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Development of vaccines against MERS coronavirus and Merbecovirus
prototype pathogens

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A dissertation
submitted in partial fulfillment of the
Requirements for the degree of

Doctor of Philosophy

University of Washington

2025

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Abstract

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Abstract

Middle East Respiratory Syndrome is a severe respiratory infection caused by the MERS coronavirus (MERS-CoV), a member of the Merbecovirus subgenera in the Betacoronavirus genus. There are no licensed vaccines against MERS-CoV, furthermore our understanding of the members within the Merbecovirus subgenera is still within early stages. As global surveillance efforts continue to unveil new members within this subgenera, analysis of receptor utilization and viral tropism reveal the broad use of ACE2 and DPP4 receptor orthologues, some of which are capable of utilizing human receptors for cell entry. This underscores the need for not only continued viral surveillance to monitor for future outbreak potentials but also the development of vaccine therapeutics against MERS-CoV, which could help guide further advances to address broadly protective Merbecovirus vaccines.

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ACKNOWLEDGMENTS

This work would not have been possible without the mentorship, guidance, inspiration, and endless support from many distinguished individuals throughout my life and career. I am lucky to have a strong community spread throughout the world.

I would first like to thank Dr. John Bracht at American University for giving me my very first research opportunity. That experience, paired with the infectious zeal from Dr. Victoria Connaughton and Dr. Jeffrey Kaplan for anatomy and physiology, and microbiology, inspired me to pursue a career in medicine and therapeutics, which took me to the Vaccine Research Center within the National Institute of Allergy and Infectious Diseases. To my next mentors, Peter Kwong and Baoshan Zhang, thank you both for introducing me to the field of protein design and vaccinology. My time as a post baccalaureate student was life-changing, and instilled a passion for developing therapeutics that carried me forward to pursuing my PhD at the University of Washington.

To my thesis primary thesis advisor, Dr. Neil King, thank you for being a source of support and for years of mentorship that have not only helped me grow as a scientist, but also instilled many lifelong lessons in determination, rigor, and adaptability. Your unwavering positive outlook on life is inspirational and a constant reminder to push forward despite drawbacks and during times of uncertainty. I would also like to thank members of my thesis committee, David Veessler, Kelly Lee, Frank DiMaio, and especially Deborah Fuller for your mentorship, valuable insights and guidance.

Thank you to all of the collaborators who were instrumental in my thesis work and who were all a joy to work with: Tyler Starr, Ashley Taylor, Caroline Craig, Ralph Baric, Alexandra Schäfer,

Nick Cantanzaro, Miranda Hubbard, David Veessler, Kaitlin Sprouse, Amin Addetia, Jack Brown, Cameron Stewart, Francois Villinger, Bali Pulendran, and Jesse Bloom.

To the members of the King lab and the IPD, it has been an absolute privilege to work and grow with you all. The King lab is a special place with outstanding individuals and scientists, from whom I have learned so much from within the past 5 years and continue to learn from every day. To Annie Dosey, thank you for being my mentor from day 1 until now. I have grown so much as a scientist learning from you, and your determination and creativity are inspiring. I am very lucky to have Grace Hendricks, Marti Tooley, Chloe Adams, and Naveen Jasti – thank you all for being pillars of support, sanity, friendship, and scientific confidants. I am thankful to all of my colleagues at the IPD for creating a warm, fun, and creative environment: Maggie Ahlrichs, Richmond Boateng, Abby Burtner, Priya Christensen, Jung-Ho Chun, Cameron Criswell, Helen Eisenach, Dan Ellis, Chelsea Fries, Cyrus Haas, Bianka Haro, Dan Humphrys, Geoff Hutchinson, Yuhan Jiang, Zac Jones, Hyojin Kim, Susan Kleinfelter, Mark Langowski, Meg Lunn, Sebastian Ols, Audrey Olshefsky, Sanela Rankovic, Felicitas Ruiz, Sam Tipps, Erin Yang, John Wang, Adam Wargacki, Dane Zambrano, Suna Cheng, Adian Valdez, Elizabeth Soberg, Kandise van Wormer, and Rashmi Krishnamurty. I also thank the MCB graduate program staff, Maia Low, Denise Barnes, and Alice Ven for all of your support.

Next I want to thank all of my friends, new and old, who have supported me throughout different stages of my life. To Nina Dinh and Raghav Muralidharan, I am so grateful for our enduring friendship. You two have always been a place of respite and home, and some of my biggest cheerleaders. Thank you to Myungjin Lee and Yiran Wang for keeping me sane through some of the most uniquely challenging times including the COVID-19 lockdown. I also want to thank members from my MCB cohort, Tim Yu and Jodiene Johnson. Moving to a new city during the middle of a pandemic was difficult, and finding new friends at the time was even more of a

challenge, so I am very thankful that I met you two so early on after moving to Seattle and for the adventures we've shared. To Cherokee Toole, thank you for all of the life-long memories and for being a part of my community. To Jess Tan and Ella Carlander, playing volleyball with you two was a highlight of graduate school. To Jessie Kulsuptrakul and Carley Gray, I am immensely grateful for our found kinship. Our annual Hawaii trips were a blessing and always gave me something to look forward to during the long Seattle winters. It is a tradition that I look forward to keeping as we move towards the next stage of our lives.

Lastly to my family, my very first mentors and biggest cheerleaders who have provided unconditional love and support throughout all of my endeavors. To Thomas, I couldn't have asked for a better brother. You've always been a rock for me, patiently keeping me grounded during every step of my life. To my father, Huai-En, thank you for instilling in me a love and appreciation for the arts and sciences, and for teaching me early lessons on determination and perseverance. And to my mother, Fang-Ying, thank you for giving me the space to try anything and everything, and for ingraining in me the importance of being adaptable. Lastly, I am deeply grateful to my grandparents for teaching me resilience. Your combined lessons and support allowed me to be bold and helped me navigate through my life.

CHAPTER 1: INTRODUCTION

Coronaviruses and their prevalence as mammalian pathogens

Coronaviruses (CoVs) are enveloped viruses with positive-sense RNA genomes, composed of four genera within the family Coronaviridae, including Alpha-, Beta-, Delta-, and Gammacoronavirus (**Figure 1**). Alpha- and Betacoronaviruses are known to infect mammals, Deltacoronaviruses can infect avian and mammalian species, while Gammacoronaviruses infect avians, causing respiratory, enteric, and systemic disease.^{1,2} Of the four genera there have historically been seven members known to be pathogenic in humans, two of which belong to Alphacoronavirus (HCoV-229E and HCoV-NL63) while Betacoronavirus comprise the other five (HCoV-OC43, HCoV-HKU1, the Middle East Respiratory Syndrome (MERS) CoV, the Severe Acute Respiratory Syndrome (SARS) CoV, and SARS-CoV-2). The first two documented human coronaviruses prior to 2003 are 229E, an Alphacoronavirus, and OC43, a Betacoronavirus, which were both identified in the 1960s.³ In 2002, SARS-CoV was discovered in 29 countries with over 8,000 reported cases and a ~10% case fatality rate, causing widespread attention towards coronaviruses.⁴ Shortly after the emergence of SARS-CoV, two other CoVs were characterized. In 2004 another Alphacoronavirus called NL63 was reported from a patient with conjunctivitis, and in 2005 yet another Betacoronavirus, HKU1, was found in a child with bronchitis and conjunctivitis.^{5,6} Later in 2012, the next highly pathogenic Betacoronavirus, MERS-CoV, was isolated in Saudi Arabia in a patient hospitalized with severe pneumonia with over 2,500 confirmed cases and a fatality rate of ~35%.⁷ Recent reports further illuminated two additional potential human coronavirus infections within Haiti and Malaysia in 2014 and 2017, respectively.^{8,9} The newest reported human CoV is SARS-CoV-2 in 2019, the causative agent of the COVID-19 pandemic, that's been found reported in 240 countries with over 700 million documented cases and a mortality rate of < 2%.¹⁰ This chronology of CoV identification and outbreaks over the course of the past two decades underscores the significance of this viral

Family as a threat to human health, and necessitates further research towards developing therapeutics.

