using differential pH conditions, while IgG2 does not have affinity for protein G column (Daley et al., 2010, 2005). Following the elution of IgG1 and IgG3 from the protein G column, unbound IgG2 was absorbed onto a protein A affinity column and subsequently purified (Figure 4.4). Confirmation of the content of each fraction was performed by comparing reducing and nonreducing Western blots for each IgG isotype.
Figure 4.3: Total IgG and neutralizing antibody response in alpacas infected with MERS-CoV.

Total IgG response in alpacas infected with MERS-CoV was analyzed by ELISA and microneutralization assay. 4.3A). MERS-CoV S1 IgG ELISA on alpaca serum samples collected at the indicated timepoint. 4.3B). MERS-CoV (EMC/2012) microneutralization assay on serum samples collected at the indicated timepoint. Serum samples were collected at day -7, 0, 7-, 14-, 21- and 28-dpi. Each point represents 1 alpaca at given timepoint with line indicating geometrical mean. Error bars are indicating standard deviation.
Under non-reducing conditions, IgG1 migrated at approximately 150kDa when detected with an anti-IgG1 monoclonal antibody (Clone 27E11) (Figure 4.5A). The IgG3 and IgG2 purified fractions did not show evidence of the presence of IgG1 under non-reducing conditions. Of note, IgG1 could not be detected under reducing conditions with the anti-IgG1 antibody.

Under reducing conditions, the purified IgG3 fraction collected from the protein G column was observed as a band at approximately 50kDa, while in purified IgG2 and IgG1 fractions the IgG3 was undetected when tested using anti-IgG3 antibody (Clone 8E11) (Figure 4.5C). The IgG2 fraction was observed as a single band at 55 kDa under reducing conditions when detected with an anti-IgG2 antibody (Clone 19D8) (Figure 4.5B). IgG1 and IgG3 isotypes did not have any detectable IgG2 when assessed under reducing conditions. Western blot analysis of purified IgG1, IgG2 and IgG3 indicates that the purified isotypes did not appear to be contaminated with other IgG isotypes.

Following purification, each IgG isotype concentration was determined by using the protein estimation assay. Each IgG isotype was diluted separately to obtain a final concentration of 2 μg/ml and further serially diluted 2-fold. Each IgG isotype dilution was subsequently incubated with MERS-CoV at a concentration of 100 TCID<sub>50</sub> / 50μl to obtain an EC<sub>50</sub> value for neutralization. Both purified IgG1 and IgG3 had neutralizing activity against MERS-CoV with EC<sub>50</sub> values of 0.20 μg/ml and 0.05 μg/ml, respectively at 21 dpi. These findings suggests that the comparably lower level of IgG3 binding in the S1 ELISA does not affect the neutralizing activity of IgG3 compared to IgG1. As expected, neutralizing activity was not observed for IgG2 at 21 dpi as IgG2 was absent to very low in the S1 binding ELISA (Figure 4.6B).
Figure 4.4: Affinity chromatography of IgG isotypes from alpacas infected with MERS-CoV.

Serum samples collected from IT/IN challenged alpacas at 21dpi were diluted 1:5 in 20mM phosphate buffer and bound to a protein G column. IgG3 and IgG1 was eluted from protein G using different pH conditions. The unbound fraction was subjected to 2 additional rounds of binding to the protein G column. The final flow through from the protein G column was then absorbed onto a protein A column and IgG2 was subsequently purified. The chromatography trace is showing individual peak of absorbance for each IgG isotype in real time.
Figure 4.5: Western blot analysis of purified IgG isotypes from alpaca A15-5 at day 21-post infection.

The purified IgG isotypes from protein G and protein A column were subjected to Western blot analysis. Reference ladder (in kDa) was separated under same conditions and on the same gel. Purified IgG1, IgG2 and IgG3 as indicated were separated under non-reducing (4.5A) or reducing (4.5B, 4.5C) conditions and probed with anti-IgG1 (4.5A), anti-IgG2 (4.5B) or anti-IgG3 (4.5C) monoclonal antibodies to demonstrate that specific isotypes were free from contamination with other isotypes. Unfractionated serum was used as a positive control.
Figure 4.6: IgG isotype response against MERS-CoV infection in alpacas.

4.6A) IgG isotype ELISA against MERS-CoV S1 protein on serum samples collected at the indicated timepoint from alpacas infected via the IT/IN route. Individual IgG isotypes were detected with anti-IgG1 (27E11), anti-IgG2 (19D8) and anti-IgG3 (8E11), respectively. The optical density was measured at 450nm.

4.6B) Percent neutralization of purified IgG isotypes against MERS-CoV at 21 dpi. Each IgG isotype was 2-fold diluted from 2 μg/ml and subjected to microneutralization assay against EMC/2012. EC$_{50}$ values were calculated using GraphPad 9.2 and the antibody concentration resulting in 50% neutralization was estimated. Each point represents the mean of % neutralization at a given concentration.
4.1.3 **Binding affinity of purified IgG isotypes to MERS-CoV S1.**

The binding kinetics of each IgG isotype to the S1 protein of MERS-CoV was determined by surface plasmon resonance analysis (SPR). The kinetics and equilibrium of S1 and each IgG isotype reaction was measured with varying concentrations of analyte (each IgG isotype) over a given time. The purified IgG isotype was diluted 2-fold in 20mM phosphate buffer and binding was performed at room temperature. Purified MERS-CoV S1 protein was captured onto the surface of a GLC chip at density of ~2000 response unit (RU). Purified IgG1, IgG2 and IgG3 were injected separately over immobilized S1 at flow rate of 30 μl/min.

The initial interaction between antibody and spike was measured as an association constant (ka) and a dissociation constant (kd). IgG1 had mean ka of 2.43 ± 2.2x10^3 1/Ms and mean kd of 1.16 ± 3.08x10^-5 1/s. IgG3 had mean ka of 1.03±1.45x10^4 1/Ms and a kd of 1.32 ± 1.68 x10^-5 1/s. The comparatively higher ka of IgG3 indicates a faster association with S1 as compared to IgG1; however, the difference was not significant. The ratio of the ka and kd in an equilibrium reaction is described as the equilibrium dissociation constant (KD). The mean KD for IgG1 was 1.60 ±1.17 x10^-9 M while the mean KD for IgG3 was 4.27 ±1.90x10^-9 M. However, there was no significant difference between the KD of IgG1 or IgG3 binding in all tested animals (Figure 4.7A&B) (Table 4.1). Binding kinetics for IgG2 were not reported as no interaction was observed from any of the animals, consistent with the lack of signal in the IgG2 S1-binding ELISA at 21 dpi.
Figure 4.7: Surface plasmon resonance between IgG isotypes and MERS-CoV S1 at day 21 post-infection.

Surface plasmon resonance by IgG isotypes collected infected and reinfected alpacas and MERS-CoV S1 protein. A representative sensogram depicting binding kinetics of purified IgG1 (4.7A) and IgG3 (4.7B) against the S1 protein of MERS-CoV. The S1 protein was immobilized on a GLC chip using amine coupling reagents and a 2-fold dilution of purified IgG1 or IgG3 starting from upper line at 100nmole (Red), 50 n mole (Green), 25nmole (blue), 12.5nmole (yellow), 6.25 nmole (pink) (each colored line indicates 1 dilution) was absorbed. The association constant (ka) and dissociation rate constant (kd) were calculated using a 1:1 Langmuir method global fitting model. The dissociation (binding) constant (KD) was estimated from the ka and kd for each IgG isotype.
Table 4.1: Binding kinetic of IgG1 isotype to MERS-CoV S1 protein by Surface plasmon resonance.

Association rate(ka), dissociation rate(kd) and dissociation constant (KD).

<table>
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<tr>
<th>Animal ID</th>
<th>IgG isotype</th>
<th>ka (1/MS)</th>
<th>kd (1/s)</th>
<th>KD (M)</th>
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Mean ±SD

<table>
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<th></th>
<th>IgG1</th>
<th></th>
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<tr>
<td>Mean ±SD</td>
<td>2.43 ±2.2x10^3</td>
<td>1.16 ±3.08x10^-5</td>
<td>1.60 ±1.17x10^-9</td>
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<tr>
<td>Mean ±SD</td>
<td>1.03 ±1.45x10^4</td>
<td>1.32 ±1.68 x10^-5</td>
<td>4.27 ±1.90x10^-9</td>
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</table>
4.1.4 Alpacas are protected against MERS-CoV re-infection following initial infection.

Alpacas infected via the IT/IN route were re-infected with 1x10^6 TCID_{50}/ml via the intranasal route 61 days post-infection. As expected, no clinical signs were observed following re-infection in any of the alpacas. Post- reinfecion viral loads were determined in nasal as well as oral swabs using RT-PCR and infectious virus titration. No viral RNA or infectious virus was detected in oral swabs in any of the alpacas following re-infection up to day 21. Very low levels of viral RNA were (10^1 - 10^2 TCID_{50} eq./ml) were observed up to day 4 post-reinfection in all alpacas; however, no infectious virus was recovered from nasal washes at any timepoint (Figure 4.8). To assess the total IgG response following re-infection, serum samples collected on day 7, 14, and 21 post-reinfection (67, 74 and 81 dpi) were assessed using the MERS-CoV S1 ELISA. The IgG levels were increased following re-infection above the levels observed in initial infection in all three alpacas (Figure 4.9A). Similarly, the neutralizing activity was also determined in serum collected at day 7,14 and 21 post-re-infection. The neutralizing titer increased at 14 days post-reinfection and remained comparable on day 21 post-reinfection in all alpacas (Figure 4.9B).

4.1.5 Characterization of the IgG isotype profile following MERS-CoV re-infection.

The IgG isotype response following re-infection with MERS-CoV was characterized. The IgG isotype-specific ELISA showed an increase in the level of IgG1 at day 7 post-reinfection, which peaked at day 21 post-re-infection. Similarly, IgG3 levels also increased after re-infection and reached at moderate level as compared to IgG1 at day 21 post-infection. Interestingly, IgG2 levels started to increase on day 7 post-re-infection and peaked at day 21 post-reinfection; however, IgG2 levels remained low when compared to the levels of IgG1 and IgG3 (Figure 4.10A).

The binding kinetics post-re-infection were assessed by purifying each IgG isotype at day 21 post-re-infection from all alpacas against immobilized S1 protein. Similar to results from the primary infection reported in earlier sections, IgG3 showed comparatively faster association (ka) mean (1.23±8.34x10^4 1/Ms) with S1 compared to IgG1(1.48±9.37x10^3 1/Ms) at day 21 post reinfection. The dissociation means (kd) of IgG1 (2.08±2.99x10^{-5} 1/s) and IgG3 (8.17±0.58x10^{-5} 1/s) were not significantly different from each other. Similarly, IgG1 (1.22±2.66x10^{-9} M) and IgG3 (2.22±5.85x10^{-9} M) showed comparable equilibrium dissociation constant (KD) at day 21 post reinfection against S1 (Table 4.2).
Figure 4.8: Viral load from nasal swabs of alpacas following re-infection with MERS-CoV.

MERS-CoV viral RNA in nasal washes from alpacas re-infected with MERS-CoV, 60 days following the initial infection, was determined by RT-qPCR. TCID$_{50}$ equivalents were calculated by using viral RNA from stock with a known TCID$_{50}$/ml titers and subsequently diluted to generate a standard curve.
Figure 4.9: Total IgG binding and neutralizing antibody response following re-infection with MERS-CoV in IT/IN inoculated alpacas.

Total IgG and neutralizing antibody response in alpacas re-infected with MERS-CoV. 4.9A) Total IgG was determined following re-infection by MERS-CoV S1 ELISA. Serum samples were diluted 2-fold and detected by anti-llama IgG HRP tagged antibody. 4.9B) neutralizing antibody response against MERS-CoV in IT/IN infected alpacas following re-infection with MERS-CoV. Each dot represents geometrical mean and error bars indicating standard deviation at given time.
Table 4.2: Binding affinity by SPR analysis on IgG isotypes at day 21 post-reinfection.

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<tr>
<th>Animal ID</th>
<th>IgG isotype</th>
<th>$K_a$ (1/Ms)</th>
<th>$kd$ (1/s)</th>
<th>$K_D$ (M)</th>
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<tr>
<td>A15-1</td>
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<td>2.32x10^4</td>
<td>2.29x10^{-5}</td>
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<td>A15-5</td>
<td>IgG1</td>
<td>1.65x10^4</td>
<td>2.22x10^{-5}</td>
<td>6.55x10^{-9}</td>
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<td>7.56x10^3</td>
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<tr>
<td>Mean±SD</td>
<td>IgG1</td>
<td>1.48±9.37x10^4</td>
<td>2.08±2.99x10^{-5}</td>
<td>1.22±2.66x10^{-9}</td>
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<tr>
<td>Mean±SD</td>
<td>IgG3</td>
<td>1.23±8.34x10^4</td>
<td>8.17±0.58x10^{-5}</td>
<td>2.22±5.85x10^{-9}</td>
</tr>
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</table>
IgG isotype response and neutralizing antibody response was measured by IgG isotype ELISA and EC$_{50}$ assay respectively in alpacas reinfected with MERS-CoV. 4.10A) Each IgG isotype was detected at day 0, 7 and 14 post re-infection using monoclonal antibodies against each IgG isotype. 4.10B) EC$_{50}$ assay was calculated on purified IgG isotypes against EMC/2012. The EC$_{50}$ values were estimated in GraphPad prism 9.2.
Since the IgG isotypes were increased upon re-infection indicated by ELISA, each IgG isotype from day 21 post-re-infection was purified using protein G and protein A affinity column chromatography. Following purification, each IgG isotype was analyzed for purity by Western blot analysis and quantified by protein assay as mentioned in section 3.1.2. Neutralization against MERS-CoV was assessed in a microneutralization assay and the neutralizing activity of IgG1 and IgG3 were increased following re-challenge (EC$_{50}$: IgG1- 0.06 μg/ml, IgG3- 0.02 μg/ml). However, both IgG1 and IgG3 had comparable neutralizing activity. Interestingly, IgG2 now had a low level of neutralizing activity (EC$_{50}$=0.23 μg/ml); however, its activity remained much lower than that of other two isotypes. These findings suggest IgG1 and IgG3 are primarily involved in providing neutralizing activity against the MERS-CoV spike protein (Figure 4.10B).
4.1.6 A S1 subunit vaccine induces a strong neutralizing antibody response against MERS-CoV in alpacas.

Previously, the IgG1 and IgG3 isotype response following MERS-CoV infection and re-infection was determined. Here the IgG isotype following vaccination was also investigated to determine if vaccination induces a similar IgG isotype profile as observed from infection. Three groups of alpacas were immunized using a prime-boost regimen with 25 μg of purified MERS-CoV S1 protein expressed in a mammalian expression system formulated with S1+TriAdj delivered via intramuscular (IM) or intranasal (IN) route. A control group received PBS by the IM route. Alpacas were subsequently challenged with MERS-CoV 56 days following the boost. Serum samples were collected weekly post-vaccination and post-challenge. The total anti-S1 IgG response was detected following vaccination and challenge in alpacas by ELISA. The alpacas vaccinated with the IM route detected anti-S1 IgG response as early as 14 days post-vaccination and peaked at day 42 post-vaccination (following the boost). In contrast, the anti-S1 IgG response in IN immunized alpacas was not detected at any timepoint following vaccination. The anti-S1 IgG response was detected at 7 dpc (63-day post-vaccination) in alpacas immunized with IN route at similar levels to those observed in PBS control.

The neutralizing antibody response was correlated with an anti-S1 IgG response and neutralizing antibody response in IM group following vaccination. The neutralizing antibody response in IM immunized alpacas was detected as early as 28 days post vaccination with a significant increase following both boost and following MERS-CoV challenge. In contrast, the IN route of vaccination completely failed to induce a neutralizing antibody response, consistent with the lack of an anti-S1 response. Following challenge, a neutralizing antibody response was detected in IN vaccinated alpacas 14 dpc that was of a similar magnitude as control animals further, supporting the lack of effectiveness of this route of vaccination (Figure 4.11B).
Figure 4.11: Total IgG and neutralizing antibody response following S1+TriAdj vaccination and MERS-CoV challenge in alpacas.

Total IgG and neutralizing antibody response was measured following Tri-Adj vaccination and MERS-CoV challenge in alpacas. 4.11A) Anti-S1 endpoint titer of serum samples collected following vaccination and MERS-CoV challenge. 4.11B) Neutralizing antibody titer against MERS-CoV induced by an S1+tri-adjuvant vaccine and MERS-CoV challenge. Alpacas were vaccinated intranasally (IN) (n=3), intramuscularly (IM) (n=3) or PBS control (n=4) and challenged with MERS-CoV. Serum samples were collected at every 7 days following vaccination, challenge and subjected to anti-S1 ELISA and microneutralization assay to detect total IgG response. The vertical line indicates timepoints from vaccination to challenge and horizontal dotted line indicates the limit of detection (LOD).
Figure 4.12: IgG isotypes response in alpacas vaccinated with an S1+TriAdj vaccine and subsequently challenged with MERS-CoV.

The IgG isotype response from alpacas immunized with S1+TriAdj by IM or IN route or sham PBS control. 4.12 A). IgG1 response 4.12 B). IgG2 response 4.12 C). IgG3 response Serum samples were collected every 28 vaccination and 14 dpc. Further, serum samples were diluted 1:100 and were assessed by IgG isotype response against MERS-CoV S1 protein using monoclonal antibodies specific for each IgG isotype in ELISA. The optical density was measured at 450nm. The dotted line indicates the challenge on day 56 post-vaccination.
4.1.6.1 Characterization of IgG isotype profile following vaccination and MERS-CoV challenge in alpacas.

The anti-S1 IgG isotype response was detected MERS-CoV in serum collected at different timepoints post-vaccination and MERS-CoV challenge. The IgG1 in IM vaccinated alpacas was detected as early as day 28 post-vaccination and increased up to day 28 post-challenge (85 days post-vaccination) (Figure 4.12A). In contrast, IgG2 levels were detected at day 56 post-vaccination. The IgG3 isotype was detected on day 28 post-vaccination which peaked at 7dpc (Figure 4.12B). Notably, the anti-S1 IgG2 and IgG3 were present in low levels at each timepoint as compared to full length IgG1 antibody in IM immunized and MERS-CoV challenged alpacas (Figure 4.12C). The alpacas vaccinated by the IN route and PBS control did not have detectable anti-S1 IgG isotypes response following vaccination.