Betacoronaviruses can be further broken down into five subgenera: Sarbecovirus, Merbecovirus, Embecovirus, Hibicovirus, and Nobecovirus.² Embecovirus comprises HCoV-OC43 and HCoV-HKU1 in addition to many mammalian CoVs of veterinary relevance including porcine, canine, feline, and bovine CoVs (**Figure 1**).^{2,8,9} Highly pathogenic SARS-CoV and pandemic-causing SARS-CoV-2 are both members of the Sarbecovirus subgenus, and the Merbecovirus subgenus consists of the lethal MERS-CoV.¹¹ After the emergence of SARS-CoV-2, global efforts mobilized to research this virus for rapid vaccine development. With this influx in COVID-19-related work came an expansion of Sarbecovirus knowledge and broadened interest in creating a pan-Betacoronavirus vaccine. The Merbecovirus subgenus remains largely understudied with no available vaccines, despite historically deadly sporadic outbreaks of MERS-CoV with a 35% mortality rate and continuous spillover infections into humans. In order to work towards a pan-Betacoronavirus vaccine, more must be done to comprehend the nuances of each subgenus, starting with viruses with known global health risks such as MERS-CoV.

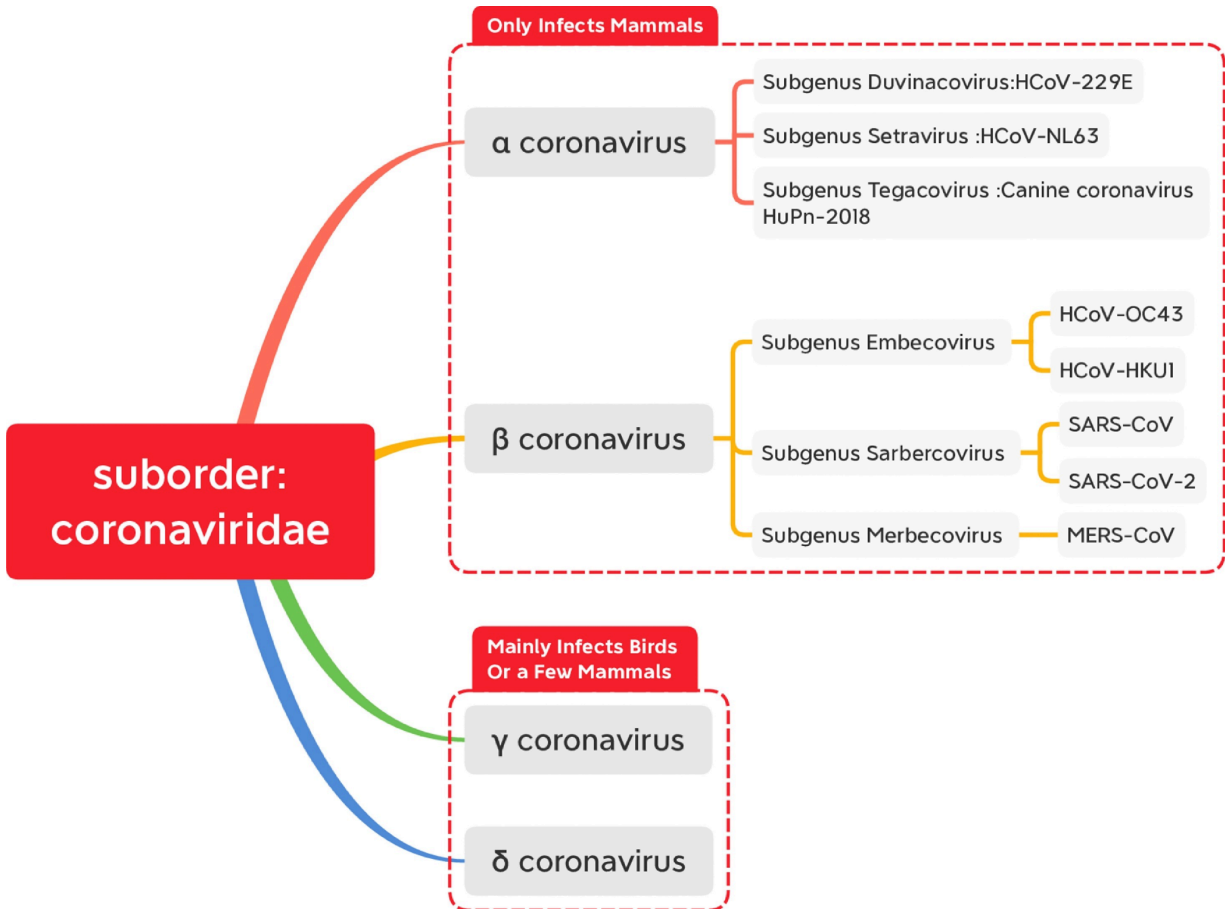


Figure 1. Classification of coronaviruses into four subgenera.

Division of coronaviruses into Alpha-, Beta-, Gamma-, and Deltacoronavirus genera. Betacoronavirus is further partitioned into Embecovirus, Sarbecovirus, and Merbecovirus subgenera with representative human pathogens from each subgenus.

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Coronaviruses inhabit a broad host range and have been known to adapt to new environments through recombination of the viral genome, facilitating cross-species expansion and frequent host-shifting events.^{12–19} Bats make up approximately 1/5th of mammalian biodiversity are known to be a primary reservoir of Alpha- and Beta-CoVs.^{20–32} Due to their broad geographic distribution and species diversity, bats are often facilitators of interspecies transmission

events.^{13,30,33} In addition to being causative agents in multiple deadly human infections, CoVs are also a major burden as animal pathogens. Cross-species transmission from bats into animals and humans has been well documented in multiple instances, including: porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), SADS-CoV, HCoV-OC43, HCoV-229E, SARS-CoV, MERS-CoV, and possibly SARS-CoV-2.^{14,20,34–36}

As global surveillance and sampling increase and improve, novel CoVs are gradually being identified, including a canine CoV (CCoV-HuPn-2018) isolated in Malaysia from a child with pneumonia, a porcine CoV spillover in Haitian children, the discovery of a new HKU5-CoV-2 capable of utilizing human ACE2 for viral entry, and other CoVs capable of utilizing mammalian receptors for viral entry.^{9,27–29,37–39} The discovery of CoVs capable of using orthologues of human receptors involved in viral infection, such as angiotensin converting enzyme 2 (ACE2) and dipeptidyl peptidase 4 (DPP4), demonstrates promiscuity of receptor use and the potential for more future zoonotic infections. A highly pathogenic zoonotic infection that utilizes DPP4 is MERS-CoV.