Furthermore, to determine the contribution that each IgG isotype made toward the neutralizing antibody response, serum samples collected from day 56 post-vaccination (following a prime/boost vaccination and prior to challenge) as well as 28 dpc in serum from alpacas immunized IM were subjected to protein G and protein A affinity chromatography to purify IgG1, IgG2 and IgG3 isotypes. Following purification, the relative neutralizing antibody activity was determined by microneutralization assay to determine the EC₅₀ assay. Each purified IgG isotype was diluted 2-fold with a starting concentration of 2 μg/ml. On day 56, IgG1 and IgG3 neutralizing activity was comparable and IgG2 activity was calculated minimal (IgG1 EC₅₀: 0.34 μg/ml, IgG2 EC₅₀: 1.95 μg/ml, IgG3 EC₅₀: 0.11 μg/ml) (Figure 4.13A). Similarly, on the day 28 post-infection (day 85 post-vaccination) the neutralizing antibody response showed increased neutralizing antibody titer of IgG3 (EC₅₀: 0.04 μg/ml) and comparable to neutralizing activity of IgG1(EC₅₀: 0.08 μg/ml). The neutralizing activity of IgG2 activity was still minimal even at day 28 post-challenge (IgG2 EC₅₀: 0.30 μg/ml) (Figure 4.13B).
Figure 4.13: Neutralizing activity of IgG isotypes following S1+TriAdj vaccination and MERS-CoV challenge in alpacas.

Neutralizing antibody response following Tri-Adj vaccination and MERS-CoV challenge was detected by EC$_{50}$ assay. 4.13A) IgG1, IgG2 and IgG3 antibodies were purified from serum collected at day 56 post-vaccination from alpacas immunized via the IM route. 4.13 B). % Neutralization of IgG isotypes purified from serum samples at 28 dpc with MERS-CoV. EC$_{50}$ values were calculated in GraphPad prism 9.2.
4.14A) Viral load (TCID$_{50}$/mL) against Days post-challenge.

4.14B) Virus titer (TCID$_{50}$/mL) against Days post-challenge.

Figure 4.14: Quantification of infectious virus following S1+TriAdj vaccination and MERS-CoV challenge in alpacas.

Viral RNA and infectious virus titer were determined in nasal washes collected from alpacas at various timepoints following MERS-CoV challenge. 4.14A) Viral RNA in nasal washes collected from alpacas challenged with MERS-CoV. TCID$_{50}$ equivalents were calculated by using viral RNA from stock with a known TCID$_{50}$/ml titers and subsequently diluted to generate a standard curve. 4.14 B) Infectious virus titer in nasal washes collected from MERS-CoV challenged alpacas. Each dot representing geometric mean and error bars are indicate standard deviation.
4.1.6.2 Viral RNA and infectious virus in S1+ Tri-Adj vaccinated alpacas following MERS-CoV challenge.

Alpacas vaccinated with S1+TriAdj IM or IN were challenged intranasally with 1x10^6 TCID_{50} dose of MERS-CoV on day 56 post vaccination. The viral RNA was peaked at 1 dpc and undetected by 4 dpc in nasal washes of alpacas vaccinated with IM route. In contrast, the high levels of viral RNA (10^7 TCID_{50} eq/ml) were detected in alpacas immunized with IN route as early as 2 dpc. The levels of viral RNA peaked at 8 dpc and decreased to undetectable levels by 10 dpc in nasal washes of alpacas immunized IN route. Similarly, the levels of viral RNA in nasal washes of PBS control peaked at 8 dpc (10^7 TCID_{50} eq/ml) and decreased by 10 dpc (Figure 4.14A).

The infectious virus titers were correlated with viral loads in nasal washes with no infectious virus was recovered in alpacas immunized with IM route at any timepoint following MERS-CoV challenge. In contrast, the IN immunized alpacas (10^7 TCID_{50}/ml) high levels infectious virus titer peaked at 4 dpc (Figure 4.14B). The infectious titer in nasal washes of alpacas immunized with IN route were negative for infectious titer by 8 dpc. Similarly, the infectious virus in nasal washes of alpacas in PBS control peaked at 7 dpc (10^7 TCID_{50}/ml) and undetected at 8 dpc.
4.1.7 Cross-neutralization activity of IgG isotypes against MERS-CoV viral isolates from Saudi Arabia.

Most MERS-CoV vaccine candidates tested in either humans or camels, including our own S1+TriAdj, contain the spike protein (S) (Choi et al., 2020; Du et al., 2017a). However, the spike protein undergoes mutations that result in multiple variants of MERS-CoV that circulate in the population (Kim et al., 2016; Kleine-Weber et al., 2019). Characterization of the neutralization ability against isolates from dromedary camels in Saudi Arabia will help inform whether an S antigen derived from a single virus isolate will be sufficient for a vaccine to protect against all MERS-CoV virus isolates. As such, virus isolates were obtained from camels in Saudi Arabia. Infectious virus was isolated from seven of ten MERS-CoV RT-qPCR positive dromedary camel nasal swabs. These virus isolates were subsequently sequenced by next generation sequencing. Limited heterogeneity in the amino acid sequence of S1 and S2 was noted compared to the EMC/2012 genome sequence deposited at (NCBI Acc. JX869059) at indicated amino acid positions (Table 4.3). Among the 7 isolates, only a single isolate (MERS-CoV/2018/SA/3-38) possessed an amino acid change in the receptor binding domain (RBD). Interestingly, phylogenetic analysis of S revealed that this viral isolate was a unique outgroup within the clade B, while the other 6 viral isolates grouped with other previously sequenced isolates (data not shown). Based on presence of amino acid changes we selected 4 isolates for further assays (Table 4.3).

For the cross-neutralization assay, sera from 3 groups of alpacas were used: 1.) 21 days post infection (day 21) 2.) 21 days post-reinfection with MERS-CoV from section 4.1.1 and 3) sera from alpaca vaccinated with an S1+TriAdj vaccine (day 56 post-vaccination and IM route) was used from section 4.1.6. Serum samples were diluted 2-fold starting from 1:20 dilution and subsequently 1:1 with 100 TCID50/50 μl of 4 of the indicated isolates and incubated for 1 hr 37°C. After 1 hr, Vero76 cells were infected with antibody-virus mixtures and monolayers were observed on day 3 and 5.
Table 4.3: Amino acid changes in the spike protein of MERS-CoV isolate obtained from dromedary camels in Saudi Arabia compared to EMC/2012.

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>EMC/2012</th>
<th>Amino acid change</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-38</td>
<td>Phe</td>
<td>Leu</td>
<td>473</td>
</tr>
<tr>
<td>4-125</td>
<td>Gln</td>
<td>Arg</td>
<td>1020</td>
</tr>
<tr>
<td>9-2197</td>
<td>Gln</td>
<td>Arg</td>
<td>1020</td>
</tr>
<tr>
<td>4-125</td>
<td>Lys</td>
<td>Asn</td>
<td>1322</td>
</tr>
</tbody>
</table>
Figure 4.15: Cross-neutralization activity of serum from MERS-CoV infected and MERS-CoV S1+TriAdj vaccinated alpacas against MERS-CoV camel isolates from Saudi Arabia.

Cross-neutralizing activity of serum from infected and vaccinated alpacas against MERS-CoV dromedary camel isolates from Saudi Arabia. 4.15A) alpaca serum from MERS-CoV infection (21 dpi), or 4.15B) alpaca serum from MERS-CoV reinfection (21 days post reinfection), 4.15C) alpaca serum from S1+TriAdj vaccination (day 56 post-vaccination). Serum samples were diluted 1:2 and incubated with the indicated MERS-CoV isolate. Each dot is representing serum from an individual alpaca. Statistical significance t-test ** p=0.01.
Cross-neutralization data revealed that there were no significant differences in the neutralization titer between the 4 camel isolates from Saudi Arabia and the original EMC/2012 isolate when analyzed with sera from infected as well as re-infected alpacas (Figure 4.15A&B). The cross-neutralizing antibody response was minimal following infection which increased subsequently following re-challenge with MERS-CoV. However, sera from alpacas vaccinated with S1+TriAdj showed significant difference between neutralizing antibody response against 3-38 virus isolate as compared to EMC/2012 (Figure 4.15B). These findings suggest antibodies generated in infection do not have any effect on antibody binding to amino acid changes in virus isolates and serum raised against EMC-2012 can effectively neutralize virus strains isolated from Saudi Arabia. On the other hand, antibody response developed in Tri-Adj vaccinated alpacas may have comparatively less sensitivity to (MERS-CoV/2018/SA/3-38) virus isolates (Figure 4.15C).

In summary, both experimental infection and vaccination with S1+TriAdj result in induction of IgG antibodies that are capable of mediating neutralization, including of multiple MERS-CoV isolates that were obtained from camels. In both scenarios, both IgG1 and IgG3 are induced and both isotypes can mediate a high level of neutralizing activity with comparable binding kinetics. In contrast, IgG2 was not induced by the primary infection nor following a prime/boost immunization, but it is induced following re-infection or following infection after a prime/boost vaccination – suggesting that its induction differs from that of IgG1 and IgG3. These findings suggest that the reduced size and distinct structure of IgG3 does not negatively impact its capacity to neutralize virus. In contrast, IgG2 appeared to be less efficient in neutralization.

Moreover, the cross-neutralizing activity against MERS-CoV circulating isolates in camels showed minimal effect of amino acid changes on the neutralizing response while the neutralizing antibody response developed in S1+Tri-Adj vaccinated alpaca had comparatively lower cross-neutralization against 3-38 isolate with mutations in spike protein. However, the overall titer was remained relatively high against 3-38 virus isolate.
4.2 Development of reagents for the characterization of camel IgG isotypes.

Similar to alpacas, dromedary camels possess both conventional IgG1 antibodies as well as IgG2 and IgG3 heavy chain-only antibody isotypes. These heavy chain-only antibodies constitute approximately 75% of the IgG in camel serum compared to approximately 45% in alpacas and llamas. While monoclonal antibodies that bind to alpaca and llama IgG isotypes are available (Daley et al., 2005) and were utilized to characterize the isotype response to MERS-CoV infection and vaccination, comparable reagents are not available for camels. Furthermore, alpaca and llama monoclonal isotype-specific antibodies do not recognize specifically the camel IgG isotypes, suggesting there is some divergence between new-world and old-world camelids (Daley et al., 2010).

Most studies on camel immune responses to MERS-CoV have only focused on evaluating the presence of MERS-CoV specific antibodies (seroprevalence studies); while the kinetics and IgG isotype response of antibody responses in camels have not been clearly defined. Understanding the IgG isotype response from natural infection and vaccination would possibly provide insight in immunity to infection and protection from re-infection. However, there is a need to develop reagents to detect the specific camel IgG isotypes. Therefore, reagents to detect camel IgG isotypes were developed.

4.2.1 Purification of IgG isotypes from camel serum.

Similar to alpacas, dromedary camel IgG isotypes have differential affinity for protein G and protein A column chromatography (Daley et al., 2005). To purify IgG isotypes, commercially available camel serum was subjected to protein G and protein A column chromatography to purify IgG1, IgG2 and IgG3 under differential pH conditions and analyzed by SDS-PAGE. Each purified IgG isotype was analyzed under reducing and non-reducing conditions (Figure 4.16). Under reducing conditions (with β-mercaptoethanol), a single band representing the heavy chain was observed at 45kDa and 42kDa corresponding to IgG3 and IgG2, respectively. IgG1 was detected at 50 kDa and 29kDa corresponding to heavy and light chains, respectively. Under non-reducing conditions IgG1, IgG2 and IgG3 were observed at an apparent molecular weight of 150kDa, 95kDa and 93kDa respectively (Figure 4.16). Various bands corresponding to unknown serum proteins were also detected at lower intensity.
Figure 4.16: SDS-PAGE and Coomassie staining of purified camel IgG isotypes.

Purified fractions from affinity column were separated under reducing and non-reducing conditions separately on SDS gels. Lane1 – IgG3 (reducing), Lane2 – IgG3 (non-reducing), Lane3 – IgG1 (reducing), Lane4 – IgG1 (non-reducing), Lane5 – IgG2 (reducing) and lane 6 – IgG2 (non-reducing). Markers were separated under similar conditions in SDS-PAGE analysis.
4.2.2 Development of anti-dromedary camel IgG isotype-specific monoclonal antibody.

BALB/C and B57L/6 mice were immunized with purified dromedary camel IgG3 for the purpose of generating an anti-dromedary camel IgG3 isotype specific monoclonal antibody. Test bleeds were collected after each immunization and analyzed by ELISA. Each mouse serum sample was analyzed against IgG1, IgG2 and IgG3 at a starting concentration of 1:1000. The C57BL/6 mice showed higher specificity and reactivity to the camel IgG3 isotype. Hybridoma cells were prepared from 1 C57BL/6 splenocytes and each clone was screened with ELISA. Five highly anti-IgG3 isotype-specific clones were selected for subsequent steps. Out of 5 clones, clone 227, 228 and 229 showed higher cross-reactivity against camel IgG1 and IgG2. Finally, clone 230 with comparatively low cross-reactivity against IgG2, IgG1 and high specificity against IgG3 was selected for scale up and purified antibody was delivered by Genscript (Figure 4.17). Based on ELISA analysis, the optimal concentration was selected to be used in subsequent assays to determine the IgG isotype response against MERS-CoV infection and vaccination. The Western blot analysis using anti-IgG3 monoclonal antibody detected purified IgG3 at 95 kDa. However, the low intensity bands were detected in purified IgG1 and IgG2 elutes at 95 and 150kDa suggesting minimal cross-reactivity of anti-IgG3 antibody (Figure 4.17B).

4.2.3 Purification of IgG1 from camel serum.

Using a similar approach as for the alpaca IgG isotype purification, dromedary camel IgG1, was purified using a protein G column. A commercially available anti-camel IgG1 antibody (ABIN1981265, clone Cam 17-2) purified IgG1 was detected in Western blot analysis. The purified camel IgG1 under non-reducing conditions was detected at 150kDa in a fraction collected from protein G column. However, anti-camel IgG1 did not detect camel IgG1 under reducing conditions (Figure 4.18A). Further, purified IgG2 and IgG3 fractions were also subjected to Western blot analysis to detect cross-reactivity of anti-camel IgG1 antibody. Under non-reducing and reducing conditions, camel IgG1 was not detected when tested with anti-camel IgG1 antibody suggesting absence of contamination and cross-reactivity with other IgG isotypes.
C57BL/6 mice were immunized with purified dromedary camel IgG3 and serum was analyzed after 3 immunizations. IgG isotype ELISAs were performed by coating purified IgG1 (B), IgG2 (C) and IgG3 (A) and incubation with serum from individual mice at the indicated dilution. Binding was detected using an anti-mouse IgG HRP conjugated IgG and developed with TMB; absorbance was read at 450nm. Number mentioned on top of each graph denoted represents an individual mouse hybridoma clone.

Figure 4.17: IgG isotypes for selection of anti-IgG3 positive clones.
Figure 4.18: Western blot analysis of purified camel IgG isotypes by anti-IgG1 and anti-IgG3 antibodies.

Purified camel IgG isotypes were diluted in 4x loading buffer without beta-mercaptoethanol and loaded onto an SDS-PAGE gel followed by Western blotting with 4.18A) anti-camel IgG1; or 4.18B) anti-camel IgG3 antibody. Purified IgG1 (lane 1), IgG2 (lane 2) and IgG3 (lane 3) purified from camel serum was loaded along with whole camel serum (lane 4). Camel IgG1 was detected by anti-camel IgG1 monoclonal antibody at 150kDa, while camel IgG3 was detected by anti-camel IgG3 monoclonal antibody at 95kDa.
The camel IgG isotype ELISA was established using anti-IgG1 and anti-IgG3 monoclonal antibodies. The indicated dilutions of 4.19 A) Anti-camel IgG1; and 4.19B) anti-camel IgG3 antibody was added to plates previously coated with purified camel IgG1, IgG2 and IgG3. The optical density was measured at 450nm. Individual dots representing geometric mean and standard errors are representing standard deviation.

Figure 4.19: Development of dromedary camel IgG1 and IgG3 isotype specific ELISA.
4.2.4 Development of a camel IgG isotype ELISA.

ELISA plates were coated with purified camel IgG1, IgG2 and IgG3. Anti-IgG3 and anti-IgG1 monoclonal antibodies were diluted 1:100 and absorbed on coated wells. Anti-IgG3 antibody reactivity was optimal at 1:800 concentration in ELISA against purified camel IgG isotype (Figure 4.19A&B). Further, anti-camel IgG1 and IgG2 reactivity was minimal at 1:800 concentration of anti-camel IgG3 MAbs. Furthermore, anti-IgG1 antibody was diluted 1:10 and the optimal concentration was determined at 1:100 with high specificity against camel IgG1 and low cross-reactivity against camel IgG2 and IgG3. In conclusion, we have developed and characterized monoclonal antibodies specific for IgG1 and IgG3 of from camels. These antibodies are applicable to characterize the IgG isotype response in camels following MERS-CoV infection and vaccination.

4.3 Serum from MERS-CoV vaccinated or infected alpacas does not have neutralization activity against SARS-CoV-2.

The spike protein from SARS-CoV-2 shares 79% and 51.8% sequence similarities with SARS-CoV and MERS-CoV, respectively (Ren et al., 2020). The initial reports suggest the antibody response may play a major role in protection against SARS-CoV-2 and MERS-CoV and the details of whether there was cross-reactivity between these viruses were not known at the beginning of the pandemic. Like MERS-CoV, SARS-CoV-2 also uses its spike protein to enter into the host cells (Hoffmann et al., 2020b); however, MERS-CoV uses DPP4 as a receptor while SARS-CoV-2 binds to ACE-2 to enter the host cells. Here, serum from MERS-CoV infected alpacas was used to perform analysis on SARS-CoV-2 cross reactivity. To do so, the SARS-CoV-2 isolated at VIDO was incubated with serum samples from alpacas re-infected with MERS-CoV and analyzed by microneutralization assay. The antibodies induced against MERS-CoV did not show cross-reactivity against SARS-CoV-2 even at 1:10 dilution. Furthermore, the 56- days post-vaccinated alpaca serum induced by S1 Tri-Adj against MERS-CoV showed minimal cross-reactivity against SARS-CoV-2 (Figure 4.20A). However, the overall titers were relatively low against SARS-CoV-2. Several reports suggests that antibody responses generated against MERS-CoV may not cross-protect against SARS-CoV-2 (Anderson et al., 2020; Maani et al., 2021). As our data is consistent with others, we only focused on determining the neutralizing antibody response developed against SARS-CoV-2 infection and vaccination using a hamster model.
A microneutralization assay was performed using sera from MERS-CoV infected/reinfected and S1+TriAdj vaccinated alpacas against 4.20A) Cross neutralization assay against SARS-CoV-2 using MERS-CoV S1 Tri-Adj vaccinated (day 56 post vaccination) and MERS-CoV reinfected serum (day 21 post reinfection) or 4.20B). Microneutralization assay against MERS-CoV using MERS-CoV S1 Tri-Adj vaccinated (day 56 post vaccination) and MERS-CoV reinfected serum (day 21 post reinfection).
4.4 Differential neutralizing antibody response and its correlation with pathology in a hamster model of SARS-CoV-2 B.1.1.7 and B.1.351 infection.

Since the emergence of SARS-CoV-2, several VOCs have been evolved, some which have widely circulated in the human population. In Dec 2020, a large cluster of cases were reported in the United Kingdom with emergence of the B.1.1.7 lineage (Also known as 20I/501Y. V1 or alpha variant). The B.1.1.7 possesses 9 mutations in the spike protein including 2 deletions (69/70 del, Y145 del, N501Y, A570D, D614G, P681H, T7161, S982A and D1118H). The N501Y mutation in the RBD region enhances the ACE-2 receptor binding (Washington et al., 2021b). Furthermore, the other mutations in the spike protein of B.1.1.7 are presumed to increase infectivity as well as transmissibility in humans. Shortly, thereafter another VOC, B.1.351 emerged in lineage 501Y.V2 (also known as beta variant) which became dominant in South Africa. The B.1.351 contains a total of 8 mutations in spike (L18F, D80A, D215G, R246I, D614G, A701V) with three in the RBD region (K417N, E484K, N501Y). These mutations in the B.1.351 lineage also appears to increase infectivity, transmissibility, and pathogenesis (Ramanathan et al., 2021; Wang et al., 2021a).

With several mutations in the RBD, both B.1.1.7 and B.1.351 VOCs were presumed to have the potential to escape the neutralizing antibody response from previous infection as well as vaccination (Planas et al., 2021a; Supasa et al., 2021; Wang et al., 2021a). The mutation in RBD region at position Y501 is present at the ACE-2 interface however it is not proposed to interfere with neutralizing antibodies. In contrast, E484K in RBD of B.1.351 present in ACE-2 interface and is located in epitopes that are targeted by neutralizing antibody. The presence of these mutations is responsible for immune escape from neutralizing antibodies developed in previous infections and by vaccination (Planas et al., 2021a; Wang et al., 2021a).

4.4.1 B.1.1.7 and B.1.351 infected hamsters show comparable weight loss and viral loads in nasal washes.

The Syrian hamsters are susceptible to infection with SARS-CoV-2 (ancestral) with presence of high lung pathology and immune responses. However, the mutations in spike protein of B.1.1.7 or B.1.351 may change the building affinity for hamsters ACE-2. To establish an infection model with the B.1.1.7 and B.1.351 VOCs variants in the hamster model, 2 groups of hamsters were infected with either B.1.1.7 or B.1.351 in separate studies.
Eight hamsters (for each VOC) were divided into two cages (n=4 each cage) and weight was recorded each day throughout the study. 4.21A) weight loss in B.1.1 infected hamsters. 4.21B) weight loss in B.1.351 infected hamsters. Percent weight loss was calculated from the weight of each hamster one day prior to infection in both VOCs. The dotted line indicating weight on day before the start of the study.

Figure 4.21: Percent weight loss in hamsters infected with B.1.1 and B.1.351.
Weight loss was monitored daily used to track clinical disease following infection. Hamsters infected with either B.1.1.7 or B.1.351 showed progressive weight loss, reaching peak weight loss on 5 or 6 dpi.

Notably, the weight loss from one cage of hamsters (n=4) infected with B.1.1.7 was severe and consistent with previous observations for SARS-CoV-2 infection in hamsters. These hamsters showed approximately 12% weight loss by 5 dpi reaching to 15% on 6 dpi (Figure 4.21A). The other cage containing four hamsters did not show any weight loss as reported following B.1.1.7 infection. The weight change in hamsters infected with the B.1.351 was similar to observed weight loss in B.1.1.7. In all hamsters infected with B.1.351 at 5 dpi, the mean of % body weight loss was approximately 15% (Figure 4.21 B). The overall weight loss throughout the study was ranging between 5-20%, which was consistent with findings reported in hamsters upon infection with SARS-CoV-2. All hamsters returned to their original weight by day 10 post-infection.

Furthermore, viral RNA and infectious virus in nasal washes were determined from samples taken at days 1, 3, 5, 7 and 10 post-infections. Interestingly, there were no significant differences in viral RNA levels in hamsters that did not show body weight loss in B.1.1.7 group. Similarly, when compared nasal viral RNA in B.1.1.7 and B.1.351 similar kinetics were recorded with peak at 3 dpi in both groups of VOCs. The viral RNA was decline on 7 dpi in B.1.1.7 and B.1.351 infected hamsters (Figure 4.22A). The infectious viral titers were correlated with nasal RNA levels with gradual decline by day 7 dpi in B.1.1.7 infected as well as B.1.351 infected hamsters.

4.4.2 Infection of hamsters with B.1.1.7 or B.1.351 replicate leads to high viral loads in the lungs of hamsters.

The total viral RNA and infectious titer were quantified in hamster lungs at day 5 and day 10 upon infection with B.1.1.7 and B.1.351. There were no significant differences in viral RNA amongst both VOCs infected groups at 5 dpi in lung as well nasal turbinates and trachea. The viral RNA in trachea showed lower replication on 5 dpi and minimal by 10 dpi in both groups (Figure 4.23A, B, C). Further, the highest viral load was detected at day 5 post-infection in nasal turbinates which was comparable between B.1.1.7 as well as B.1.351 (Figure 4.23A, C).
Nasal washes were collected on day 1-3-5-7 and 10 post-infection to quantify viral RNA by RT-PCR targeting the upE gene of SARS-CoV-2 and infectious virus by TCID$_{50}$ assay. 4.22A). The viral RNA was determined as copies/reaction from a standard curve of a plasmid dilutions containing E gene of SARS-CoV-2. 4.22 B) Similarly, the infectious virus was determined on day 1-3-5-7 and 10 post-infection by virus titration. The infectious titer was recorded as TCID$_{50}$/ml. Each dot is representing each hamster at given dpi and line indicating geometric mean.

Figure 4.22: Viral RNA and infectious virus titer in nasal washes of hamsters infected with B.1.1.7 and B.1.351.
Hamsters infected with B.1.1.7 and B.1.351 were euthanized on 5 dpi (n=4) or 10 dpi (n=4) and trachea, nasal turbinates and lung lobes were harvested to detect viral RNA and infectious virus titer. 4.23A). Viral RNA detection on tissues collected at 5 dpi. 4.23B). Viral RNA detection on tissues collected at 10 dpi. 4.23C) infectious virus titer was detected using tissue homogenates collected at 5 dpi from hamsters infected with B.1.1.7 or B.1.351. Horizontal dotted line indicating limit of detection (LOD).

**Figure 4.23:** Viral burden in tissues on 5 and 10 dpi following B.1.1.7 and B.1.351 infection.
This viral load was decreased by $10^4$ copies/ml on day 10 in B.1.1.7 infected hamsters while B.1.351 showed a decrease of $10^2$ copies/ml on day 10 when compared to 5 dpi (Figure 4.23B). Similarly, the 4 lung lobes showed high viral RNA as well as infectious virus titer on 5 dpi with decrease on 10 dpi. The 4 lung lobes had high loads of virus on day 5 which were comparable between B.1.1.7 as well as B.1.351 infected hamsters. At 10 dpi, there were comparable viral RNA levels in lung lobes; however, no infectious virus was detected on 10dpi in any of the tissues (Figure 4.23C). To determine whether these VOCs have altered pathogenesis histology and immunohistochemistry analysis on the left lung lobe was performed (Figure 4.24). The lung pathology consistent with the previously reported SARS-CoV-2 ancestral isolate was observed at 5 dpi in B.1.1.7 as well as B.1.351 infected hamsters. On day 5, marked interstitial pneumonia and bronchiolitis was observed in B.1.1.7 as well as B.1.351 infected hamsters (Figure 4.24A&B). The bronchiolitis was characterized by hyperplasia, syncytia formation and epithelial cell necrosis. The interstitial pneumonia in B.1.1.7 as well as B.1.351 infected lungs was marked by expanded alveolar septa, infiltration of monocytes and alveolar pneumocytes and in some cases complete destruction of alveolar architecture. The B.1.351 infected lungs showed presence of alveolar hemorrhages compared to B.1.1.7 infected lungs on 5 dpi (Figure 4.24C&D). The interstitial pneumonia and inflammatory cells infiltration were varying from moderate to severe regardless of infection with variants. Based on presence of interstitial pneumonia all lungs were scored from 1–4 on 5dpi. All hamster lungs regardless of VOCs showed moderate (score =2) to severe (score=3 or 4) pathology on 5 dpi (Figure 4.24E). On 10 dpi the lung lobes from B.1.1.7 infected hamsters showed mild interstitial pneumonia and inflammatory cells infiltration indicative of disease progression towards recovery (score raging between 1-2). In contrast, on 10 dpi, 2 of the 4 euthanized hamsters from the B.1.351 group still showed evidence of severe interstitial pneumonia (score=3) suggesting delayed recovery from infection in comparison with B.1.1.7 (Figure 4.24E). The immunohistochemistry analysis showed presence of SARS-CoV-2 nucleocapsid protein within bronchiolar epithelia, macrophages and in type I and type II pneumocytes in B.1.1.7 as well as B.1.351 infected lungs on 5 dpi.
**Figure 4.24: Histopathology of lungs collected at 5dpi from hamsters infected with B.1.1.7 and B.1.351.**

Formalin fixed paraffin embedded tissues were stained with Hematoxylin & Eosin on tissues collected at 5 dpi. 4.24 A &B). B.1.1.7 infected tissues at 10X and 40X respectively. 4.24 C&D). B.1.351 infected tissues at 10X and 40X. Lung were scored based on pneumonia, inflammatory cells infiltration and pneumocytes (blue arrow). Some hamsters were showed alveolar hemorrhages (black arrow). 4.24 E) lung scores based on pathology 0-25%: 1, 26-50%: 2, 51-70%: 3, 76-100%: 4. Statistical significance * p= 0.05.
Figure 4.25: Immunohistochemistry against SARS-CoV-2 nucleocapsid protein (N) from lung lobes of B.1.1.7 and B.1.351 infected hamsters.

Formalin-fixed paraffin-embedded lung tissues were stained against SARS-CoV-2 nucleocapsid protein (N) using anti-N monoclonal antibody at concentration 1:800. 4.25A). IHC on lung lobe collected from hamster infected with B.1.1.7 at 5 dpi and 4.25B). IHC on lung lobe collected from hamster infected with B.1.351 at 5 dpi. Following staining, the nucleocapsid protein (N) was detected in epithelial cells (brown spots indicated by black arrow) in hamster lung lobes infected with either B.1.1.7 or B.1.351.
On 10 dpi, the parenchyma and bronchiolar epithelial cells showed few nucleocapsid protein stains in B.1.1.7 as well as B.1.351 indicating viral clearance (Figure 4.25A&B).

4.4.3. Differential S1 and RBD binding antibody from B.1.1.7 and B.1.351 infected hamsters.

Serum from hamsters infected with either B.1.1.7 or B.1.351 VOCs were analyzed to determine the binding capacity to VOC-specific RBD and S1 proteins using ELISA. RBD proteins from Wuhan-Hu-1 (referred as Wuhan isolate), B.1.1.7 and B.1.351 were used as coating antigen in an ELISA and subsequently incubated with serum samples collected from B.1.1.7 or B.1.351 infected hamsters. Sera from B.1.1.7-infected hamsters on 5 dpi, showed comparable binding to Wuhan, B.1.1.7 and B.1.351 S1 and RBD proteins. However, differences were more prominent on 10 dpi with decreased binding to S1 derived from B.1.351 when tested with serum collected from B.1.1.7 infected hamsters (p= 0.05) (Figure 4.26A&Figure 4.27A). The binding antibody response against homologous as well as heterologous RBD from antibodies induced in B.1.1.7 hamsters showed no statistical differences on day 5 post-infection. However, on day 10 dpi, the binding was decreased against Wuhan RBD (p=0.005) as well as B.1.351 (p=<0.001) when tested with serum induced in B.1.1.7 infected hamsters.

The serum isolated from hamsters infected with B.1.351 showed no significant differences in binding against S1 protein from B.1.1.7 and Wuhan isolate on 5 dpi. On day 10, sera from B.1.351 infected hamsters showed significant decreased binding to Wuhan and B.1.1.7 S1 (p=0.001). The reduction in heterologous binding of antibody response was marked on 10 dpi when tested with antibodies induced in B.1.351 infected hamsters. The binding of B.1.351 specific antibodies against RBD from Wuhan and B.1.1.7 (p=0.005) isolate was decreased significantly on day 10. Interestingly, the overall binding antibody response against homologous S1 as well as RBD was consistently lower on day 5 and day 10 post-infection in B.1.351 infected hamsters (10^1 -10^2) compared to B.1.1.7 (10^3-10^4) as well as previously reported in Wuhan isolate (Figure 4.26B&Figure 4.27B).
Figure 4.26: IgG binding against Wuhan, B.1.1.7 or B.1.351 derived S1 by ELISA.

Serum samples were collected on day 5 and day 10 post B.1.1.7 or B.1.351 infection. 4.25A) Anti-S1 IgG response in B.1.1.7 infected hamster serum 4.25B) Anti-S1 IgG response in B.1.351 infected hamster serum. To quantify anti-S1 IgG antibodies, purified S1 protein from either Wuhan, B.1.1.7 or B.1.351 was coated on to the ELISA plate and 1:2 dilutions of serum samples were absorbed on S1. The hamster IgG was detected by HRP tagged secondary antibody and titers were determined from negative samples. The optical density was measured at 450nm. Statistical significance * p=0.05, **p=0.01, ***p=0.001, ****p=<0.001.
The serum collected from B.1.1.7 & B.1.351 infected hamsters at day 5 and day 10 post-infection were diluted 1:2 and absorbed on purified RBD protein from three isolates. 4.26A) Anti-RBD IgG response in B.1.1.7 infected hamster serum 4.26B) Anti-RBD IgG response in B.1.351 infected hamster serum. The anti-RBD IgG response was detected by HRP tagged Similarly, the cross-neutralization assay on day 10 with serum samples from either challenged hamsters showed diverse fold change against 3 virus isolates mentioned above. The polyclonal secondary antibody and titers were determined from negative samples. The optical density was measured at 450nm. Statistical significance * p=0.05, * p=0.05, **p=0.01, **p=0.001, ****p=<0.001.

Figure 4.27: IgG binding against Wuhan, B.1.1.7 or B.1.351 derived RBD isolates by ELISA.
4.4.4 B.1.351 infected hamsters consistently show reduced neutralization when tested against other SARS-CoV-2 isolates.

The impact of amino acid changes on neutralizing antibodies in either B.1.1.7 or B.1.351 infected polyclonal serum was assessed on 5 and 10 dpi. Here we used vero76 and three virus isolates hCoV-19/Canada/ON/VIDO-01/2020 (referring as VIDO-01 isolate and 100% sequence similarity with Wuhan-hu-1 isolate), B.1.1.7 & B.1.351 containing wild type spike or N501Y mutations (B.1.1.7), or N417T, E484K & N501Y (B.1.351) in the receptor binding domain. When performed cross-neutralization assay with B.1.1.7 infected hamster serum against VIDO-01 isolate no difference in neutralizing activity was observed on day 5 post-infection (Figure 4.28). The cross-neutralization activity was decreased by 4-fold against B.1.351 on day 5 dpi when tested with serum isolated from B.1.1.7 infected hamsters (Figure 4.28). Further, serum neutralizing antibody response on 10 dpi, the cross-neutralizing activity against VIDO-01 was decreased 2-3-fold (p=0.020). Notably, the cross-neutralizing activity against B.1.351 was reduced 7-fold on 10 dpi (p=<0.001) when assessed with serum isolated from B.1.1.7 infected hamsters (Figure 4.28). Furthermore, we performed similar cross-neutralization assay using serum infected from B.1.351 hamsters on day 5 against VIDO-01 isolate or B.1.1.7 and B.1.351 virus isolates (Figure 4.29).

On day 5, B.1.351 reactive antibodies did not show any fold change in cross-neutralization against B.1.1.7 or VIDO-01 isolate on 5 dpi (Figure 4.29). However, the overall neutralizing antibody response was lower in B.1.1.7 or B.1.351 infected hamsters, this may be due to low antibody response on 5 dpi in hamster model of infection (Figure 4.29). The cross-neutralization and fold change against VIDO-01 and B.1.1.7 was marked even at 5 dpi when tested with B.1.351 infected hamsters (p=0.009 and p=<0.001 respectively). Furthermore, on day 10 serum samples from B.1.351 infected hamsters showed a 4-fold reduction in cross-neutralization titer against VIDO-01 isolate (p<0.0001) (Figure 4.29). Notably, these findings were consistent with 4-fold decrease against B.1.1.7 isolates when tested with serum from B.1.351 infected hamsters (p<0.0001) (Figure 4.29). Here we observed the overall neutralizing titers on hamster infected with B.1.351 were consistently lower in all assays compared to B.1.1.7 infected hamster serum samples (Figure 4.28 & Figure 4.29).
Figure 4.28: Neutralizing antibody against VIDO-01, B.1.1.7 and B.1.351 following B.1.1.7 infection in hamsters.

Hamsters were infected with B.1.1.7 and serum collected on 5 and 10 dpi was screened for cross-reactivity against VIDO-01, B.1.1.7 or B.1.351. Neutralization activity against B.1.1.7 and cross neutralization activity against VIDO-01 and B.1.351 was measured by microneutralization assay. The dotted line indicating limit of detection (LOD). The neutralizing activity was calculated as a fold change by running two-way repeated measures p= *0.05, **p=0.005 ***p=0.001 ****p=<0.001.
Figure 4.29: Neutralizing antibody against VIDO-01, B.1.1.7 and B.1.351 following B.1.351 infection in hamsters.