Middle East Respiratory Syndrome Virus (MERS)

The causative agent of the Middle East Respiratory Syndrome (MERS) is MERS-CoV, which was first identified in a patient in Jeddah, Saudi Arabia, in 2012.⁷ Since its discovery there have been reported cases of MERS from 27 countries, with a case-fatality rate of 36%.⁴⁰ MERS has primarily impacted countries from the Eastern Mediterranean regions but has also been found in South and East Asia, with the second largest known outbreak occurring in South Korea in 2015.^{40–42} Human infection with MERS-CoV is often linked to direct contact with dromedary camels with nosocomial outbreaks in healthcare facilities being sources of major outbreaks.^{42–44} MERS-CoV is not as infectious as SARS-CoV-2 is from human-to-human exposure, but local outbreaks from close contact with camels and animal produce are the primary sources of new

MERS-CoV cases that can cascade into nosocomial infections.⁴⁵ While dromedary camels are the primary source of human infection, MERS-CoV originated in bats but circulated within camels as an intermediary host before crossing over into humans.⁴⁵ Further evidence of bat origins were discovered from other related Merbecoviruses sharing similar genomes.^{46,47}

For MERS-CoV infection to occur the virus must first infiltrate target cells. The human receptor used by MERS-CoV is CD26 or Dipeptidyl-peptidase-4 (DPP4). DPP4 is a type II transmembrane homodimer expressed in a wide range of tissue, including on the surface of epithelial, endothelial, and lymphoid cells. Receptor binding via the S1 alone is not sufficient for receptor-mediated entry, and requires activation with the help of host proteases.⁴⁸ Type II transmembrane serine protease (TMPRSS2) and members of the cathepsin family (L and K) cleave and activate the spike (S) protein, priming it for membrane fusion. TMPRSS2 is widely expressed in epithelial cells of the human respiratory and gastrointestinal tracts, and is known to enhance viral replication by many viruses including influenza virus, SARS-CoV, NL63-CoV, 229E-CoV, and PEDV.^{49,50} While TMPRSS2-mediated S1/S2 cleavage facilitates viral entry via DPP4 binding, MERS-CoV can utilize an alternative to infect cells with additional cleavage by cathepsin proteases. A separate S2' site can be cleaved by cathepsin L proteases found within endosomal compartments, allowing membrane fusion and subsequent viral entry.^{48,50} Utilization of both DPP4 and proteases enables MERS-CoV to enter cells via two distinct pathways: the cell surface through activation of S by TMPRSS2 and DPP4 binding, and the endosome by cathepsins (**Figure 2**). Another mechanism involved in receptor binding is cell-surface sialoglycoconjugates between the N-terminal domain (NTD) and sialic acid, a feature that is shared with HKU1-CoV, a Beta-CoV endemic in humans, and influenza virus.⁵¹⁻⁵³

Although protease inhibitors have been shown to inhibit viral entry of MERS-CoV *in vitro* there are no clinically available vaccines or therapeutics on the market against this pathogen with

multiple candidates undergoing phase 1 clinical trials.^{49,50,54,55} Despite the development of multiple vaccine candidates, the correlates of protection and immunity remain unclear, with no definitive target antigen identified. Neutralizing antibodies directed against different epitopes along the spike protein have been associated with protection within animal models but protection within humans remains unclear.

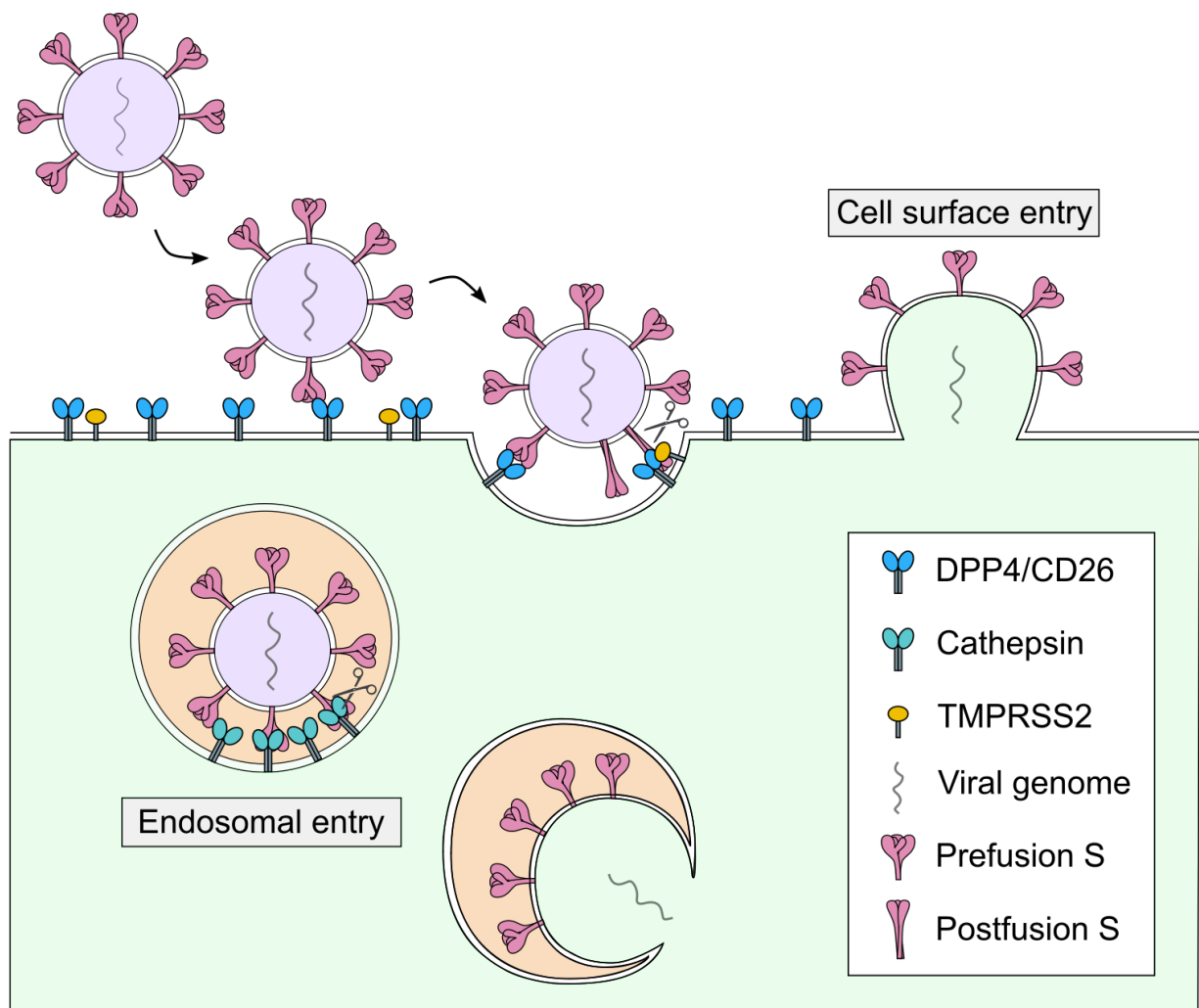


Figure 2. Overview of MERS-CoV infection.

Steps of MERS-CoV infection are shown. Cell surface viral entry can be mediated by binding of S to DPP4 and cleavage by TMPRSS2 protease resulting in the fusion of viral and host

members and subsequent infection. An alternative TMPRSS2 independent pathway is through the endosome where host cathepsins cleave S, enabling membrane fusion and viral entry.

Strategies implemented in vaccine development

Vaccination is one of the most effective ways of preventing and controlling the spread of disease, which is particularly critical for those with preexisting health conditions and comorbidities that leave groups of people vulnerable to severe disease. Prophylactic vaccines have historically eradicated deadly or incapacitating viral and bacterial pathogens, infections such as polio, smallpox, measles, and tetanus. Not only have vaccination campaigns saved well in excess of 10 million lives globally from viral infections, with roughly 2-3 million childhood lives annually reducing childhood mortality rates, but they have also reduced economic burdens by preventing medical expenditures and associated costs that come with healthcare.⁵⁶⁻⁵⁹

The goal of vaccines is to educate and train the immune system to recognize, combat, and remember foreign pathogens when infection occurs, and they often utilize components of pathogens to achieve these goals. Traditional vaccine approaches using whole pathogens have prevented many diseases, however shortcomings in efficacy, safety, antigen stability, and manufacturing underscore the need for diverse vaccine platforms. There are multiple types of vaccine approaches that each have their own benefits and drawbacks that must be considered, such as selecting the right target protein on a pathogen, delivery to specific tissue compartments, and stimulating a particular type immune response, all of which are pathogen-dependent (**Figure 3**). The COVID-19 pandemic in 2019 was another remarkable example of multiple successful therapeutics produced as a result of monumental efforts implemented across the world to rapidly develop and scale the production of vaccines against the SARS-CoV-2 virus. Global efforts to combat the recent pandemic utilized every approach

used in vaccine design including whole inactivated virus, recombinant proteins, peptides, DNA and mRNA (Moderna and Pfizer), viral vectors and live attenuated virus (AstraZeneca, Johnson and Johnson, Gamaleya, and CanSino Biologics) and virus-like particles.^{60–64} Although there are a multitude of vaccine approaches that can be taken, all of these modalities aim to elicit long-lasting protective antibody responses.