Hamsters were infected with B.1.351 and serum collected on 5 or 10 dpi was screened for cross-reactivity against VIDO-01, B.1.1.7 or B.1.351. following B.1.351 infection. Neutralization activity against B.1.351 and cross neutralization activity against VIDO-01 and B.1.1.7 was measured by microneutralization assay. The dotted line indicates limit of detection (LOD). The neutralizing activity was calculated as a fold change by running two-way repeated measures Anova p= *0.05, **p=0.005 ***p=0.001 ****p=<0.001.
4.5 Vaccination with an ancestral S1 vaccines leads to reduced neutralization and protection against VOC challenge in hamsters.

In the last study, the reduced activity of neutralizing and cross-neutralizing antibodies was determined following experimental infection with B.1.1.7 and B.1.351 in a hamster model. All of the currently approved vaccines are based on the spike sequences from the original Wuhan-Hu-1 strain, which was circulating in the population in early 2020. The emergence of VOCs with enhanced transmissibility and in some cases increased resistance to neutralization was highly concerning. Given these concerns, the neutralizing antibody response induced following vaccination with S1 subunit vaccine formulated with TriAdj (COVAC-1) or SWE (COVAC-2) was evaluated against B.1.1.7 and B.1.351 in hamsters. This vaccine is also based on the S1 protein sequence from the original Wuhan-Hu-1 strain of SARS-CoV-2.

4.5.1 The IgG response induced by COVAC-1 or COVAC-2 vaccination and followed by B.1.1.7 challenge in hamsters.

To determine the effect of mutations acquired in the spike protein of two VOCs on the protective efficacy of a subunit vaccine, hamsters were immunized with two doses, 28-days apart of S1+TriAdj (COVAC-1) or S1+SWE (COVAC-2) and then challenged 28 days following the second dose with the B.1.1.7. The S1 protein used in vaccination was purified from mammalian expression system and showed 100% sequence identity to Wuhan SARS-CoV-2 isolate. Serum samples were collected on days 0, 28- and 48-days post-vaccination as well as on days 5 and 10 post-challenge (dpc). The binding IgG response was measured against Wuhan S1 and Wuhan RBD by ELISA following vaccination and challenge. All vaccinated hamsters exhibited minimal binding titers against Wuhan S1 as early as 28 days post-vaccination and increased following second vaccination on day 48 post-vaccination. Notably, the anti-S1 IgG response was 1-2 log higher than in COVAC-2 group compared to COVAC-1 group on 48 days post-vaccination (Figure 4.30A). The anti-Wuhan RBD IgG titers were detected at day 28 post-vaccination in COVAC-2 vaccinated hamsters while COVAC-1 immunization did not induce anti-RBD IgG antibodies on day 28 post-vaccination. Following second vaccination on day 48, the anti-RBD IgG titers were increased in COVAC-2 vaccinated hamsters and now anti-RBD IgG titers were detected in COVAC-1 vaccinated hamsters (Figure 4.30B).
Figure 4.30: Quantification of S1 and RBD binding antibodies after COVAC-1 and COVAC-2 vaccination following B.1.1.7 challenge in hamsters.

To determine the levels of S1 and RBD-specific antibodies, serum samples were collected at day 0, 28 and 48 from hamsters immunized with COVAC-1 or COVAC-2 and at day 53, day 58 post vaccination (5 and 10 dpc) with B.1.1.7. 4.30A) Anti-S1” Wuhan” ELISA and 4.30B) Anti-RBD “Wuhan” ELISA was performed on serum samples collected and optical density was measured at 450nm. The horizontal dotted line indicating limit of detection (LOD) and vertical dotted line separating vaccination timepoints from days post-challenge. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
Figure 4.31: Neutralizing antibody response induced from COVAC-1, COVAC-2 vaccination and following B.1.1.7 challenge in hamsters.

Neutralizing titers against the VIDO-01 isolate was determined by using microneutralization assay with serum collected from hamster immunized with COVAC-1 and COVAC-2 (48 days post-vaccination) and challenged with B.1.1.7 (53- and 56-days post vaccination) at 5 dpc and 10 dpc. The horizontal dotted line indicating limit of detection (LOD) and vertical dotted line separating vaccination timepoints from days post challenge. Statistical significance Two-way Anova ***p=0.001.
Although the COVAC-2 vaccinated hamsters had significantly higher levels of anti-RBD responses compared to COVAC-1 immunized hamsters on day 48 post-vaccination (Repeated measures mixed effect model; \( p<0.001 \)). Furthermore, following challenge with B.1.1.7, the anti-Wuhan RBD as well as anti-Wuhan S1 IgG response did not show significant increases in titer at day 5 as well as day 10 post-challenge in COVAC-2 vaccinated group. In contrast, the COVAC-1 vaccinated hamsters showed 1 log increase in anti-RBD titers at day 58 (day 10 post-challenge). The COVAC-1 (Repeated measures mixed effect model; when day 53 \( p=0.01 \) and day 58 \( p=0.05 \)) as well as COVAC-2 (day 53, \( p=0.05 \) and day 58 \( p<0.001 \)) vaccination had significantly higher anti-S1 titers as well as anti-RBD (COVAC-1 vs PBS control day 53 and day 58, \( p=0.05 \); COVAC-2 vs PBS control day 53 \( p=0.05 \), day 58 \( p<0.001 \)) titers on day 53 as well as day 58 post-vaccination compared to PBS control (Figure 4.30B).

Furthermore, the neutralizing activity of antibodies induced from vaccination as well as following challenge was determined against as VIDO-01 isolate (Figure 4.31). Prior to challenge (day 48 postvaccination) both vaccines induced very low to no neutralizing antibodies (titers of 1:20 or 1:32), which rapidly increased in comparison to control hamsters, following B.1.1.7 challenge reaching 1:128 (COVAC-1) and 1:256 (COVAC-2). However, the difference between PBS control and vaccinated groups was not significant at day 53 post-vaccination (5dpc). In comparison, this difference was more prominent at 10 dpc with increase in the neutralizing antibodies (titer of 1:512) in COVAC-2 vaccination was higher than PBS control (titer of 1:128) \( (p=0.006) \). The COVAC-1 group also induced increase in neutralizing antibody responses at 10 dpc however, the statistical significance was not established compared to PBS control (Figure 4.31).
4.5.2 The IgG response from vaccination COVAC-1 or COVAC-2 vaccination followed by B.1.351 challenge in hamsters.

In a separate study, hamsters were vaccinated with two-doses, 28 days apart, of COVAC-1 and COVAC-2 and then challenged with B.1.351 on day 48 post-vaccination. The anti-Wuhan S1 and anti-Wuhan RBD binding IgG response was measured by ELISA following vaccination and challenge (Figure 4.32 A&B). Both vaccines induced a low anti-S1 antibody response to minimal levels at day 28 post-vaccination with a 1-2 log increase following the second vaccination dose on day 48 (Figure 4.32A). In comparison, the anti-RBD IgG was detected as early as day 48 post-vaccination. The anti-RBD IgG titer was significantly higher in COVAC-2 immunized hamsters compared to COVAC-1 immunized hamsters on day 48 post-vaccination (p=0.001) (Figure 4.32B).

Furthermore, the challenge of hamsters with B.1.351 vaccinated with COVAC-1 or COVAC-2 did not induce increase in anti-Wuhan S1 as well as anti-Wuhan RBD response and maintained at a similar level reported prior to challenge. However, the COVAC-2 vaccinated hamsters had significantly high anti-Wuhan S1 (p=0.05) and anti-Wuhan RBD (day 53 p=0.05 and day 58 p=<0.001) than PBS control on 5 as well as 10 dpc. Also, the COVAC-1 as well as COVAC-2 vaccination showed significant differences in anti-RBD titers on day 10 dpc (p=0.01). Furthermore, the neutralizing antibody response following vaccination and B.1.351 challenge was measured against the VIDO-01 isolate. Prior to challenge at day 48 post-vaccination, COVAC-1 and COVAC-2 immunization induced minimal to no neutralizing antibodies (1:20 or 1:32 respectively), with minimal increase on 5dpc following challenge compared to PBS control (1:32 and 1:64 respectively). However, the differences were not significant on 5 dpc as well as 10 dpc between the vaccinated and in PBS control groups with mean titer of 1:128 in all 3 groups following challenge with B.1.351 (Figure 4.33).

4.5.3 COVAC-2 was more effective than COVAC-1 in reducing levels of virus in the lungs following infection with either B.1.1.7 or B.1.351 in hamsters.

At day 5 and 10 post-challenge, lung tissues were collected from vaccinated and challenged hamsters with B.1.1.7 and B.1.351 to detect viral RNA as well as infectious viral titers. Higher viral RNA copies were detected in lungs of PBS control (10^5 copies/mg) hamsters compared to the viral copies in COVAC-1 (10^4 copies/mg) as well as COVAC-2 (10^3 -10^2 copies/mg of tissues) in hamsters on 5 dpc with B.1.1.7 challenge (Figure 4.34).
Figure 4.32: Quantification of S1 and RBD binding antibodies after COVAC-1 and COVAC-2 vaccination following B.1.351 challenge in hamsters.

To detect circulating IgG antibodies, serum samples were collected at day0, 28 and 48 from hamsters immunized with COVAC-1 and COVAC-2 and at day 53, day 58 post challenged (Day 5 and day 10 post-challenge) with B.1.351. 4.32A) Anti-S1” Wuhan” ELISA 4.32B) Anti-RBD “Wuhan” ELISA. Optical density was measured at 450nm. The vertical dotted line separating vaccinated from challenge timepoints. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
Figure 4.33: Antibody response in COVAC-1, COVAC-2 vaccinated and B.1.351 challenge in hamsters.

Neutralizing titers against the VIDO-01 isolate was determined by using microneutralization assay with serum collected from hamster immunized with COVAC-1 and COVAC-2 (48 days post-vaccination) and challenged with B.1.351 (53- and 56-days post vaccination) at 5 dpc and 10 dpc. The horizontal dotted line indicating limit of detection (LOD) and vertical dotted line separating vaccination timepoints from days post challenge. Statistical significance Two-way Anova when p=0.05.
COVAC-2 vaccination resulted in a significant reduction of viral RNA copies in the lungs on 5 dpc in B.1.1.7 challenged hamsters compared to the PBS control group (right middle p=0.05, right bottom p=0.05 and right accessory p=0.01) (Figure 4.34A). Similarly, COVAC-1 vaccination resulted in a significant reduction in viral RNA in the lungs of B.1.1.7 challenged hamsters compared to PBS control on 5 dpc (right middle p=0.05, right bottom p=0.05 and right accessory p=0.01). On 10 dpc the viral RNA in lung lobes of all hamsters challenged with the B.1.1.7 were overall low. Further analysis of infectious virus in the B.1.1.7 challenged hamsters showed a significant reduction in COVAC-2 vaccinated hamsters compared to the PBS control group on 5 dpc (right top p= <0.001, right middle p=<0.001, right bottom p=0.05 and right accessory p=0.01) (Figure 4.34C). On 10 dpc no infectious virus was detected in any of the hamsters challenged with the B.1.1.7 isolate.

In contrast to the B.1.1.7 challenge, COVAC-1 vaccination did not reduce viral RNA copies following challenge with B.1.351 on 5 dpc when compared to the PBS control group (Figure 4.34A). Similarly, COVAC-2 vaccination reduced viral RNA in only one lung lobe compared to the PBS control (right bottom p=0.01. In comparison, on 10dpc the viral RNA in the lungs of all hamster lungs were overall low, indicative of virus being cleared in both vaccinations and the PBS control hamsters in the B.1.351 challenge study (Figure 4.34A&Figure 4.35B). Furthermore, detection of infectious virus following the B.1.351 challenge in hamsters showed a reduction in only 1 lung lobe of COVAC-2 vaccinated hamsters compared to the PBS control (right top p=0.01) (Figure 4.34C). The other lung lobes collected from COVAC-1 as well as COVAC-2 vaccinated hamsters did not show reduction infectious virus titers following B.1.351 challenge on 5 dpc. On 10 dpc no infectious virus was detected in any of the lung lobes in hamsters challenged with B.1.351.
Figure 4.34: Tissue viral load and infectious virus in hamsters challenge with B.1.1.7.

4 hamsters from each group were euthanized either at 5 dpc or 10 dpc and respiratory tissues were collected to detect viral RNA and infectious virus. 4.34A) Viral RNA in tissues at day 5 from hamsters challenged with B.1.1.7 4.34B) Viral RNA in tissues at day 10 from hamsters challenge with B.1.1.7. 4.34C) Infectious virus titer in tissues collected at day 5 from B.1.1.7 challenged hamsters. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
Figure 4.35: Tissue viral load and infectious virus in hamsters challenge with B.1.351.

4 hamsters from each group were euthanized either at 5 dpc or 10 dpc and respiratory tissues were collected to detect viral RNA and infectious virus titer. 4.35A) Viral RNA in tissues at 5 dpc from hamsters challenge with B.1.351. 4.35B) Viral RNA in tissues at day 10 from hamsters challenge with B.351. 4.35C). Infectious virus titer in tissues collected at 5 dpc from B.1.351 challenged hamsters. Statistical significance Two-way annova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
4.5.4 Reduced neutralizing antibody response in COVAC-1 or COVAC-2 immunized hamsters against B.1.351 challenge.

Neutralizing antibody response against homologous (challenge) virus were analyzed on day 48, 53 (5 dpc) and day 58 (10 dpc) post-vaccination in B.1.1.7 as well B.1.351 challenged hamsters. The neutralizing antibody response was not detected at 48 days post-vaccination. The rapid increase in neutralizing antibody titers were observed in COVAC-2 vaccinated hamsters on day 5 post-B.1.1.7 challenge compared to COVAC-1 (titer of 1:128 and 1:64 respectively, p=0.05) as well PBS control (titer of 1:128 and 1:20, p=0.01) (Figure 4.36A). Similarly, the overall neutralizing antibody response was higher in COVAC-2 vaccinated hamsters compared to COVAC-1 immunized hamsters (1: 512 and 1:256 respectively, p=0.01) and PBS control (1:512 and 1:128 respectively p=<0.001) at 10 dpc with B.1.1.7 challenge. In contrast, the neutralizing antibody response was comparatively lower in B.1.351 challenged hamsters and were similar between groups with a titer of (1:32 on 5 dpc and 1:128 on 10 dpc). The significant difference between COVAC-1, COVAC-2 and PBS control was not observed on 5dpc as well as 10dpc in B.1.351 challenged hamsters (Figure 4.36B).
Figure 4.36: Neutralizing antibody response against homologous virus in hamsters vaccinated with either COVAC-1 or COVAC-2 and subsequently challenged with B.1.1.7 or B.1.351.

Serum samples collected at day 48, 53 (5dpc) and 58(10dpc) from hamsters challenged with 4.36A) B.1.1.7 were incubated with B.1.1.7 and neutralizing titer was determined. 4.36B) Serum samples collected at day 48, 53 (5 dpc) and 58 (10 dpc) from hamsters challenged with B.1.351 were incubated with B.1.351 and neutralizing titer was determined. Vertical dotted line separating vaccinated from challenge timepoints. Horizontal dotted line indicating limit of detection. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
4.5.4 Clinical and pathological changes in COVAC-1 and COVAC-2 vaccinated hamsters challenged with B.1.1.7 and B.1.351.

Histopathological analysis was performed on the left lung lobe collected at 5 dpc and 10 dpc from hamsters vaccinated and challenged with B.1.1.7 or B.1.351. The PBS control hamsters challenged with B.1.1.7 or B.1.351 underwent histopathological changes primarily marked by bronchiolitis indicated by hyperplasia, epithelial cell necrosis, interstitial pneumonia with the presence of pneumocytes and in some cases alveolar hemorrhages on 5 dpc (Figure 4.37A & Figure 4.38 A). In comparison, at 5 dpc, the overall pathological changes for the COVAC-1 and COVAC-2 groups were decreased with reduction in interstitial pneumonia as well as in immune cell infiltrate and absence of alveolar hemorrhages upon challenge with B.1.1.7 (Figure 4.37 B&C).

Overall pathological alterations were more severe upon vaccination and B.1.351 challenge hamsters compared to B.1.1.7 with severe pneumonia indicated by pneumocytes, macrophage infiltration and alveolar hemorrhages on 5 dpc. The COVAC-2 immunized hamsters showed minimal reduction of pathological alteration (reduced bronchiolitis and interstitial pneumonia) on day 5 dpc compared to PBS control and COVAC-1 vaccinated hamsters when challenged with B.1.351 (Figure 4.38 C). At 10 dpc all hamsters showed similar histopathological alterations and reduced signs of pneumonia in vaccinated as well as PBS control in B.1.1.7 while the recovery was delayed in B.1.351 challenged hamsters.

Furthermore, the immunohistochemistry analysis was performed on the left lung lobe to detect the presence of SARS-CoV-2 nucleocapsid (N) protein. The lung lobes from PBS control and COVAC-1 immunized hamsters lung lobes showed the presence of N protein at 5 dpc in B.1.1.7 challenged hamsters, Figure 4.39 & Figure 4.40), while the COVAC-2 vaccinated hamsters showed no N protein in the lungs of B.1.1.7 challenged hamsters. At 10 dpc the presence of N protein was not detected in B.1.1.7 challenged hamsters. Overall, the intensity of N protein was marked in B.1.351 challenged hamsters compared to B.1.1.7 challenged animals. The lung lobes collected from PBS control; COVAC-1 vaccinated hamsters showed presence of N protein in B.1.351 challenged hamsters on 5 dpc. The COVAC-2 vaccinated hamsters showed no to minimal presence of N protein on 5 dpc following B.1.351 challenge. At 10 dpc the presence of N protein was not detected in B.1.351 challenged hamsters.
Changes in body weight were monitored through weight loss changes throughout the course of challenge. At 6 dpc, the mean of % body weight loss peaked and was approximately 10.6% for the PBS control, 6.4% for the COVAC-1 and 3.9% for the COVAC-2 vaccine groups (Figure 4.41C). Noticeably, the vaccine groups recovered body weights at 10 dpc while the PBS control still had a 5.1% body weight loss at 10 dpc. Over the duration of the study there were significant differences observed amongst the vaccine groups and PBS control with respect to percent body weight change (Repeated Measures Mixed Effects Model Anova; p=0.0038). In comparison, the hamsters challenged with B.1.351 showed peak weight loss with at 6dpc approximately 13.1% for PBS control, 10.1 % for COVAC-1 and 10.8% for COVAC-2 vaccine groups (Figure 4.41B). Over the duration of the study none of the hamsters completely returned to normal by 10dpc.

The lung: body weight ratio in healthy hamsters is ~0.5 -0.6 %. Higher lung: body weight ratio is indicated by fluid accumulation in lung lobes of hamsters. The differences in lung: body weight ratio at 5 dpc the COVAC-2 measured around 0.6 % while COVAC-1 and COVAC-2 were had lung: body weight ratio around 1.0 following B.1.1.7 challenge which was returned to the lung: body ratio of 0.6% by 10 dpc (Figure 4.41B). In contrast, the lung: body weight ratio in hamsters challenged with B.1.351 did not show any difference among the vaccinated and PBS control groups. The lung: body weight ratio and measured around 0.8- 1 on 5 dpc as well as at 10 dpc in COVAC-1, COVAC-2 immunized hamsters and in PBS control (Figure 4.41D).
Figure 4.37: H&E staining of the left lung lobe from hamsters vaccinated with COVAC-1 or COVAC-2 and subsequently challenged with B.1.1.7.

The hematoxylin and Eosin staining was performed on 4% Formalin fixed tissues. The scoring of lung pathology was based on presence of pathology. 4.37A) Left lungs harvested from PBS control hamsters. Histology score: 3. 4.37B) Lung lobe from COVAC-1 vaccinated and B.1.1.7 challenged hamsters with score: 2. 4.37C) Left lung lobe harvested from COVAC-2 vaccinated and B.1.1.7 challenged hamsters with score: 2. All lung lobes showed presence of pneumocytes, inflammatory cells infiltration and Signs of alveolar hemorrhages indicated by black arrow and blue arrow respectively.
Figure 4.38: H&E staining of left lung lobe in hamsters vaccinated with COVAC-1 or COVAC-2 and subsequently challenged with B.1.351.

The hematoxylin and Eosin staining was performed on 4%Formalin fixed tissues. The scoring of lung pathology was based on presence of pathology. 4.38A) Left lungs harvested from PBS control hamsters. Histology score: 4. 4.38B) Lung lobe from COVAC-1 vaccinated and B.1.351 challenged hamsters with score: 3. 4.38C) Left lung lobe harvested from COVAC-2 vaccinated and B.1.351 challenged hamsters with score: 2. All lung lobes showed presence of pneumocytes, inflammatory cells infiltration and Signs of alveolar hemorrhages indicated by black arrow and blue arrow respectively.
Figure 4.39: Immunohistochemistry analysis on lungs following B.1.1.7 challenge in hamsters.

Lung tissues from B.1.1.7 challenged animals collected on 5dpc were stained for the nucleocapsid protein (N) of SARS-CoV-2. 4.39A) IHC on lung lobes collected from hamsters in PBS control challenged with B.1.1.7 group 4.39B) IHC on lung lobes from COVAC-1 vaccinated and B.1.1.7 challenged hamsters. 4.39C) IHC on lung lobes from COVAC-2 vaccinated and B.1.1.7 challenged hamsters. The N protein is indicated by black arrow.
Figure 4.40: Immunohistochemistry analysis on lungs following B.1.351 challenge in hamsters.

Lung tissues from B.1.351 challenged animals collected on 5dpc were stained for the nucleocapsid protein (N) of SARS-CoV-2. 4.39A) IHC on lung lobes collected from hamsters in PBS control challenged with B.1.351 group. 4.39B) IHC on lung lobes from COVAC-1 vaccinated and B.1.351 challenged hamsters. 4.39C) IHC on lung lobes from COVAC-2 vaccinated and B.1.351 challenged hamsters. The N protein is indicated by black arrow.
Figure 4.41: Weight loss in hamsters following immunization with COVAC-1 or COVAC-2 and subsequent B.1.1.7 or B.1.351 challenge.

Percent weight loss was calculated from the initial day weight for each hamster. 4.41A) Hamsters challenged with B.1.1.7, 4.41C) and hamsters challenged with B.1.351 were weighted daily. Each dot representing geometrical mean and error bars representing standard deviation. The horizontal dotted line indicating weight of hamsters on the first day of study and vertical line indicating statistical significance when p=0.05. 4.41B) the ratio of lung: body weight in hamsters challenged with B.1.1.7, 4.41D) ratio of lung: body weight in hamster with B.1.351 challenge. Each dot representing individual hamster and line indicating geometric mean.
4.6 Neutralizing monoclonal antibodies provide protection against SARS-CoV-2 in a hamster model.

Previously the protective role of neutralizing antibodies induced in infection as well as by vaccination was determined. The monoclonal antibody treatment is another subject that determined the perspective of neutralizing antibodies against SARS-CoV-2. Here two monoclonal antibodies that bind the RBD of SARS-CoV-2 were identified at the University of Pittsburgh from a large human phage-display library that was screened by panning against RBD of SARS-CoV-2. A high-affinity Fab region of IgG1 against SARS-CoV-2 RBD region was identified and subsequently engineered to full length IgG1 antibody (150kDa) by conjugating the human Fc portion, called Ab1.

Apart from full length IgG1, the antibody fragments such as variable region of the heavy chain (VH) (15kDa) are shown to exhibit strong antigen binding and high stability. To identify potent neutralizing activity of VH against SARS-CoV-2, the large library of (10¹¹ clones) and diverse phage-displayed human VH antibody library against recombinant SARS-CoV-2 RBD was panned. The VH binders were isolated with high affinities to RBD, competing with ACE-2 binding and for high stability. One of these binders, Ab8 was fused with human Fc region (of human IgG1) and VH Ab8-Fc was engineered (referred as Ab8).

4.6.1 The neutralizing activity of Ab1 against SARS-CoV-2 challenge in a hamster model.

The hamster model of SARS-CoV-2 infection was used to allow the evaluation of both the prophylactic and therapeutic efficacy of Ab1 against SARS-CoV-2 infection. The full length Ab1 was administered intraperitoneally (i.p) at a concentration of 10mg/kg, 24 hours prior to challenge with 1x10⁵ TCID₅₀/ml of SARS-CoV-2/Canada/ON/VIDO-001/2020 (sequence similarity with Wuhan-Hu-1 isolate). The control group of hamsters were only challenged with SARS-CoV-2 only. Upon challenge, the percent weight loss was calculated up to 5 days throughout the study (Figure 4.42A&B). The prophylactic administration of Ab1 showed reduction in weight loss by 5-6 % up to 2 dpc and returned to their original weight by 5 dpc (Figure 4.42A). In contrast, the untreated but challenged (SARS-CoV-2) (control) hamsters showed 15-20% weight loss throughout the study which was consistent with findings in previous hamster trials with SARS-CoV-2 challenge.
Clinical weight of each hamster was recorded daily, and percent weight loss was calculated. The percent weight loss in 4.42A) 10mg/kg of Ab1 prophylaxis group hamsters and 4.42B) in 3mg/kg as well as 10mg/kg of Ab1 treatment group hamsters. Each hamster was weighed first day prior to the challenge and the percent weight loss was calculated by taking on the first day of study and denoted it as 100%.

Figure 4.42: The percent weight loss following administration of Ab1 and SARS-CoV-2 infection in hamsters.
Further, in a separate study the two groups of hamsters were infected with \(1 \times 10^5\) TCID\(_{50}\)/ml of SARS-CoV-2 and treated intraperitoneally with 3mg/kg of Ab1 or 10mg/kg of Ab1 6 hours following infection (therapeutic administration). Hamsters therapeutically treated with Ab1 showed weight loss in a dose dependent manner with 10% weight loss in hamsters treated with 3mg/kg of Ab1 and 5% weight loss in 10mg/kg of Ab1 treated hamsters (Figure 4.42B). All hamsters in the treatment group were returned to their original weight on day 5 regardless of treatment dose. The control hamsters showed 15-20% weight loss throughout the study.

The infectious virus titer as well as viral RNA in lung lobes was recorded on day 5 post-challenge upon prophylactic as well as therapeutic administration of Ab1. In prophylaxis, all 5 lung lobes a 10- fold reduction in viral RNA was observed in four out of 5 hamsters when compared to the control group (Figure 4.43 A). Similarly, the infectious virus was significantly reduced to below the limit of detection in all lung lobes compared to control hamster lungs on day 5 post-challenge and prophylactic administration of Ab1 (right top, right bottom and accessory p= 0.05 and right middle and left lobe p=0.01) (Figure 4.43 A&B). Further, the therapeutic intraperitoneal administration of 10mg/kg of Ab1 resulted in a 1 -2 log decrease in viral RNA on 5 dpc compared to control group (Figure 4.43B).

These viral RNA levels were correlated with infectious virus titer in which showed reduction in a dose dependent manner where 10mg/kg administration significantly reduced infectious virus in all lung lobes compared to control hamster lungs (right middle, bottom, accessory p=0.05 and right top and left lobe p=0.01) (Figure 4.44B).
Figure 4.43: Viral load in hamster lung lobes following administration of Ab1 prophylactically or therapeutically.

The hamster lung lobes were harvested on day 5 post-challenge and subjected to viral RNA detection with RT-PCR. 4.43A) hamsters were prophylactically administered with 10mg/kg of Ab1 before challenge with 1x10^5 TCID_{50}/ml of SARS-CoV-2. Viral RNA levels were calculated from known standards and determined as TCID_{50} eq./mg of lung lobe. 4.43B) Following SARS-CoV-2 infection, 3mg/kg, or 10mg/kg of Ab1 was administered at 6 hours post-challenge. Viral RNA levels were calculated from known standards and determined as TCID_{50} eq./mg of lung lobe. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
4.44A)

![Graph showing TCID50/mg in different tissues for Ab1 prophylaxis (10mg/kg) and control.

4.44B)

![Graph showing TCID50/mg in different tissues for Ab1 (10mg/kg) and Ab1 (3mg/kg) and control.

Figure 4.44: Infectious virus in hamster lung lobes following administration of Ab1 prophylactically or therapeutically.

The hamster lung lobes were harvested on day 5 post-challenge and subjected to infectious virus detection. 4.44A) hamsters were prophylactically administered with 10mg/kg of Ab1 before challenge with 1x10⁵ TCID₅₀/ml of SARS-CoV-2. Infectious virus levels were determined as TCID₅₀/mg of lung lobe. 4.44B) Following SARS-CoV-2 infection, 3mg/kg, or 10mg/kg of Ab1 was administered at 6 hours post-infection. Infectious virus levels were determined as TCID₅₀/mg of lung lobe. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
The prophylactic as well as therapeutic administration of Ab1 was also resulted in reduced lung pathology and decreased viral antigen in the lung (Figure 4.45A-D). Hematoxylin and eosin (H&E) stain of lung tissues showed that Ab1 treatment remarkably decreased interstitial pneumonia marked by pulmonary congestion, alveolar septal thickening, and hyaline membrane formation caused by SARS-CoV-2 infection (Figure 4.45A-B). The H&E images were scored based on inflammatory cell infiltration and alveolar hemorrhage in infected lung lobes (clinical score 0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia). The Ab1 prophylactic and therapeutic administration reduced the interstitial pneumonia and monocytes and inflammatory cells infiltration (score= 1 to 2) compared to severe pneumonia in control lungs (score=3 to 4). In addition, the immunohistochemistry analysis (IHC) showed marked reduction in nucleocapsid positive alveolar epithelial cells upon prophylactic as well as therapeutic administration of Ab1 compared to control hamster lungs (Figure 4.46A-D).

Ab1 not only decreased viral burden in the hamster lung, but also reduced viral shedding in hamster nasal washes and oral swabs (Figure 4.47 A-E). In control hamsters, viral loads in nasal washes were higher than those in oral swabs, and viral shedding waned faster in oral swabs. Both prophylactic and therapeutic treatments with Ab1 decreased viral RNA and infectious viral titers in nasal washes and oral swabs at 3 and 5 dpi with the exception of viral RNA levels in nasal washes from the therapeutic group. The reduction at 1 dpi was not as significant as that at 3 and 5 dpi, likely due to the infection peak occurring before 3 dpi, as reported in hamsters. As expected, the prophylactic treatment decreased viral loads more effectively than the therapeutic treatment. Overall, the viral RNA decrease in hamster shedding was not as obvious as the decrease observed in the lung tissue, consistent with a recent finding in hamsters (Roberts, 2005; Sia et al., 2020; Tostanoski et al., 2020). The decreased viral shedding in the upper airways could potentially reduce transmission of SARS-CoV-2.
Figure 4.45: H&E staining on lung lobes following administration of Ab1 prophylaxis and treatment against SARS-CoV-2 in hamsters.

Left lung lobes were collected at day 5 post-infection from hamsters either prophylactically or therapeutically administered Ab1. 4.45A) H &E staining on lung lobes from hamsters prophylactically administration with Ab1. 4.45B) H&E staining on lung lobe from control hamsters in prophylactic study. 4.45C) H&E staining on lung lobes from hamsters treated with 10mg/kg of Ab1. 4.45 D) H&E staining on lung lobes from control hamsters in treatment study. All left lung lobes were fixed in 4% formalin and stained with hematoxylin and eosin to detect pathological changes upon treatment with Ab1. All lung lobes were scored based on the presence of inflammatory cell infiltration (black arrows), pneumocytes and, alveolar hemorrhages (black arrow).
Figure 4.46: Immunohistochemistry staining for nucleocapsid (N) protein of SARS-CoV-2 in lung lobes Ab1 administered in hamsters.

All formalin fixed and paraffin embedded lung lobes were stained with 1:800 of SARS-CoV-2 N specific antibody. 4.46A) IHC on lung tissues from hamsters prophylactically administered with Ab1. 4.46B) IHC on lung tissues from control hamsters in prophylaxis study. 4.46C) IHC on lung tissues from hamsters treated with 10mg/kg of Ab1. 4.46D) IHC on lung tissues from control hamsters in treatment study. The presence of N protein indicated by black arrow.
Figure 4.47: Viral load and infectious virus in nasal and oral swabs following Ab1 prophylaxis and treatment and SARS-CoV-2 infection.

Viral RNA and infectious virus titer were determined in nasal and oral swabs following Ab1 administration. 4.47A) Nasal RNA load in hamsters prophylactically administered with Ab1. 4.47B) Nasal RNA load in hamsters treated with Ab1. 4.47C) Infectious virus titer in hamsters prophylactically administered with Ab1. 4.47D) Infectious virus titer in hamsters treated with Ab1. 4.47E&F) Oral infectious titer in hamsters administered prophylactically (4.47 E) or treated (4.47F) with Ab1. Nasal and oral swabs were collected on day 1,3 and 5 post-infection.
4.6.2 High neutralizing antibody activity of Ab8 in a hamster model upon prophylaxis or therapeutic administration.

In the previous section the high neutralizing activity of full-length monoclonal antibody Ab1 was evaluated in a hamster model upon challenge with SARS-CoV-2. To evaluate the smaller fragment Ab8 and its neutralizing activity in hamsters, the administration by IP route either 24 hours prior to SARS-CoV-2 or 6 hours post-challenge (1x10^5 TCID_{50}/ml IN) was performed. The prophylactic administration (10mg/kg) of Ab8 reduced viral RNA by 1-2 logs in all lung lobes at day 5 post-challenge compared to control hamster lungs (right middle p=0.05, right accessory p=0.01) (Figure 4.48A&B). Similarly, the infectious virus levels were also decreased significantly in all lung lobes when compared to control hamster lungs on day 5 post-challenge and upon prophylactic administration of Ab8 antibody. The reduction in viral RNA levels in the lung lobes from the therapeutically treated groups was slightly lower (by 1.2 log) than after prophylactic administration of Ab8 (Figure 4.48A&C). These findings were correlated with infectious viral levels in lung lobes upon treatment with 10mg/kg of Ab8 and challenge with 1x10^5 TCID_{50}/ml compared to control group at day 5 post-infection. Interestingly, the lower dose at 3mg/kg of Ab8 also significantly reduced infectious virus levels in lung lobes compared to control hamster lungs (1-2 log) (p=0.05) (Figure 4.49 A&B). Further, consistent with Ab1, Ab8 also alleviated hamster pneumonia and reduced the viral nucleocapsid protein in the lungs (Figure 4.50A-F). The control hamsters exhibited severe interstitial pneumonia characterized by extensive inflammatory cell infiltration, presence of type II pneumocytes, alveolar septal thickening, and alveolar hemorrhage as seen in the previous studies (Figure 4.50A-B). Both prophylactic and therapeutic treatment of Ab8 reduced the lesions in alveolar epithelial cells as well as reduced focal hemorrhage, interstitial pneumonia, and inflammatory cell infiltration (Figure 4.50A-D) (score= 1 prophylaxis, 2 treatment and, control score=4). The immunohistochemistry analysis of N protein of SARS-CoV-2 also showed reduced staining in alveolar epithelial cells in prophylactic and treatment groups compared to control lungs lobes (Figure 4.50A-D).

Furthermore, the Ab8 also reduced the viral shedding from mucosal membranes including the nasal washes and oral swabs (Figure 4.51A-F).
4.48A) The hamster lung lobes were harvested on day 5 post-challenge and subjected to viral RNA detection with RT-PCR. 4.48A) hamsters were administered with 10 mg/kg of Ab8 before challenge with $1 \times 10^5$ TCID$_{50}$/ml of SARS-CoV-2. Viral RNA levels were calculated from known standards and determined as TCID$_{50}$ eq./mg of lung lobe.

4.48B) Following SARS-CoV-2 infection, 3 mg/kg, or 10 mg/kg of Ab8 was administered at 6 hours post-infection. Viral RNA levels were calculated from known standards and determined as TCID$_{50}$ eq./mg of lung lobe.

Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).

Figure 4.48: Viral load in hamster lung lobes following administration of Ab8 prophylactically or therapeutically.

The hamster lung lobes were harvested on day 5 post-challenge and subjected to viral RNA detection with RT-PCR. 4.48A) hamsters were administered with 10 mg/kg of Ab8 before challenge with $1 \times 10^5$ TCID$_{50}$/ml of SARS-CoV-2. Viral RNA levels were calculated from known standards and determined as TCID$_{50}$ eq./mg of lung lobe. 4.48B) Following SARS-CoV-2 infection, 3 mg/kg, or 10 mg/kg of Ab8 was administered at 6 hours post-infection. Viral RNA levels were calculated from known standards and determined as TCID$_{50}$ eq./mg of lung lobe. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
Figure 4.49: Infectious virus in hamster lung lobes upon administration of Ab8 prophylactically or therapeutically.