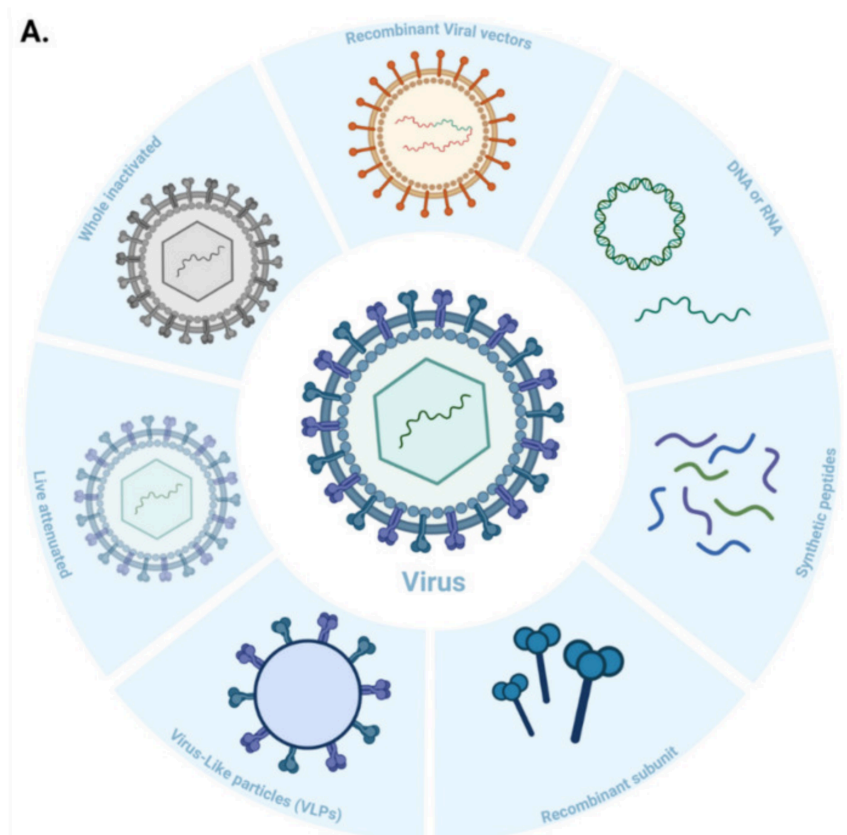


Figure 3. Vaccine approaches against viral pathogens.

Different types of approaches taken towards developing vaccines against viral pathogens. These include recombinant viral vectors, nucleic acid delivery (DNA & mRNA), small peptides and epitope scaffolding, recombinant proteins as soluble antigens, VLPs and self-assembling protein nanoparticles, live attenuated viruses, and inactivated virus.

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B cells (BCs) are the primary mediator in humoral immunity through the antibody production and memory but also play major roles in antigen uptake and processing, as well as presentation for T cell (TC) activation.^{65–67} Upon immunization or infection, naive BCs migrate into secondary lymphoid organs and differentiate into short-lived antibody-producing plasmablasts. BCs can also migrate into germinal centers (GCs) within B cell follicles where they undergo rounds of proliferation and clonal selection, affinity maturation, and somatic hypermutation to generate antigen-specific memory BCs and long-lived plasmablasts that produce antibodies to combat infection. Neutralizing antibodies are a known correlate of protection, thus a primary goal in vaccine development is the production of protective high-affinity antigen-specific antibodies. Self-assembling protein nanoparticles have become widely adopted as a vaccine platform due to their repetitive presentation of antigens and other proteins of interest on a single particle. This approach mimics the spatially repetitive structures that are often seen in nature (viruses, bacteria, and parasites) which immune systems have evolved to recognize. Having the ability to exploit this tailorable method of antigen presentation has shown to enhance lymph node trafficking of vaccinated immunogens, improve B Cell activation, and elicit potent immune responses.^{65,68–72}

Computationally engineered protein nanoparticles unlock another dimension of tailorability to accommodate different symmetries of antigens being presented, such as trimeric viral glycoproteins including the Respiratory Syncytial virus fusion protein, the S protein from coronaviruses, HIV envelope, and influenza virus hemagglutinin surface proteins.^{73–77} Smaller subunit antigens can also be presented on these nanoparticles to obtain benefits seen from multivalent presentation that would otherwise be poor immunogens if left as soluble proteins.⁷⁸ A major milestone that was developed during the pandemic was SKYCovione™, the first computationally-designed protein nanoparticle vaccine clinically approved for use in humans

(**Figure 4**), which elicited higher live-virus neutralizing activity than ChAdOx1-S, the viral vector vaccine encoding the SARS-CoV-2 spike protein developed by AstraZeneca, after two doses.⁷⁹ Another advantage of this approach is the production of mosaic and cocktail nanoparticle formulations, containing antigens of different strains and variants, that are capable of eliciting broadly reactive immune responses within CoV subgenera.^{73,80–82} Recent advances in protein nanoparticle vaccines make this a versatile tool for future vaccine campaigns.

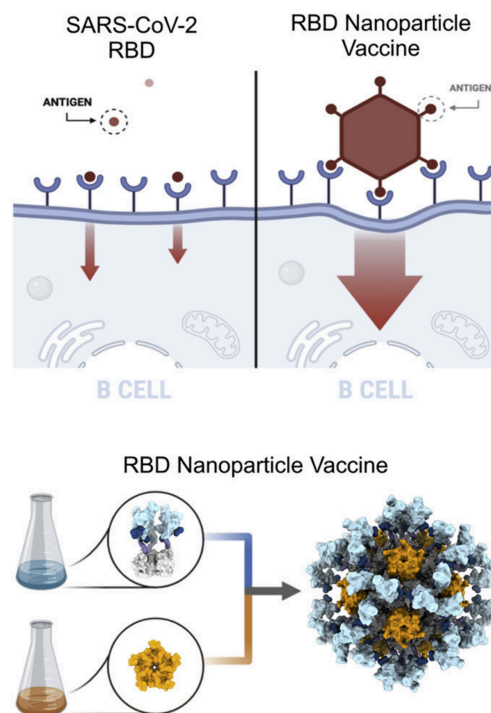


Figure 4. Nanoparticle presentation of subunit antigens enhances immune responses.

Nanoparticle immunogens promote BC receptor clustering due to multivalent presentation of antigens. A self-assembling nanoparticle displaying the SARS-CoV-2 RBD made of trimeric components displaying the RBDs and a pentameric protein.

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An overview of CoV Spike proteins

CoVs are enveloped viruses with positive-sense RNA genome and have three structural surface proteins, the spike (S), envelope (E), and membrane (M) proteins. The surface of the virion is decorated with multiple copies of the S protein, which has diverse receptor usage and is responsible for determining host specificity and tissue tropism, binding host cell receptors, membrane fusion, and viral entry.¹ The spike is a homotrimeric class I fusion glycoprotein organized as the receptor binding and membrane fusion domains, S1 and S2 respectively, capable of adopting “open” and “closed” conformational states (**Figure 5**).