The hamster lung lobes were harvested on day 5 post-challenge and subjected to infectious virus detection 4.49A) hamsters were administered with 10mg/kg of Ab8 before challenge with 1x10^5 TCID_{50}/ml of SARS-CoV-2. Infectious virus levels were determined as TCID_{50}/mg of lung lobe. 4.49B) Following SARS-CoV-2 infection, 3mg/kg, or 10mg/kg of Ab8 was administered at 6 hours post-infection. Infectious virus levels were determined as TCID_{50}/mg of lung lobe. Statistical significance Two-way Anova *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001).
Figure 4.50: Pathology and histological analysis upon Ab8 prophylaxis and treatment and challenge with SARS-CoV-2.

Left lung lobes were collected at day 5 post-infection from hamsters either prophylactically or therapeutically administered Ab8. 4.50A) H&E staining on lung lobes from hamsters administered prophylactically with Ab8, 4.50B), H&E staining on lung lobe from control hamsters in prophylactic study. 4.50C) H&E staining on lung lobes from hamsters treated with 10mg/kg of Ab8. 4.50 D) H&E staining on lung lobes from control hamsters in treatment study. All left lung lobes were fixed in 4% formalin and stained with hematoxylin and eosin to detect pathological changes upon treatment with Ab8. All lung lobes were scored based on presence of inflammatory cells infiltration (black arrow), pneumocytes, alveolar hemorrhages (blue arrow).
Figure 4.51: Immunohistochemistry analysis for nucleocapsid (N) protein of SARS-CoV-2 in lung lobes of Ab8 administered in hamsters.

All formalin fixed and paraffin embedded lung lobes were stained with 1:800 of SARS-CoV-2 N specific antibody. 4.51A) IHC on lung tissues from hamsters prophylactically administered with Ab8. 4.51B) IHC on lung tissues from control hamsters in prophylaxis study. 4.51C) IHC on lung tissues from hamsters treated with 10mg/kg of Ab8. 4.51D) IHC on lung tissues from control hamsters in treatment study. The presence of N protein in lung lobes indicated by black arrow.
Viral RNA and infectious virus titer were determined in nasal and oral swabs following Ab1 prophylaxis and treatment and SARS-CoV-2 infection.

Viral RNA and infectious virus titer were determined in nasal and oral swabs following Ab1 administration. 4.52A) Nasal RNA load in hamsters prophylactically administered with Ab1. 4.52B) Nasal RNA load in hamsters treated with Ab8. 4.52C) Infectious virus titer in hamsters prophylactically administered with Ab8. 4.52D) Infectious virus titer in hamsters treated with Ab8. 52E&F) Oral infectious titer in hamsters administered prophylactically (4.52E) or treated (4.52F) with Ab8. Nasal and oral swabs were collected at day 1,3 and 5 post-infection. Statistical significance ** p=0.01, ***p=0.001.
The decrease in viral RNA in nasal washes and oral swabs were not as large as the decrease observed in the lung tissue, similar to a recent finding in hamsters. Overall, prophylactic treatment was more effective than therapeutic treatment in decreasing viral load in nasal washes and oral swabs (Figure 4.52 A-F). Notably, prophylactic administration of Ab8 effectively reduced the infectious virus in oral swabs at 1 dpi, while the post-exposure treatment did not (Figure 4.52 E-F). Interestingly, reduction in viral RNA as well as infectious virus (except the viral titer in the oral swab at 1 dpi) was more effective at 3 and 5 dpi compared to that at 1 dpi, likely due to the infection peak occurring before day 3 as reported in hamsters. A striking finding is that Ab8 given therapeutically at as low dose as 3 mg/kg can still decrease viral loads in the lung, nasal washes, and oral swabs (Figure 4.52 E&F).

4.6.3 Ab1 and Ab8 disseminate into the lung tissues and serum in hamsters upon administration:

The concentration of Ab8 and Ab1 were detected in hamster serum samples and lung lobes in following SARS-CoV-2 infection following Ab1 or Ab8 treatment. To detect both antibodies, serum samples were collected at day 1 and day 5 post-infection in both studies and subjected to ELISA. Upon detection of both antibodies using anti-human IgG antibodies, the concentration of Ab8 was significantly higher (40 µg/ml) than that of full length Ab1(26 µg/ml) at 1 dpi and 5 dpi in spite of same dose of treatment of monoclonal antibodies (10 mg/kg) (p=0.01). Interestingly, the Ab8 antibody (22 µg/ml) was detected at high concentration even at 5 dpi compared to no to low concentration of Ab1 in hamsters administered therapeutically (Figure 4.53 A). Following therapeutic administration, Ab8 and Ab1 was detected in lung lobe at 5 dpi using Tissue-ELISA. The Ab8 concentration in all hamster lung lobes was significantly higher in that of Ab1 antibody on 5 dpi (right top p=.05 (ns), right middle p=>0.05 (ns), right bottom p=0.05, right accessory p=0.001 and left lobe p=0.01) (Figure 4.53 B). This finding is consistent with the low concentration of Ab1 in serum samples at 5 dpi.
Figure 4.53: Comparison of Ab8 and Ab1 concentration following SARS-CoV-2 infection in the lungs and serum samples in hamsters.

Lung lobes were collected on 5 dpi and supernatant from lung homogenates were subjected to and S1 ELISA to determine the amount of either Ab1 or Ab8 in the lung lobes. 4.53A). Serum Ab1 and Ab8 levels in hamsters collected at 1 and 5 dpi following treatment. 4.53B) S1 ELISA on lung homogenate collected from hamsters following therapeutic administration at dose of 10mg/kg. The concentration of antibodies was determined by running known concentrations of purified Ab1 or Ab8 in the same assay. Statistical analysis- two-way Anova analysis followed by Tukey test, Statistical significance *p=0.05, **p=0.01, ***p=0.001 and ****p=<0.001.
5 Discussion:

Current evidence for respiratory coronaviruses supports the hypothesis that neutralizing antibodies raised as a result of either infection and vaccination constitute at least part of the protective mechanisms that prevent pathology and clinical disease (Mercado et al., 2020a, 2020b). Both MERS-CoV and SARS-CoV-2 contain a surface glycoprotein that protrudes from the surface of virus particles and engages with the host cell receptor to facilitate virus entry (Du et al., 2017b; Li, 2016). Although the two spike glycoproteins of MERS-CoV and SARS-CoV-2 have low sequence similarities (50.8%), the antibody response is comparable following infection in humans (Ansariniya et al., 2021). Current evidence suggests that the majority of the humoral response in SARS-CoV-2 targets the spike protein which positively correlates with neutralizing antibody titers (Du et al., 2017b; Konwarh, 2020). However, it is unknown if neutralizing antibody responses induced in infection and by vaccination are correlated with protection against MERS-CoV and SARS-CoV-2.

This study contributed to the understanding of the IgG response and its neutralizing activity in infection, vaccination, and when administered therapeutically were correlated with reduced viral load in two animal models against MERS-CoV and SARS-CoV-2.

5.1 Alpacas are protected from re-infection upon induction of IgG1 and IgG3 following prior infection of MERS-CoV.

The transmission of MERS-CoV occurs sporadically as a result of close contact with infected patients, primarily in healthcare settings and in limited cases in household settings (Barry et al., 2020; Hui et al., 2018; Ki, 2015b; Omrani et al., 2013; Oraby et al., 2020). Apart from human-to-human transmission, about 54% of human cases occurs after the exposure to infected Dromedary camels (Alagaili, 2014; Conzade et al., 2018; Bart L. Haagmans et al., 2014; Meyer et al., 2016b; Miguel et al., 2017). Currently there are no approved vaccines or therapies available against MERS-CoV. Several challenges make development of a MERS-CoV vaccine for human challenging. This includes the long duration required for pre-clinical and clinical testing and licensing of human vaccine candidates, the small number of cases and deaths to date and selection of target population for vaccination; for example, should all people in the Middle East be vaccinated, travelers to areas where MERS-CoV is predominated or should vaccination be directed to camel handlers or healthcare workers. Additionally, the older population with co-morbidities, which tend to be at the highest risk of poor outcomes also tend to be in some cases poor responders
to vaccinations. In addition, some vaccine candidates in pre-clinical trials have documented possibilities of enhanced disease as a result of vaccination against SARS-CoV (Jaume et al., 2012; Kam et al., 2007; Luo et al., 2018; Wang et al., 2014). Given the central role in MERS-CoV transmission that dromedary camels play as one of the major reservoirs, strategies disrupting the transmission cycle from camels to humans may serve as an effective means of preventing human MERS-CoV outbreaks. A similar strategy has been implemented successfully in Australia where a vaccine used in horses against Hendra virus has largely stopped its transmission to humans and also protects horses from the disease (Middleton et al., 2014).

Several efforts have been made to develop a MERS-CoV vaccine in camels; however, experiments with virus challenge generally requires containment level 3 facilities and limited immunological reagents make it difficult to study the efficacy of vaccines in camels. Thus, additional animal models are necessary which can recapitulate what is observed in camel to allow better understanding of MERS-CoV infection in camels. Current data of on MERS-CoV infections of alpacas finds that they largely mimic what is observed in camels – namely that virus predominantly infects the upper respiratory tract, and that no disease is observed (Adney et al., 2019; Adney et al., 2016; Crameri et al., 2016). The greater availability in North America and comparably smaller size make the alpacas model highly suited to study MERS-CoV infection and vaccination in containment level 3 labs. The current knowledge regarding the humoral response following MERS-CoV infection in camels and alpacas is limited to detection of antibodies and characterization of these immunoglobulins is lacking (Adney et al., 2019; Crameri et al., 2016). Both new-world camelids (guanacos, vicunas, alpacas, and llamas) and old-world camelids (dromedary and Bactrian camels) possess unique heavy chain antibodies – IgG2 and IgG3 (Hamers-Casterman et al., 1993). Several reports have been documented the extraordinary neutralizing activities of these heavy chain antibodies against antigens in terms of therapeutic usage; however only limited data is available on the activities of these antibodies in viral infections. Here, the alpacas that had experienced a prior experimental infection to MERS-CoV were protected against re-infection.

The initial infection in alpacas regardless of whether inoculation was via IT, IN or IT/IN route resulted in detection of infectious virus in nasal secretions and a low quantity of viral RNA in the oral cavity. The lower level of viral RNA in oral swabs maybe a result of drainage of virus from the nasal to the oral cavity. The initial infection with MERS-CoV resulted in clinically benign
disease and no signs of nasal discharge or illness were observed. These findings were consistent with previous reports in alpacas experimentally challenged with MERS-CoV (Adney et al., 2019; Adney et al., 2016; Alharbi et al., 2020a; Crameri et al., 2016). These findings were further supported by histopathological analysis on day 28 where there was no detection of signs of viral infection in IT and IN immunized alpacas. Following a primary MERS-CoV infection a total IgG response of similar magnitude was induced in all alpacas, regardless of the route of inoculation, as early as day 14 post-infection. This response included neutralizing antibodies as well as binding antibodies detected in ELISA. These observations were correlated with a clearance of virus from nasal washes by day 14 post-infection in all alpacas. Interestingly, the delayed peak viral load in nasal washes and the delayed clearance of virus in the nasal washes of alpacas inoculated via the IT route, did not appear to affect the timing of induction of IgG binding and neutralizing antibodies against MERS-CoV. Re-infection of alpacas 61 days following the initial infection with IT/IN route showed reduction in viral load. Very low levels of viral RNA were detected up to day 4 post-reinfection, while infectious virus was not recovered from nasal washes as well as oral swabs supporting that, alpacas were protected from subsequent infection. The previous reports regarding MERS-CoV infection and re-infection of alpacas have indicated that the induction of anti-S1 specific IgG response following the initial exposure protects alpacas from re-infection (Crameri et al., 2016). These findings were consistent with our data where the induction of IgG response from initial infection may protected alpacas against re-infection of MERS-CoV.

The IgG response was further characterized to determine what IgG isotypes were induced and whether the neutralizing activity was isotype dependent following infection and re-infection in alpacas. Following initial infection with MERS-CoV, both IgG1 and IgG3 spike-specific binding antibodies were detected, with a notable absence of IgG2 antibodies. Following re-infection, the amount of S1-binding IgG1 and IgG3 increased, while S1-binding IgG2 was now detectable, although it remained comparably low. Given the high challenge dose, certainly a high level of protection was conferred against experimental reinfection and given this data it would appear that protection is not mediated by IgG2 and rather may be a reliant on IgG1 and IgG3 isotypes. To determine whether the IgG response varies by isotype, we purified each IgG isotype from serum samples collected at day 21 post-infection and day 21-post-reinfection from alpacas inoculated with MERS-CoV. The neutralization antibody response was comparable between IgG1 and IgG3 at day 21 post-infection and day 21-postreinfection when studied separately. The lower
neutralizing activity of purified IgG2 was only detected at day 21-post reinfection which was consistent with levels detected in ELISA. The lack of IgG2 response from the primary infection in alpacas raises the interesting possibility that IgG2 could be used as a maker of re-infection in dromedary camels which should also indicate the similar IgG isotype response occur in camelids. Currently, this can be only studied through a combination of strategy and repeated sampling—a process that is labor intensive, expensive, and likely to miss re-infections.

In conventional IgG isotypes the distant combinations of complementary determining region (CDR) loops result in different binding site topologies (Polonelli et al., 2008; Sela-Culang et al., 2013). In the absence of light chain, an extended CDR3 on the heavy chain forms a finger like elongations to access pockets on the target antigens thereby increasing the binding repertoire of the antibody beyond that of a normal full length IgG1 (Antonacci et al., 2011; Nguyen et al., 2000, 1999). When affinities of the IgG isotypes were assessed by SPR, no significant difference was observed between the mean KD of purified IgG1 and IgG3, regardless of the timepoint analyzed – 21 days post-infection or 21 days post-re-infection. The KD is the equilibrium dissociation constant defining a ratio between association (ka) and dissociation(kd) of a ligand (antigen) and analyte (antibody) in given time. The KD and affinity are inversely related to each other and the KD value relates to the binding kinetics of antibody thus, a lower KD value indicates that an antibody has higher affinity for its antigen and that the less antibody is needed to interact with antigen in a given time (Hearty et al., 2010). However, the IgG1 and IgG3 showed comparable against MERS-CoV infection. As expected, purified IgG2 from initial infection was not found to interact with S1 when assayed by SPR, consistent with a lack of binding to S1 in the isotype specific ELISA. Following re-infection, IgG2 still was not observed to interact with S1 by SPR despite a low level of binding being observed in the IgG2 isotype ELISA. This discrepancy may be due to differences in the sensitivity of two assays (Lofgren et al., 2007). In conclusion, here it was demonstrated that alpacas were protected from subsequent re-infection with MERS-CoV in the presence a robust IgG response, that also includes neutralizing antibodies. The MERS-CoV specific IgG response was composed of IgG1 and IgG3 with no evidence of IgG2, which may require multiple exposures for its induction. Furthermore, the mucosal immunity is primarily mediated by IgA antibody, however the detection of IgG in serum samples does raise a question on how the protection mechanism of these antibodies works against MERS-CoV infection in camels and alpacas. The detection of IgG isotypes in the upper respiratory tract of these alpacas
and camels will further strengthen the role of IgG isotypes in protection against MERS-CoV infection in camels. Additionally, there are no reports that associated a Th1 or a Th2 response in camelids with their IgG isotypes, mainly due to an absence of reagents for camelids. The analysis of Th-biased response and their correlation with IgG isotypes may provide further insights on the immune responses to be targeted in vaccination.

5.1.1 Alpacas are protected by a subunit vaccine following the induction of IgG1 and IgG3 response against MERS-CoV challenge.

It was observed that induction of S1-specific IgG1 and IgG3 antibodies, with neutralizing activity, appear to correlate with protection in a MERS-CoV alpaca model of infection and reinfection. Three vaccine candidates have been in development in dromedary camels so far; an adenovirus vectored vaccine coding for spike protein, a DNA vaccine and a poxviral vector carrying spike protein vaccine candidate (Alharbi et al., 2019; Lam et al., 2016a; Muthumani et al., 2015). Current published evidence supports that anti-spike IgG antibody response in vaccines; providing protection and reducing viral load against MERS-CoV challenge in camels (Alharbi et al., 2019). However, it is not known if vaccine-induced immunity results in mechanistically similar immunity as natural infection. Here the vaccination studies were performed in a surrogate alpaca model. The IgG isotype response following Tri-Adj vaccination and MERS-CoV challenge induced in alpacas was characterized.

The antibody response induced upon immunization (IM) or (IN) with purified MERS-CoV S1 protein formulated with TriAdj was evaluated. TriAdj based vaccine candidate have been previously evaluated in mice, pigs, sheeps and koalas for other respiratory as well as gastrointestinal viruses and are known to induce strong humoral responses along with induction of innate immune arms (Garg et al. 2015; Levast et al., 2014; Nyari et al., 2018; Polewicz et al., 2011; Snider et al., 2014; Wasan et al., 2019a). The IM immunization in alpacas with S1 + TriAdj using a prime/boost regimen resulted in robust total IgG binding as well as neutralizing antibody response following vaccination and comparable to that observed following experimental challenge. In contrast, the IN immunization of alpacas failed to induce total IgG S1-binding response nor neutralizing antibody response following vaccination. The IN-immunization showed high levels of viral titers in nasal washes following challenge, further confirming alpacas were not protected against MERS-CoV when immunized with IN route compared to IM route of immunization. This was perhaps unexpected as the IN infection has been shown to induce IgG
response in infection/re-infection studies and the reformulation or a change in vaccine delivery methods and also increase in vaccination dose for IN route could be necessary.