The S1 subdomain can be further broken down into the receptor binding domain (RBD), the portion that functionally binds host receptors, and the N-terminal domain (NTD), which is responsible for binding sugars.^{53,83–90} One notable feature of S1 is that they are highly genetically diverse and share little sequence similarity identity from different subgenera, despite sharing structural homology, and are considered the principal determinant of host range within the spike.^{18,26,27,84,86,91,92} The RBD of CoV RBDs can be structurally split into an external subdomain, often containing the receptor binding motif (RBM), and structurally conserved core subdomain encompasses the highly conserved hydrophobic pocket, often used for linoleic acid binding.^{1,84,93,94} The S2 is the viral fusion machinery region and is highly conserved across all known CoVs, comprising the fusion peptide (FP), heptad repeat sequences 1 and 2, the transmembrane domain, and the cytoplasm domain.⁹⁵ Viral entry of some CoVs often involves proteolytic cleavage of S by host proteases, resulting in primed S1 and S2 subunits ready to engage host receptors. Further proteolytic processing at the S2 may occasionally be required for S to undergo large conformational shifts from prefusion to postfusion states during membrane fusion.^{49,96,97} In most cases, collected events of receptor binding and proteolytic cleavage of the S protein are required for viral infection, both of which play a role in navigating

species barriers. Due to the natural function of the S protein in viral infection, it is often the target of immune responses making it a prime candidate for vaccine development.

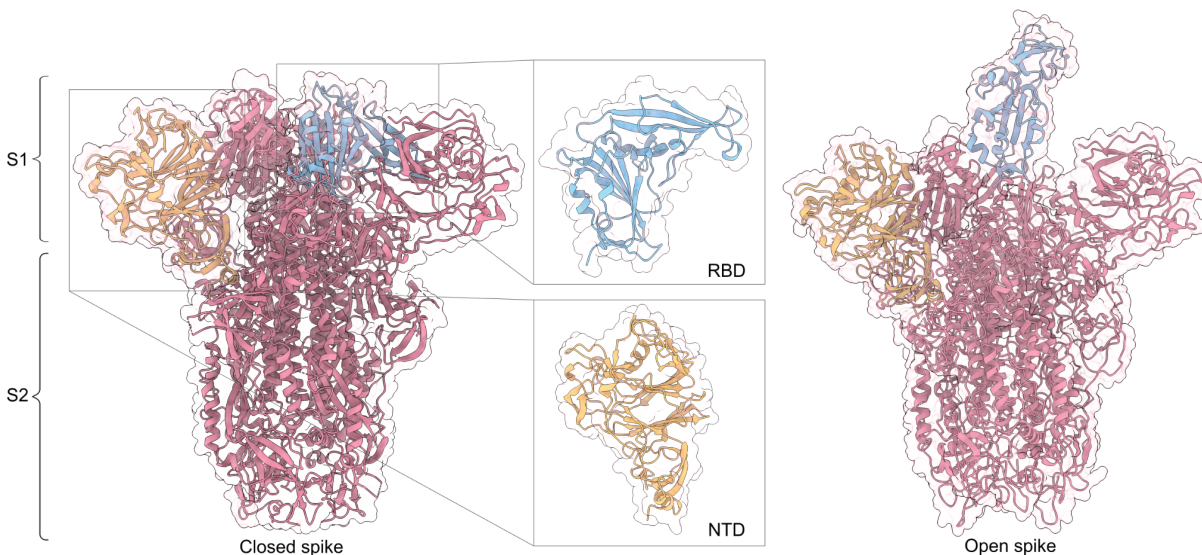


Figure 5. Coronavirus spike protein overview.

MERS-CoV spike protein depicted in a closed and open state. The S1 domain contains the RBD and NTD, and the S2 fusion machinery is found at the base and threads within the interior of S. An open spike exposes the receptor binding motif along the RBD, enabling host receptor binding and subsequent viral entry. Models were made using PDB: 6QO4 (left) and 5X5F (right).

Vaccines against MERS

As of May 2024, the WHO reported a total of 2,613 laboratory-confirmed cases of MERS infection with 943 associated deaths.⁹⁸ This alarmingly high case-fatality ratio makes MERS-CoV a great public health concern, and furthermore, after the emergence of COVID-19, global efforts were implemented to tackle a growing list of prioritized viral and bacterial pathogens. An updated list of Public Health Emergencies of International Concern (PHEICs) from the WHO in 2024 placed MERS-CoV as a priority pathogen and designated pathogens

within the Merbecovirus subgenus as high PHEIC risk.⁹⁹ A separate category of prototype pathogens was included in this list to broaden research into entire pathogen Families to bolster pandemic response strategies should and when they arise.^{100–102} Of note, Merbecoviruses were also included as a high priority on this list.

Two viral proteins that have been targets of immune responses are the spike and nucleocapsid proteins, however, the spike protein was the only target shown to elicit neutralizing antibody responses, making it a primary target for vaccine development.^{103,104} Efforts to stabilize the MERS-CoV S protein in its highly antigenic prefusion conformation demonstrate that the prefusion S is immunogenic and capable of eliciting neutralizing antibody responses.¹⁰⁵ The RBD on S is another prime target for host immune responses due to its role in receptor recognition and initiating infection.^{106,107} The most potently neutralizing antibodies found in SARS-CoV-2 patient samples have overwhelmingly been directed towards the RBD.^{108–111} It has been well-documented that the RBD of CoVs is a highly effective immunogen capable of producing potent neutralizing and protective immune responses that are also cross-reactive against other members within CoV Families.^{78,110,112–114}

There are no clinically licensed vaccines or prophylactics against MERS-CoV, however multiple candidates are undergoing clinical trials, including replication-deficient simian adenovirus (ChAdOx1 MERS) and recombinant modified vaccinia virus Ankara viral vector (MVA-MERS-S) candidates encoding the full MERS-CoV S protein and a DNA vaccine expression full-length MERS-CoV S.^{115–118} These trials demonstrated that ChAdOx1 MERS elicited cellular and humoral immune responses while MVA-MERS-S failed to elicit strong responses after a single dose. Although these two candidates were shown to be safe and well-tolerated, there is also the concern of vector immunity when working with recombinant viral vector vaccines.¹¹⁹ Although many of these candidates have demonstrated potent immune

responses, there are other avenues that require investigating to develop vaccines against MERS-CoV, including the use of subdomains to encourage immune focusing effects to known targets of neutralization, such as the RBD. Multiple RBD-specific neutralizing monoclonal antibodies have been isolated from MERS-infected convalescent patients,^{106,120} MERS-CoV immunogenicity studies,^{121,122} and antibody phage library screens.¹²³ Analysis of convalescent responses in plasma samples gathered from individuals hospitalized with MERS-CoV infection revealed that neutralizing polyclonal antibody responses were predominantly directed towards the RBD, while neutralizing antibodies against conserved S2 domain were much rarer.¹²⁴ Another potential target is the NTD, known to play an important role as an attachment factor during infection. Neutralizing antibodies targeting this subdomain can sterically clash with the RBM or inhibit the spike from adopting an open state (RBD-up).¹²⁵ Multiple neutralizing antibodies have been found to target the NTD of MERS,^{125,126} and recent results demonstrate the protective capabilities of NTD-directed antibodies against viral challenge in mice.¹²⁷

Together, these findings demonstrate that CoV RBDs are not only a primary target for neutralizing antibody responses but also make excellent vaccine immunogens when multimerized on protein nanoparticles. Having well-defined antigens and immune correlates of protection are necessary to develop effective vaccines, however, producing and characterizing candidate constructs are often large barriers that are difficult to overcome. One powerful method used to address this is structure-guided protein design.

Structure-guided and computational protein stabilization methods

Classical structure-guided rational protein design has effectively stabilized antigens from a myriad of viral, bacterial, and parasitic pathogens for therapeutic use and experimental probes. Efforts in the form of cavity-filling mutations, resurfacing exposed hydrophobics, or locking conformational states have yielded stabilized viral antigens that enhanced expression and

overall stability of viral antigens. A hallmark success of rational protein design is a two proline substitution (2P) within the MERS-CoV S protein.¹⁰⁵ The mutations not only locked S in a prefusion state, but also significantly enhanced its expression, antigenicity, and stability, and was also determined to be widely portable among other CoV S proteins.¹⁰⁵ This finding was paramount in future investigative work with other CoVs and, notably, the initial analyses of stabilized SARS-CoV-2 S proteins during the start of the COVID-19 pandemic.^{128–130} Another successful CoV antigen stabilized was the SARS-CoV-2 RBD. Through a combined effort using rational protein design and deep mutational scanning (DMS) to highlight mutations that enhanced expression, the Rpk9 cavity filling mutations were discovered within the linoleic acid binding pocket.¹³¹ These mutations significantly enhanced protein production yields, a boon for manufacturing purposes, improved thermal stability, and reduced off-target oligomers from forming. It is clear that structure-guided protein design is a powerful technique that has guided numerous vaccine design campaigns, however, it is highly dependent on published structures which are not widely available for many pathogens.

Deep learning methods in the protein space have rapidly revolutionized and expanded protein design capabilities. A prime example is AlphaFold, a neural network capable of predicting highly accurate protein structures with amino acid sequences and multiple sequence alignments as inputs.^{132–136} Accurate model predictions of protein complexes can facilitate design efforts for naturally occurring proteins with unpublished structures as well as *de novo* designs. A deep learning tool that has expanded protein sequence design capabilities is protein Message Passing Neural Network (proteinMPNN), a neural network that takes in a protein backbone and distances between C α , N, C, O as input sequences and generates designed protein sequences for selected residues (**Figure 6**).^{137,138} ProteinMPNN is a powerful tool that can stabilize *de novo* and naturally occurring proteins while retaining functionality if given the right context including sequence conservation, epitopes or paratopes of interest, and protein interfaces. These

advances in sequence design and structure prediction create an iterative process that allows for rapid protein design that can be evaluated *in silico*. As these methods have taken off, multiple groups have implemented protein design and stabilization efforts using these tools and demonstrated successes, including improved stability of protein nanomaterials, targeted binder design, production of naturally occurring enzymes, and stabilization of microbial therapeutic targets.^{137,139,140} Ongoing advances to these tools continue to hone existing methods and broaden the scope of protein design. Sequence design can now be expedited with the nuances provided with new deep learning tools. Implementing these new methods into vaccine design can ameliorate restrictions on antigen stabilization imposed by a dependence on published protein structures of high quality and atomic level detail.

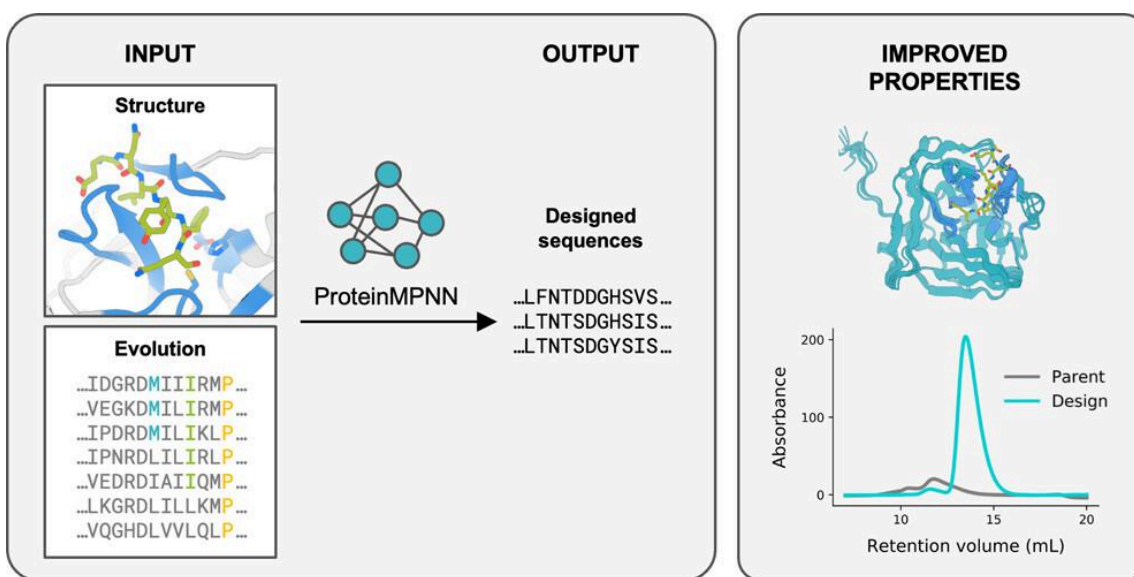


Figure 6. Improving sequence design using proteinMPNN

Overview of using proteinMPNN for sequence prediction provided a 3D structure and desired residues to optimize. Designed sequences are then outputted and can be further evaluated with other computation tools before experimental testing for improved properties.

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Summary and thesis motivation

The emergence of multiple deadly zoonotic spillovers within the past three decades underscores the need to develop therapeutic strategies against CoVs. Despite regional containment of MERS-CoV within sporadic outbreaks, the virus still remains a global threat. Protein nanoparticles bearing SARS-CoV-2 RBDs have proven to be highly potent and protective immunogens, with SKYCovione becoming the first clinically licensed computationally designed nanoparticle vaccine. With the platform being well-characterized and clinically de-risked, applying this same method to address MERS-CoV, and other members of the subgenus, may enlighten determinants of protection towards these viruses. In this thesis, I evaluate MERS-CoV nanoparticle immunogens as vaccine candidates and explore the efficacy of multiple S immunogens. I also implement computational protein design, guided by DMS, to develop a set of protein nanoparticles displaying Merbecovirus RBDs from each clade within the subgenus. My thesis work found that MERS-CoV RBD nanoparticles generated neutralizing and protective immunity in mice, and also produced an array of RBD-directed antibody responses shown from epitope mapping. My work further created a handful of novel Merbecovirus RBD nanoparticles that will be used to uncover more about viral members in the Subgenus. Additionally, I developed a hexavalent mosaic nanoparticle that will be assessed alongside the MERS-CoV RBD particle as potential vaccine candidates against MERS-CoV and possibly other Merbecoviruses in ongoing animal studies.

Chapter 2: Protein nanoparticle vaccines induce potent neutralizing antibody responses against MERS-CoV

This chapter was reproduced from the following article:

Chao CW, Sprouse KR, Miranda MR, Catanzaro NJ, Hubbard ML, Addetia A, Stewart C, Brown JT, Dosey A, Valdez A, Ravichandran R, Hendricks GG, Ahlrichs M, Dobbins C, Hand A, McGowan J, Simmons B, Treichel C, Willoughby I, Walls AC, McGuire AT, Leaf EM, Baric RS, Schaefer A, Veelsler D, King NP. "Protein nanoparticle vaccines induce potent neutralizing antibody responses against MERS-CoV." *Cell Rep* 43, 12 (24 December, 2024): 115036.

<https://doi.org/10.1016/j.celrep.2024.115036>

ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) is a betacoronavirus that causes severe respiratory illness in humans. There are no licensed vaccines against MERS-CoV and only a few candidates in phase I clinical trials. Here, we develop MERS-CoV vaccines utilizing a computationally designed protein nanoparticle platform that has generated safe and immunogenic vaccines against various enveloped viruses, including a licensed vaccine for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Two-component nanoparticles displaying spike (S)-derived antigens induce neutralizing responses and protect mice against challenge with mouse-adapted MERS-CoV. Epitope mapping reveals the dominant responses elicited by immunogens displaying the prefusion-stabilized S-2P trimer, receptor binding domain (RBD), or N-terminal domain (NTD). An RBD nanoparticle elicits antibodies targeting multiple non-overlapping epitopes in the RBD. Our findings demonstrate the potential of two-component nanoparticle vaccine candidates for MERS-CoV and suggest that this platform technology could be broadly applicable to betacoronavirus vaccine development.