The IgG isotype response was evaluated upon vaccination and challenge in IM, in alpacas using IgG isotype ELISA. The binding IgG isotype response was dominated by full length IgG1 response in alpacas vaccinated with Tri-Adj by IM route and MERS-CoV challenge. In contrast, the IgG3 was detected at moderate level while IgG2 showed minimal binding in alpacas vaccinated with IM route. An IgG2 binding antibody response was only detected only after the 2nd IM dose, which was consistent with findings from infection/re-infection study in alpacas. Furthermore, the neutralizing activity of each IgG isotype was detected at day 56 post-vaccination and day 28 post-challenge in alpacas immunized IM following affinity purification. When tested at day 56 post-vaccination, the neutralizing activity of IgG1 and IgG3 was comparable while not surprisingly, the neutralizing activity of IgG2 was minimal. Similarly, the day 28 post challenge showed comparable neutralizing activity of IgG1 and IgG3 isotypes in IM immunization in alpacas. Interestingly, the moderate levels of IgG3 detected in IgG isotype response appears to do not affect the neutralizing activity against MERS-CoV. These findings suggest two possibilities; 1. A low concentration of IgG3 isotype is sufficient to efficiently neutralize MERS-CoV compared to full length IgG1; or 2. The monoclonal antibody used for detection IgG3 isotype may not bind as efficiently as anti-IgG1 monoclonal antibody in ELISA assays.

In conclusion, the IM immunization of alpacas with S1+TriAdj vaccine induce a strong IgG1 and IgG3 response with minimal levels of IgG2 following vaccination and challenge with MERS-CoV. Vaccination induced a similar IgG isotype response as was observed from infection studies in alpacas, where the infection resulted in protection from re-infection. These findings further confirm that serum IgG1 and IgG3, with the neutralizing activity may play a major role in protection against MERS-CoV infection.

5.1.2 Antibodies induced following infection and vaccination cross-neutralize the circulating MERS-CoV strains in Dromedary camels.

Coronaviruses are unique among the RNA viruses in that they have the ability to perform proof-reading activity. However, several strains have been reported possessing numerous mutations in the RBD region of spike protein showing effect on transmissibility and reduced neutralizing activity (Gribble et al., 2021; D. W. Kim et al., 2016; Y. Kim et al., 2016b; Ogando et al., 2020). Analysis of the spike protein from virus isolates from the MERS-CoV South Korea
outbreak showed a higher evolutionary rate $6.72 \times 10^{-3}$ substitutions/year, compared to other currently circulating isolates (Kim et al., 2016). As the majority of vaccine-induced as well as naturally induced neutralizing antibodies are directed against the spike protein it is crucial to determine if the S protein from one isolate can provide cross-protection against different strains of MERS- CoV. Notably, the infection established here as well as the vaccine is based on the EMC/2012 S sequence, which is in a distinct clade that is not frequently observed circulating. Here we obtained 7 MERS-CoV isolates circulating in camels in Saudi Arabia in 2016-2017 and sequenced them by next-generation sequencing. Phylogenetically MERS-CoV is comprised of three clades: A, B and C; clade A and C contain extinct strains including EMC/2012, while clade B strains are currently circulating in both humans and dromedary camels in the Arabian Peninsula (Hemida et al., 2020; Schroeder et al., 2021; Yusof et al., 2017; Zhang et al., 2016). The 7 isolates characterized in our lab were all categorized under clade B. To represent the diversity of the mutations in these 7 isolates, we selected 4 isolates which had different mutations in spike protein (the other 3 viruses isolates had mutations at identical locations to those chosen) and the cross-neutralizing activity of serum samples collected from the infection/re-infection study were analyzed. Serum from alpacas following infection with EMC/2012 exhibited cross-neutralizing activities against all four isolates at similar levels as observed against EMC/2012. Similarly, when tested with serum induced in re-infection with EMC/2012 also exhibited comparable cross-neutralization against all 4 isolates despite the presence of mutations in spike protein in virus neutralization assay. Importantly, serum from vaccinated alpacas also showed cross-neutralization against all 4 isolates with 1 isolate containing a mutation in the RBD region (F473L) showing reduced neutralization when compared to EMC/2012; however, this only represented a 2-fold reduction in neutralization which is not expected to result in a loss of protection against the camel isolates. These results were in contrast with published studies using monoclonal antibodies which failed to cross-neutralize virus strains containing even one or two mutations in spike protein (Kleine-Weber et al., 2019). However, a recent published analysis of cross-neutralization against 15 pseudovirions containing mutation at various position in RBD were consistent with our findings (Choi et al., 2020). Cross-neutralization analysis suggests that antibodies induced against EMC/2012 can neutralize currently circulating viruses in camels possibly due to the limited changes in amino acid in the S protein or MERS-CoV. Although the presence of pre-existing antibodies to MERS-CoV seems to be not protective against reinfection in camels which may be
dependent on high titers of antibodies. In this case the vaccine design based on EMC/2012, may be sufficient to induce the immune response which will provide cross-protection against currently circulating isolates in MERS-CoV.

5.1.3 Development of reagents for detection of camel IgG isotypes.

While the presence of S1-specific IgG1 and IgG3 following MERS-CoV infection or vaccination appears to correlate with protection in alpacas, the role of IgG isotypes in camels in regard to protection from infection against MER-CoV is currently unknown. The ability to assay for this will be crucial to design and evaluate vaccination strategies for camels. Similar to alpacas, dromedary camels possess conventional IgG1 antibodies and two heavy chain antibodies: IgG2 and IgG3. These heavy chain antibodies constitute approximately 75% of the total IgG in camel serum compared to approximately 45% in alpacas and llamas (Hamers-Casterman et al., 1993). Monoclonal antibodies specific to dromedary camel IgG isotypes have yet to be characterized, limiting research on the IgG isotype response in camels. Alpaca and llama IgG isotype-specific monoclonal antibodies differ in their specificity and affinity for camel IgG isotypes, suggesting that there might be structural differences between new world and old-world camelids IgG (Daley et al., 2005). Here we developed and characterized monoclonal antibodies against camel IgG isotypes for the purpose of being able to determine the IgG isotype response in camels to infection and vaccination. Additionally, the resulting anti-camel IgG isotype monoclonal antibodies also allow a comparison between the immune responses in the experimental alpaca model to immune response following MERS-CoV infection and vaccination in camels. Here we developed an anti-IgG3 monoclonal antibody and characterized a IgG1 against camels and established ELISA-based assays to be used in camels to determine the IgG isotype response.

To guide the MERS-CoV vaccine development in camels several outstanding questions still need to be answered such as 1. what should be the target age of dromedary camels for vaccination, are calves protected from colostrum and need to be excluded, 2. the duration of immune response induced upon vaccination and infection, 3. the efficacy of vaccines in camels containing pre-existing immune response and also does the immune response and infection susceptibility different between camel species. Serosurveys find that antibodies to the MERS-CoV spike protein have been observed in up to 90% of dromedary camels assayed in Middle Eastern and African countries, including Oman, Jordan, Sudan, Somalia, Nigeria, Ethiopia, Mali, Tunisia, and Saudi Arabia (Falzarano et al., 2017a; Hemida et al., 2017a; Meyer et al., 2014; Miguel et al.,
Moreover, a recent report has also found evidence of MERS-CoV infection in Bactrian camels in China (Adney et al., 2019). Bactrian camels are generally domesticated in colder ecological regions as compared to dromedary camels which are generally found in desert areas. The initial reports on these two species have suggested differences in immune response with varying levels of innate as well as adaptive immune response to pathogens (Hussen and Schuberth, 2021). In this case, characterization of IgG isotype between Bactrian vs dromedary camels may be important to develop vaccine strategies to be used in different camel species susceptible to MERS-CoV infection.

Like other ruminants, the placenta of camels does not allow transplacental transfer of antibodies and thus newborn camel calves are born without a serum immunoglobulin repertoire (Furukawa et al., 2014; Ghazi et al., 1994). Therefore, they initially rely on IgG in colostrum transferred from their mother. Reports suggest that all IgG isotypes are present in colostrum but that maternal antibodies wane between 6 and 9 months and then calves become increasingly susceptible to infections (el sheikh et al., 2020; Salhi et al., 2015). Age-related changes in innate as well as adaptive immune response have been described in other animals suggesting that susceptibility to infection differs from birth to adulthood (Alharbi et al., 2020b; Kasem et al., 2018). Age-dependent studies in camels against MERS-CoV have suggested differential susceptibility in younger camels vs adults. In this case, the characterization of IgG isotype response and if to determine the age-related changes in calves, young and adult camels using developed monoclonal antibodies will be crucial.

The therapeutic applications of camelid heavy chain antibodies have suggested the low susceptibility to protease enzymes and comparatively high half-life when tested against viral infections (Nguyen et al., 1999; Vu et al., 1997). However, there are no reports establishing the antibody kinetics of camelids IgG isotypes induced in natural infection in camels. The previous studies in alpacas have shown that the IgG2 response require repeated exposure while an initial infection is sufficient to induce protective levels of IgG1 and IgG3. In this case, characterization of IgG1 and IgG3 using the monoclonal antibodies developed herein can be used to determine if the IgG1, IgG2 and IgG3 response wane over time with different kinetics following infection and vaccination.

In summary, we have developed and characterized monoclonal antibodies against IgG3 and IgG1, respectively, of dromedary camels. These will serve as valuable reagents to characterize
the IgG isotype response in camels against MERS-CoV infection and vaccination, as well as the response to other infectious agents such as Foot and mouth disease virus (FMDV), West Nile and pertussis. Characterization of the IgG isotype response will improve our understanding of immunity to natural infections as well as informing the development of effective vaccines for camels.

5.2 The IgG response to MERS-CoV does not provide protection against SARS-CoV-2 infection:

MERS-CoV and SARS-CoV-2 show striking differences in the sequences of their spike protein and utilize the different host cell surface receptors to enter into the cells (DPP4 and ACE-2 respectively). However, both of these coronaviruses share significant structural similarities and thus may contain some antigenic epitopes that may be capable of inducing cross-reactive antibody responses (Chen et al., 2021; Rehman et al., 2021). It has also been proposed that prior exposure to other seasonal coronaviruses may confer some level of immunity against SARS-CoV-2 (Pinto, 2020); however, initial analysis using epitope mapping between MERS-CoV, and SARS-CoV-2 suggests a low prevalence of cross-reactive antibodies. Although various analyses of naturally induced as well as monoclonal antibodies to MERS-CoV have been evaluated against SARS-CoV-2 to determine if they are cross-reactive. These antibodies have been targeted against the various regions of spike proteins including (RBD, NTD) and only a few have been confirmed to be cross-reactive against SARS-CoV-2 (Jiang et al., 2020).

Due to the unique features of heavy chain antibodies, which have been proposed to recognize a more diverse number of antigens, the cross-neutralizing activity of sera from MERS-CoV vaccinated or infected alpacas was assessed against SARS-CoV-2. The findings suggest although heavy chain antibodies may recognize diverse paratopes antigen, despite having relatively high neutralization activity against MERS-CoV, essentially no cross-neutralizing activity was observed against SARS-CoV-2 when tested with sera induced in alpacas following infection as well as vaccination. These results are consistent with the findings from others which showed a lack of cross-reactivity between these two coronaviruses. Further analysis of common epitopes and mechanism of neutralization between the two viruses may help to identify future strategies to generate cross-protective immunity and move towards pan-coronavirus vaccines. The lack of information on whether there are useful common neutralizing epitopes between these
viruses is certainly an impediment for the development of a universal vaccine against coronaviruses.

5.3 Antibodies induced by B.1.1.7 and B.1.351 show varying degrees of neutralizing and cross-neutralizing activity in hamsters.

After the emergence of SARS-CoV-2 in Dec 2019, the variant with a mutation in spike protein at position D614G was isolated and became prevalent in the human population in March 2020 (Isabel et al., 2020; Onder et al., 2020). The newly emerged variant induced higher titers in animal models and human samples suggesting high viral replication then ancestral SARS-CoV-2 isolated at the beginning of pandemic (Baric et al., 2020). Later in 2020, the B.1.1.7 became the dominant variant in the UK, outcompeting D614G (Washington et al., 2021a). Since its emergence, the B.1.1.7 lineage circulated throughout most of the world (Hodcroft et al., 2021; Washington et al., 2021b). Shortly thereafter, the B.1.351 lineage was reported in South Africa, with initially showing concerning level of resistance to neutralizing antibodies from vaccinated individuals (Tegally et al., 2021). Both VOCs have amino acid changes in the spike protein. These mutations are presumed to be involved in causing increased transmissibility, infectivity, and varying degree of immune escape in previously infected or vaccinated population (Wang et al., 2021b; Zhou et al., 2021b). B.1.1.7 and B.1.351 both possess the N501Y mutation, which confers enhanced binding to ACE-2 receptor (Ali et al., 2021; Luan et al., 2021).

Several reports on variants and antibody response pointed towards the decreased neutralizing activity against B.1.351 as compared to B.1.1.7 and D614G variant (Singh et al., 2021; Wang et al., 2021b; Zhou et al., 2021b). Structural analysis of the RBD of B.1.351 suggests that the presence of an amino acid substitution 484K reduced the neutralizing activity of therapeutic monoclonal antibodies as well as antibodies induced from previous infections and vaccination with ancestral SARS-CoV-2 (Jangra et al., 2021). The comparative analysis of viral pathogenesis and innate immune response following B.1.1.7 and B.1.351 infection showed no significant differences between these VOCs in hamster model of SARS-CoV-2 suggesting negligible impact of these amino acid changes on viral levels and innate gene expressions (O’Donnell et al., 2021). However, the longitudinal analysis of neutralizing as well as binding antibody response and its degree of cross-protection in VOCs still needs to be deciphered completely.

Syrian golden hamsters were infected intranasally with either B.1.1.7 or B.1.351. As expected, viral replication was observed in the lungs as well in nasal washes up to 5-6 dpi at levels
that were comparable to VIDO-01. Consistent with other report, no differences in gross pathology or lung viral load were recorded between B.1.1.7 and B.1.351 infected hamsters (Fischer et al., 2021; O’Donnell et al., 2021). However, the B.1.351 infected hamsters showed increased severity of interstitial pneumonia and delayed recovery compared hamsters infected with B.1.1.7. This difference in lung pathology may be due to increased dysregulated of innate immune response and can result in tissue damage as noted in other viruses. Analysis of innate gene response in hamster infected with B.1.351 has suggested that the genes involved in tissue repair were downregulated in hamsters infected with B.1.351 compared to B.1.1.7 (Abdelnabi et al., 2021).

The primary goal of this study was to determine whether the humoral immune response showed biased towards the spike protein that the hamsters were exposed to during the infection. Analysis of the humoral response showed IgG binding as well as neutralizing antibodies show bias towards the homologous isolate – B.1.1.7-infected hamsters had high titer of against B.1.1.7 than B.1.351; similarly, B.1.351 infected hamsters had higher titers against the B.1.351 than B.1.1.7.

Cross-neutralization as well as binding antibody response between these two VOCs was dependent on the VOC the hamsters were initially infected with. Serum samples collected from hamsters infected with B.1.1.7 showed the widest breadth of binding antibodies and neutralization activity when tested against VIDO-01 and B.1.351 isolate. The total IgG response induced by B.1.1.7 showed greater cross-reactivity against both S1 as well as RBD from Wuhan Hu-l and B.1.351 at 5 dpi and increased in significance at 10 dpi when the differences were more prominent. In agreement with the binding data, the cross-neutralizing activity of serum from B.1.1.7 infected hamster against the homologous as well as the VIDO-01 isolate showed comparable titers at both 5 dpi and 10 dpi; however, cross-neutralization against B.1.351 was significantly reduced to 7-fold. This is perhaps not unexpected as the reduction in neutralization between B.1.1.7 and original Wuhan-like or D614G mutants is expected to be approximately 2-fold across multiple studies (Planas et al., 2021a; Supasa et al., 2021; Wang et al., 2021a), while the reduction for B.1.351 is expected to be approximately 8-fold (Fischer et al., 2021; Planas et al., 2021a; Wang et al., 2021b).

Interestingly, the IgG response as well as neutralizing activity against B.1.351, even against homologous S1, RBD and in neutralization assays, was lower than that of B.1.1.7 – despite levels of virus titers in lungs being quite comparable. These findings certainly support that the mutations in B.1.351 dampen or delay the antibody response which is also supported by virus load clearance in the lung lobes delayed at 10 dpi, increase in pathology and delayed recovery from weight loss
in hamsters. The reduction in binding and neutralizing antibodies following B.1.351 infection even against homologous virus leads to additional possibilities that – 1.) that B.1.351 induces a smaller magnitude of immune response despite reaching comparable levels of virus or 2.) that the delayed clearance of B.1.351 is due to attenuated antibody response, suggesting B.1.351 modulates the immune response in a different manner than VIDO-01 and B.1.1.7. These findings are consistent with published data from hamster models and in clinical cases where the reduced neutralizing antibody response against B.1.351 has been noted (Abdelnabi et al., 2021; Becker et al., 2021; Caniels et al., 2021; Garcia-Beltran et al., 2021; Zhou et al., 2021a).

These data support that infection with B.1.1.7 results in broader antibody response compared to the antibody response induced by B.1.351 which results in a short delay to recovery and/or protection against B.1.351 in the hamster model. Furthermore, the impact of the decreased antibody response and relative resistance to neutralizing antibodies from heterologous isolates by B.1.351 raises the possibility that B.1.351-infected individuals may be at higher risk of re-infection with future VOCs. Although the documented evidence of re-infection suggests this is possible, the major question is how this happen and does the level of the antibodies correlates with the protection offered by previous infection against new variants (Jeffery-Smith et al., 2021; Shastri et al., 2021).

5.3.1 The antibody response following immunization with subunit vaccine showed reduced efficacy against VOCs in hamster model of SARS-CoV-2.

All currently licensed vaccines are based on the protein sequence of spike protein from Wuhan-Hu-1 isolate that dominated at the population beginning of pandemic (Al-Kassmy et al., 2020; Kaur and Gupta, 2020; Tregoning et al., 2020). Different vaccines have shown varying efficacy in clinical trials; with BTN162b2 and mRNA-1273 showing efficacy of 95% after the second dose (Baden et al., 2021; Polack et al., 2020; Tenforde et al., 2021a), Ad26.CoV2S a single dose vaccine conferring efficacy of 66.9% and ChAdOx1 with efficacy of 70.4% after the two-dose administration (Chagla, 2021; Knoll and Wonodi, 2021; Sadoff et al., 2021; Voysey et al., 2021). While the effectiveness of vaccines against symptomatic infection with ancestral SARS-CoV-2 remain high, the data suggests that vaccine induced immunity appears to decrease in some cases against some of the VOCs (Caniels et al., 2021; Hacisuleyman et al., 2021; Krause et al., 2021). The immunization of hamster with either S1+TriAdj (COVAC-1) or SWE (COVAC-2) vaccines containing spike protein sequence from ancestral SARS-CoV-2 followed by challenge
with B.1.1.7 or B.1.351 showed some degree of differences in neutralizing antibody response and protection.