INTRODUCTION

The recent SARS-CoV-2 pandemic demonstrated the human and economic toll that can accompany the spillover and spread of a zoonotic disease in humans. Although the success of vaccine development efforts in response to the pandemic were a triumph of modern vaccinology, SARS-CoV-2 remains the only coronavirus for which licensed vaccines are available. To date, nine coronaviruses are known to infect humans, three of which (SARS-CoV, MERS-CoV, and SARS-CoV-2) have caused epidemics or pandemics in the last 20 years and two of which (CCoV-HuPn-2018 and porcine deltacoronavirus) have been identified in humans in only the past three years, underscoring that more coronaviruses than previously appreciated pose zoonotic threats.^{8,9,25,141–143} Developing vaccines for additional coronaviruses, both known and unknown, is therefore a public health priority.¹⁴⁴

Among the known human-infecting coronaviruses, MERS-CoV stands out due to its high case fatality rate, estimated at 35%.^{40,42} Since its discovery in 2012, MERS-CoV infections have been reported in 27 countries, mostly from contact with dromedary camels, although sporadic human-to-human transmission has also occurred.¹⁴⁵ Beyond its immediate value in the prevention of severe respiratory disease and death caused by MERS-CoV infection, a safe and effective MERS-CoV vaccine would provide a foundation for developing broadly protective vaccines that could prevent zoonotic spillover of known or unknown members of the Merbecovirus subgenus. Three MERS-CoV vaccine candidates have entered clinical trials, although none has advanced to licensure.^{116–118}

As the major surface antigen and the target of neutralizing antibodies, the spike (S) protein has been the focus of most MERS-CoV vaccine development efforts. S is a large trimeric class I viral fusion protein that is cleaved into two subunits, S₁ and S₂.¹⁴⁶ S₁ contains the N-terminal

domain (NTD) and receptor binding domain (RBD) that mediate virus attachment to host cells by binding sialosides and the proteinaceous receptor dipeptidyl peptidase 4 (DPP4), respectively.^{53,83–86} S₂ comprises the fusion machinery that merges the virus and host membranes. Several studies have shown that the majority of serum neutralizing activity after infection or immunization, as well as the most potently neutralizing monoclonal antibodies, target the RBD and NTD,^{103,106,120,122,123,125,126,147} although neutralizing and protective antibodies targeting S₂ have also been characterized.^{148–156} Immunization with the MERS-CoV RBD, S₁, S₂, full-length S, and prefusion-stabilized S ectodomain have been evaluated in preclinical animal models and found to induce robust antibody responses and in some cases protection against challenge.^{73,103,121,122,126,147,148,150,157–160} Nevertheless, vaccine design technologies and lessons from SARS-CoV-2 vaccine development efforts may allow the generation of vaccine candidates with improved safety, immunogenicity, and manufacturability.

A major lesson of the recent SARS-CoV-2 pandemic was that pre-existing platform technologies were essential to rapid vaccine development. For example, the prefusion-stabilizing 2P mutations that were identified using MERS-CoV as a prototype pathogen in 2017¹⁵⁸ enabled essentially immediate structure determination and vaccine design based on the SARS-CoV-2 Spike.^{160–162} The readiness of mRNA as a rapid response vaccine platform also proved critical.^{163,164} Additional platform technologies were clinically de-risked during the pandemic, including computationally designed two-component protein nanoparticle vaccines.^{79,165} Over the last several years, our groups and others have shown that these immunogens elicit potent neutralizing antibody responses against a number of viral pathogens by efficiently trafficking to lymph nodes and enhancing B cell activation.^{73,74,77,166–175} In response to the SARS-CoV-2 pandemic, we developed a nanoparticle vaccine that displays 60 copies of the SARS-CoV-2 RBD on the icosahedral nanoparticle I53-50.¹⁷⁶ RBD-I53-50 induced robust neutralizing antibody responses in mice and NHPs and was found to be safe and immunogenic in clinical trials,

leading to its licensure in multiple jurisdictions under the name SKYCovione™.^{78,79,177–179} Collectively, these results suggest that two-component nanoparticles may be a robust and versatile vaccine platform.

Here, we generate two-component protein nanoparticle immunogens of diverse geometries displaying the MERS-CoV S-2P trimer, RBD, or NTD that elicit potent neutralizing antibody responses targeting antigenic sites in the S₁ subunit. Our findings identify several promising vaccine candidates for further development and establish two-component nanoparticles as a generalizable platform for betacoronavirus vaccine development.

RESULTS

Production and characterization of nanoparticle immunogens

Based on our successful previous application of two-component self-assembling nanoparticles to the development of sarbecovirus vaccines,^{73,79,178–180} we set out to evaluate a series of nanoparticle immunogens for MERS-CoV. The prefusion-stabilized MERS S-2P trimer was previously shown to elicit neutralizing antibodies in mice as a soluble trimer,¹⁵⁸ when displayed on nanoparticles,⁷³ or when delivered by mRNA.¹⁶⁰ To further explore the immunogenicity of this antigen, we displayed 4 or 20 copies of the S-2P trimer on two distinct nanoparticles, T33_dn10 and I53-50. T33_dn10 is composed of four copies each of two different designed trimeric proteins that assemble into a 24-subunit nanoparticle with tetrahedral symmetry,⁷⁵ while I53-50 is constructed from 12 pentamers and 20 trimers that form a 120-subunit complex with icosahedral symmetry.¹⁷⁶ In each case, the S-2P ectodomain trimer with a C-terminal foldon was genetically fused to a trimeric component with externally oriented N termini (**Figure 1A** and **Table S1**). Given the success of our RBD-I53-50 nanoparticle vaccine against SARS-CoV-2^{79,180} and the key role of the NTD in the attachment of MERS-CoV to cells,^{53,83} we also evaluated I53-50-based nanoparticle immunogens displaying the MERS-CoV RBD or NTD. We genetically

fused these domain-based antigens to the N terminus of the I53-50A trimer to enable display of 60 copies on the I53-50 nanoparticle in a manner analogous to our previously described SARS-CoV-2 RBD nanoparticle vaccine.¹⁸⁰

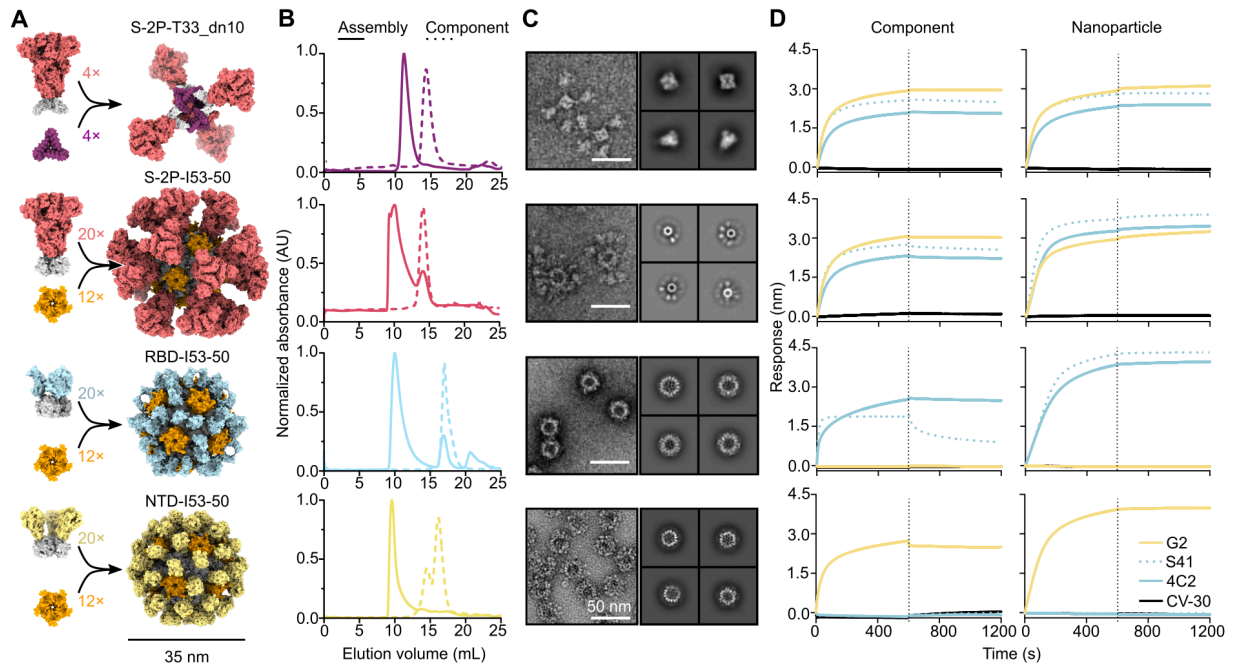


Figure 1. Production and characterization of nanoparticle immunogens

(A) Schematics of MERS-CoV nanoparticle immunogen assembly.