The binding antibody response against Wuhan-S1 and Wuhan-RBD was comparable following vaccination as well as challenge with indicated VOCs. The binding antibody response following B.1.1.7 challenge resulted in a response that was comparable to that induced following Wuhan (VIDO-01) infections in hamsters. In contrast, binding antibody response was reduced against Wuhan-S1 and Wuhan-RBD following B.1.351 challenge despite of same vaccines being administered in both VOCs studies.

The binding antibody response to Wuhan-S1 and Wuhan-RBD following vaccination and challenge with B.1.1.7 showed minimal effect on antibody response. In contrast, the B.1.351 challenge showed reduced binding antibodies against Wuhan-S1 and Wuhan-RBD despite of the same vaccines being administered in both VOCs studies. Several studies have reported the effect of mutations in spike protein of B.1.1.7 and B.1.351 on antibody response induced from vaccinations designed with ancestral spike protein (Emary et al., 2021a; Fischer et al., 2021; Xie et al., 2021; Zhou et al., 2021c). In general, these studies suggests that the effect of amino acid changes in B.1.1.7 is minimal compared to B.1.351 on antibody titers. The current data from ChAdOx1-CoV19(AZD-1222) vaccine as well as BTN162b2 showed minimal impact of on vaccine efficacy against B.1.1.7 predominated in UK population (Emary et al., 2021b; Liu et al., 2021). In contrast, the amino acid substitutions in B.1.351 spike protein resulted in 9-fold reductions in neutralizing antibody titers obtained from ChAdOx1 vaccination than the other circulating VOCs (Madhi et al., 2021; Zhou et al., 2021c). In agreement with the current available data, neutralizing antibody response against B.1.1.7 following COVAC-1 and COVAC-2 vaccination and challenge with B.1.1.7 was recorded as (1:256 COVAC-2 and 1:128 COVAC-1) on 5dpc and increased at 10dpc (1:320 COVAC-1 and 1:512 COVAC-2). The neutralizing antibody response induced following vaccination and challenge with B.1.1.7 were correlated with reduction in viral titers in lung lobes compared to unvaccinated hamsters on 5 dpc. The robust neutralizing antibody response in COVAC-2 were correlated with low viral titers ($10^1$- $10^2$ TCID$_{50}$/mg of tissues) and provided protection, while COVAC-1 vaccinated hamsters were partially protected following B.1.1.7 challenge ($10^3$ TCID$_{50}$/mg tissues) compared to PBS control hamsters ($10^5$ TCID$_{50}$/mg of tissues). In contrast, the low titer of neutralizing antibodies induced following COVAC-1 and COVAC-2 vaccination did not provide any protection against B.1.351
challenge in hamsters. The neutralizing antibody response against B.1.351 following COVAC-1, COVAC-2 vaccination, and B.1.351 challenge was recorded as (1:64 COVAC-2 and 1:20 COVAC-1) on 5 dpc with minimal increase on 10 dpc (1:128 COVAC-2 and 1:64 COVAC-1). Following challenge with B.1.351 high titers of viral replication was reported in COVAC-1 (10^5 TCID_{50}/mg of tissues) as well in COVAC-2 (10^4 TCID_{50}/mg of tissues) vaccinated hamsters which was similar to viral titers reported in PBS control (10^5 TCID_{50}/mg of tissues).

Notably, the COVAC-2 showed higher efficacy and a broader breadth of antibody response compared to COVAC-1 irrespective of the VOCs challenge. This may be due to differences in both magnitude or induction of varying IgG isotypes and its neutralizing activity in COVAC-1 vs COVAC-2 vaccine in hamsters. Previous data generated in mice against other viral infections show that COVAC-1 induces balanced Th1/Th2 response, with near equal amounts of S1-specific IgG1 and IgG2a being generated, while COVAC-2 induces a response more biased towards IgG1 (Garg et al., 2017b; Wasan et al., 2019; Younis et al., 2018). Unfortunately, the absence of IgG isotype specific antibodies for hamsters limit the level of detail of the response to vaccination.

The relatively short time interval following challenge with B.1.1.7 or B.1.351 limits how applicable this data is in a larger context. The absence of later timepoints in study limit the understanding of duration and kinetics of antibodies against B.1.1.7 and B.1.351 variants. The analysis of efficacy of subunit vaccine suggests that the current argument that the individuals who have been vaccinated against the ancestral isolate are may not be equally protected against certain VOCs with reduction of neutralizing antibody response. However, it is possible in humans that the existing memory B cells stimulated from vaccination are re-stimulated and provide partial protection against VOCs (Gaebler et al., 2021; Muecksch et al., 2021b). Additionally, it has been observed that the despite of declining titers of neutralizing antibodies, the neutralizing breadth against VOCs actually improved over time as a result of maturation of the antibody response (Muecksch et al., 2021b, 2021a; Wang et al., 2021). Similarly, the prior vaccination associated with increase in neutralizing and cross-neutralizing antibody titers highlighting the repeated vaccination appears to increase the antibody breadth against various variants (Planas et al., 2021b; Yue et al., 2021). Based on the current findings, the strategies or vaccines focusing on prevention of severe disease and hospitalizations following infection with VOCs are essential. Several studies on VOCs in vaccinated versus unvaccinated patients suggests the reduction of hospitalizations and mild disease in patients with vaccination following infection with VOCs including B.1.351 (Haas
et al., 2021; Tenforde et al., 2021b). With the ongoing evolution and emergence of VOCs the rapid pre-clinical testing of neutralizing as well as cross-neutralizing activity induced in vaccination will provide further knowledge to strengthen the future vaccine designs against SARS-CoV-2 VOCs.

5.4 Neutralizing monoclonal antibodies confer protection in the SARS-CoV-2 in hamster model.

Neutralizing monoclonal antibodies remain a promising countermeasure that has the possibility for prophylactic and therapeutic use for SARS-CoV-2 infections (Chen, 2020; Wang et al., 2020). Recently, many potent neutralizing antibodies from COVID19 patients have been identified that neutralize pseudoviruses with IC$_{50}$ values ranging from 1 to 300 ng/mL, and replication-competent SARS-CoV-2 with IC$_{50}$ values from 15 to 500 ng/mL (Pinto et al., 2020; Wang et al., 2020). While most of the current approved monoclonal antibodies are isolated from convalescent plasma, full length Ab1 was derived directly from SARS-CoV-2-naïve human library by a rapid phage-display selection method. Similarly, the Ab8 was synthesized using variable region reactive against spike protein of SARS-CoV-2 which was then fused with Fc domain.

Administration of Ab1 in hamsters infected with SARS-CoV-2, viral loads and infectious virus in the lungs were reduced both when administered prophylactically as well as therapeutically. Similarly, the intraperitoneal inoculation of Ab8 in hamsters, not only reduced the viral RNA, infectious virus in the lung and reduced pathological changes, but it also reduced shedding in the upper airway (nasal washes and oral swab), which could potentially reduce transmission of SARS-CoV-2. Impressively, Ab8 was also effective therapeutically, even at the lowest dose tested (3 mg/kg). Following single administration, Ab8 persisted for 5 days post administration at significant levels indicates that the pharmacokinetics of Ab8 is better to that of a Ab1 antibody; these findings were consistent with other studies where the half-lives of Fc fusion proteins to antibodies were reported to vary from those of IgG1s and can range from hours to days (Unverdorben et al., 2016). The molecular weight of Ab8 (80 kDa) is approximately half of that of a full-size IgG1, suggesting an advantage in terms of smaller quantities needed to be produced compared to those for IgG1s to reach similar number of molecules and efficacy. Thus, decreasing the size by 2-fold can theoretically increase its diffusion through tissues by 4-fold. Interestingly, following administration at the same dose, the concentration of Ab8 was higher than that of Ab1 in serum as well as in lung tissues. This result suggests that Ab8 diffusion from the peritoneal cavity to the blood and penetration of lung may be faster than that of Ab1. This may further explain
its efficacy at low doses in SARS-CoV-2 mouse model used in the study against the high dose SARS-CoV-2 challenge. Although the low dose (3mg/kg) showed efficacy in the mice as well as hamster models, it should be noted that in humans, higher doses may be required to achieve comparable degree of efficacy. Another caveat is that in the hamster post-exposure experiment, the Ab1 and Ab8 were administered at time (6h), a time which likely represents just a single round of virus replication. Because these antibodies are thought to function by preventing infection of new cells, administration at the peak of infection or a later time point may not be as effective unless these antibodies are capable of mediating important effector functions in vivo, which is under investigation.

5.5 Neutralizing antibodies are immune correlates of protection.

Our data supports that the neutralizing antibody response appears to be an important correlate of protection for respiratory coronaviruses- both for MERS-CoV and SARS-CoV-2-when assessed under a number of different scenarios including 1). Infection/reinfection of MERS-CoV in alpacas 2). Vaccination (in both MERS-CoV and SARS-CoV-2) and 3). When administered as a monoclonal antibody treatment (SARS-CoV-2). In all cases, neutralizing antibodies were consistently correlated with a reduction in viral RNA as well as infectious virus in respiratory tract of alpaca and hamster model when challenged with MERS-CoV and SARS-CoV-2 respectively. The antibodies induced following a primary infection with MERS-CoV protect from re-infection; however, the specific type of antibody response has yet to be deciphered. Protection against re-infection with MERS-CoV in alpacas was associated with the presence of neutralizing antibodies that developed following the primary infection. Here we determined that this neutralization activity was provided by IgG1 and IgG3 isotypes. This finding extended to vaccination with the S1+TriAdj vaccine in alpacas where again, in the presence of neutralizing antibodies, alpacas were protected from challenge. A similar profile of IgG1 and IgG3, with neutralizing activity was also observed. Interestingly, parenteral vaccination with and adjuvanted subunit vaccine was able to modulate protection of the upper respiratory tract. However, further studies on determining the role of IgM and IgA antibodies against MERS-CoV in alpacas will be important, which is currently limited due to the lack of lab reagents for camelids.

Similarly, infection and vaccination (using S1 from ancestral virus of SARS-CoV-2) against the B.1.1.7 variant showed decreased viral loads in the presence of neutralizing antibodies, that rapidly increased following challenge. In contrast, the same vaccine regimen provided
incomplete protection against B.1.351 challenge and was indicated in the absence of a neutralizing antibody response, which was also not rapidly induced following challenge. Several published studies have evaluated the relationship between protection and neutralizing antibody responses using clinical as well as pre-clinical data, suggesting neutralizing antibodies are one of the possible mechanisms of protection against SARS-CoV-2 (McMahan et al., 2020; Vanshylla et al., 2021). Adoptive transfer of convalescent serum showed protection against SARS-CoV-2 challenge in non-human primates in dose dependent manner suggesting that at least in this model protection is antibody mediated (McMahan et al., 2020). Similarly, the therapeutic as well as prophylactic administration of full length IgG1 and engineered Ab8 monoclonal antibodies reduced the viral RNA and infectious virus in the lungs of hamsters following challenge with SARS-CoV-2. The detection of these monoclonal antibodies in lungs further supports a role for these antibodies in mediating protection. The administration of therapeutic Ab1 and Ab8 antibodies provides a proof-of concept, and mechanistically supports that neutralizing antibody alone can provide protection at least in a hamster model of SARS-CoV-2 infection. Collectively, these data support that neutralizing antibody and its role in protection against respiratory coronaviruses such as MERS-CoV and SARS-CoV-2 have important implications for the development of better vaccines candidates and therapeutic agents.

**Conclusion:**

The protective role of neutralizing antibodies in both MERS-CoV and SARS-CoV-2 infections, induced from either infection or vaccination, as well as when delivered as monoclonal antibodies was demonstrated in alpaca and hamster models, respectively. In alpacas, IgG1 and IgG3 isotypes appears to mediate almost all the neutralizing activity against MERS-CoV infection/re-infection. Lower viral burden, both viral RNA and infectious virus in nasal washes and lung lobes in alpacas and hamsters, respectively, correlated with the presence of neutralizing antibodies whether induced from infection or vaccination. For SARS-CoV-2 these findings were further confirmed by administering two monoclonal antibodies with a high level of neutralizing activity in a hamster model. The prophylactic as well as therapeutic administration of antibodies alone reduced viral titers and pathogenesis following SARS-CoV-2 challenge in hamsters at early timepoints when natural immunity hamsters would not have been induced. The detection of Ab8 in lungs of hamsters further supported the involvement of this antibody in reducing of viral loads upon therapeutic administration. Taken together, these data suggest that protection from infection
from either MERS-CoV or SARS-CoV-2 requires a neutralizing antibody response and that a certain threshold would provide protection from viral loads and that neutralizing antibody are correlates of protection or one of the protective adaptive immune mechanisms (Figure 5.0.1).
Neutralizing antibodies can be induced following 1) MERS-CoV natural infection; 2) SARS-CoV-2 natural infection; 3) therapeutic monoclonal antibodies; 4) vaccination; or 5) previous infection with heterologous viruses—These neutralizing antibodies bind to spike protein of MERS-CoV or SARS-CoV-2 and interferes with its ability to bind its host receptor, (DPP4 or ACE-2, respectively, ) (indicated by inhibitor arrow) and prevent or reduce viral burdens—and decreased or eliminate pathological processes—in respiratory tissues. Cross-reactive neutralizing antibodies against other coronaviruses, such as MERS-CoV- induced response providing cross-reacting antibodies against SARS-CoV-2. However, current research and our study suggest that such cross-reactivity is low or not determined clearly (dotted by arrow).

Figure 5.0.1: Neutralizing antibody response and its role in protection.
**Future directions:**

Although our data, as well as data from others, seems to support that neutralizing as well as binding IgG isotypes are major correlates of protection, these antibodies are not necessarily responsible for providing the mechanism of protection. To provide stronger evidence that neutralizing antibodies are mechanistically protective the serum samples from alpacas previously infected with MERS-CoV could be delivered to naïve animals intravenously and these animals would be subsequently challenged with MERS-CoV. From our findings we have determined that IgG1 and IgG3 appear to be immune correlates of protection in alpacas against MERS-CoV. Future studies could involve transfer of purified IgG1 or IgG3 alone to determine if a single IgG isotype can provide protection from infection. Furthermore, here we developed and characterized IgG isotype specific monoclonal antibodies for dromedary camels. However, the high sequence similarity between IgG3 and IgG2 make it challenging to develop reagents against IgG2. Future studies could focus on development on monoclonal antibodies specifically recognizing IgG2 of camels using similar methods as established for IgG3. We have access to 570 naturally infected camel serum samples that are currently stored at NIAID, NIH. In the future, we will determine if the IgG isotype response to natural infection in camels is dominated by IgG1 and IgG3 response.

Furthermore, current vaccine data including our own, suggest that vaccine based on the ancestral (Wuhan) SARS-CoV-2 spike protein may show reduced efficacy against the VOCs. Here I proposed that, the repeated vaccination of hamster model with vaccine containing heterologous S1 will have increased breadth and neutralizing antibody response against challenged variant of concerns. The longer interval between doses will further increase the antibody breadth as a result of antibody maturation over time. At various timepoints the RBD binding activity and neutralization breadth can be assessed with ELISA based assays. The Ig next generation sequencing on B cells collected at initial and later timepoints could be used to evaluate how maturation leads to increase the breadth and neutralizing antibodies at later timepoints.
6 Appendix:

10% resolving SDS gel

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<th>Volume</th>
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<tr>
<td>4.4 ml</td>
<td>sterile H₂O</td>
</tr>
<tr>
<td>3.0 ml</td>
<td>40%Acrylamide/Bis (BioRad)</td>
</tr>
<tr>
<td>2.5 ml</td>
<td>1.5M Tris-HCl (pH 8.8)</td>
</tr>
<tr>
<td>100 µl</td>
<td>10% SDS in dH₂O</td>
</tr>
<tr>
<td>50 µl</td>
<td>10% ammonium persulphate</td>
</tr>
<tr>
<td>8 µl</td>
<td>TEMED</td>
</tr>
</tbody>
</table>

4% Stacking gel

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 ml</td>
<td>sterile dH₂O</td>
</tr>
<tr>
<td>0.5 ml</td>
<td>40% Acrylamide/Bis (BioRad)</td>
</tr>
<tr>
<td>1.25 ml</td>
<td>0.5M tris-HCl (pH 6.8)</td>
</tr>
<tr>
<td>50 µl</td>
<td>10% SDS in H₂O</td>
</tr>
<tr>
<td>25 µl</td>
<td>10% ammonium persulfate</td>
</tr>
<tr>
<td>5 µl</td>
<td>TEMED</td>
</tr>
</tbody>
</table>

Anode buffer

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 ml</td>
<td>0.67M boric acid</td>
</tr>
<tr>
<td>200 ml</td>
<td>methanol</td>
</tr>
<tr>
<td>725 ml</td>
<td>sterile dH₂O</td>
</tr>
</tbody>
</table>

Cathode buffer

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 ml</td>
<td>0.67M boric acid</td>
</tr>
<tr>
<td>50 ml</td>
<td>methanol</td>
</tr>
<tr>
<td>725 ml</td>
<td>sterile dH₂O</td>
</tr>
</tbody>
</table>
**Phosphate buffer saline (PBS) (pH 7.4)**

800ml dH₂O  
8g NaCl  
200g KCl  
1.44g Na₂HPO₄  
245g KH₂PO₄

**Wash buffer**

1 l. PBS (pH 7.4)  
500 µl. Tween-20

**Blocking buffer**

1 l PBS (pH 7.4)  
500 µl Tween-20  
50g. skimmed milk

**4x loading buffer**

2ml 1M Tris-HCl (pH 6.8)  
0.8g SDS  
4ml 10% glycerol  
0.4ml 14.7M β-mercaptoethanol  
1ml. 0.5M EDTA  
8mg bromophenol blue

**Coomassie brilliant blue**

1.25g Coomassie brilliant blue R250  
50ml glacial acetic acid  
225ml methanol  
225ml dH₂O
**Coomassie destain**

- 50ml glacial acetic acid
- 225ml methanol
- 225ml dH$_2$O

**Chromatography priming buffer (pH 7.0)**

- 1 l phosphate buffer
- 500µl Tween-20
7 References:


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