(B) Size exclusion chromatograms of MERS-CoV nanoparticle components (dotted lines) and assembled nanoparticles (solid lines) on a Superose 6 10/300 GL.

(C) *Left*, raw micrographs and *right*, 2D class averages of negatively stained SEC-purified nanoparticles. For S-2P-T33_dn10, class averages of the nanoparticle core and displayed S-2P trimer are shown on top and bottom, respectively. Scale bar, 50 nm.

(D) Binding of *left*, antigen-bearing components prior to assembly and *right*, SEC-purified nanoparticle immunogens to mAbs G2 (NTD-specific), S41 (RBD-specific), 4C2 (RBD-specific), and CV-30 (SARS-CoV-2 S RBD-specific; negative control).

We secreted these glycoprotein antigen-bearing components from transfected Expi293F cells and purified them from clarified supernatants using immobilized metal affinity chromatography. We also expressed and purified the soluble MERS-CoV S-2P trimer as a fusion to the foldon trimerization domain for use as a benchmark. Size exclusion chromatography (SEC) of the S-2P-T33_dn10A and S-2P-I53-50A trimers showed that these components eluted at the same

volume as the S-2P foldon trimer, while the RBD- and NTD-I53-50A components eluted later, as expected due to their smaller size (**Figures 1B** and **S1A**). After mixing each antigen-bearing component with the appropriate second component (i.e., trimeric T33_dn10A or pentameric I53-50B), a shift to an earlier SEC elution volume was observed, suggesting nanoparticle assembly. Small amounts of residual unassembled components were observed in the S-2P- and RBD-I53-50 assembly reactions, as expected given that each contained a slight excess of the trimeric component (see Methods). Analysis of the SEC-purified nanoparticles by SDS-PAGE confirmed that each preparation contained both of the expected protein components, and dynamic light scattering (DLS) indicated monodisperse nanoparticles with the expected hydrodynamic diameters (**Figures S1B** and **S1C**). Negative stain electron microscopy (nsEM) supported the SEC and DLS data, revealing fields of monodisperse nanoparticles of the expected sizes and shapes. S-2P-T33_dn10 clearly displayed four copies of the S-2P trimer, although these were flexibly linked to the underlying nanoparticle core: we obtained separate classes during 2D averaging corresponding to the tetrahedral nanoparticle and the displayed prefusion S trimer (**Figure 1C**). nsEM of S-2P-I53-50 revealed spherical assemblies densely decorated with Spikes that resembled micrographs previously obtained for sarbecovirus S-I53-50 immunogens.^{181–183} RBD- and NTD-I53-50 also formed well-defined nanoparticles with additional densities on the nanoparticle surface, although these were less clearly defined than displayed S-2P due to the smaller size of the RBD and NTD antigens. Having confirmed expression of the antigen-bearing components and assembly of the nanoparticle immunogens, we next sought to evaluate retention of antigenicity using bio-layer interferometry (BLI) and a panel of conformation-specific monoclonal antibodies (mAbs) (**Figure 1D**). As expected, the two RBD-specific antibodies S41¹⁸⁴ and 4C2¹²² bound to all antigen-bearing components and nanoparticles except for NTD-I53-50A and NTD-I53-50. Conversely, the NTD-directed antibody G2^{103,125} bound to all constructs except RBD-I53-50A and RBD-I53-50. Dissociation was unambiguously observed only for the RBD-I53-50A–S41 complex; as expected, the highly avid

interactions between the corresponding RBD-I53-50 nanoparticle and the S41 mAb prevented dissociation. Our negative control antibody CV-30, a SARS-CoV-2 S RBD-directed neutralizing antibody isolated from a convalescent patient,¹⁸⁵ did not bind to any of our MERS constructs. Together, these data demonstrate the production of a series of well-defined and antigenically intact nanoparticle immunogens for MERS-CoV displaying the prefusion-stabilized S-2P trimer, RBD, or NTD.

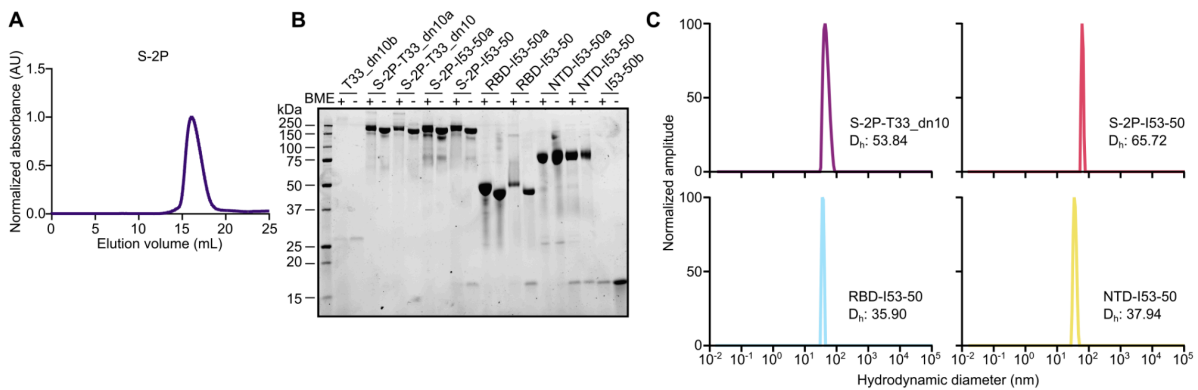


Figure S1. Characterization of MERS-CoV S-2P and nanoparticle immunogens, related to Figure 1.

(A) MERS-CoV S-2P SEC on a Superose 6 Increase 10/300 column.

(B) SDS-PAGE of nanoparticle components before and after assembly under reducing and non-reducing conditions. BME, β -mercaptoethanol.

(C) DLS of assembled nanoparticles measured on an UNcle (UNchained Labs). Hydrodynamic diameters (D_h) are indicated.

Antibody responses elicited by MERS-CoV nanoparticle immunogens in mice

We evaluated the immunogenicity of our MERS-CoV nanoparticles by measuring antigen-specific and neutralizing antibody titers in serum after immunizing groups of 10 BALB/c mice twice with AddaVax-adjuvanted immunogens four weeks apart (**Figure 2A**). Two weeks post-prime, the highest levels of S-2P-specific antibodies were observed in the sera of animals that received RBD-I53-50 (geometric mean titer (GMT) EC_{50} of 5.6×10^3), followed by S-2P-I53-50 (1.6×10^3); all other groups had GMTs $< 10^3$ (**Figures 2B and S2A**). Two weeks

post-boost, all groups but the bare I53-50 nanoparticle control showed high antibody titers, ranging from $3.2\text{--}8.4\times 10^4$, that slightly waned by week 8 ($7.0\times 10^3\text{--}1.9\times 10^4$). S-2P, S-2P-T33_dn10, and, to a lesser extent, S-2P-I53-50 elicited serum antibodies that bound to SARS-CoV and SARS-CoV-2 HexaPro-foldon trimers,¹²⁸ but we did not observe similar cross-reactivity from the nanoparticles displaying the domain-based antigens (**Figures S2B and S2C**). Upon further investigation, these sera bound similarly to an influenza hemagglutinin-foldon trimer but not to an OC43 S-2P trimer fused to the GCN4 tetramerization domain, implicating anti-foldon antibodies, which we subsequently confirmed by nsEM polyclonal epitope mapping (ns-EMPEM; **Figure S2D**).^{186,187} Antibody responses against the T33_dn10 and I53-50 nanoparticle scaffolds were also observed in sera from the expected groups (**Figures S2E and S2F**).

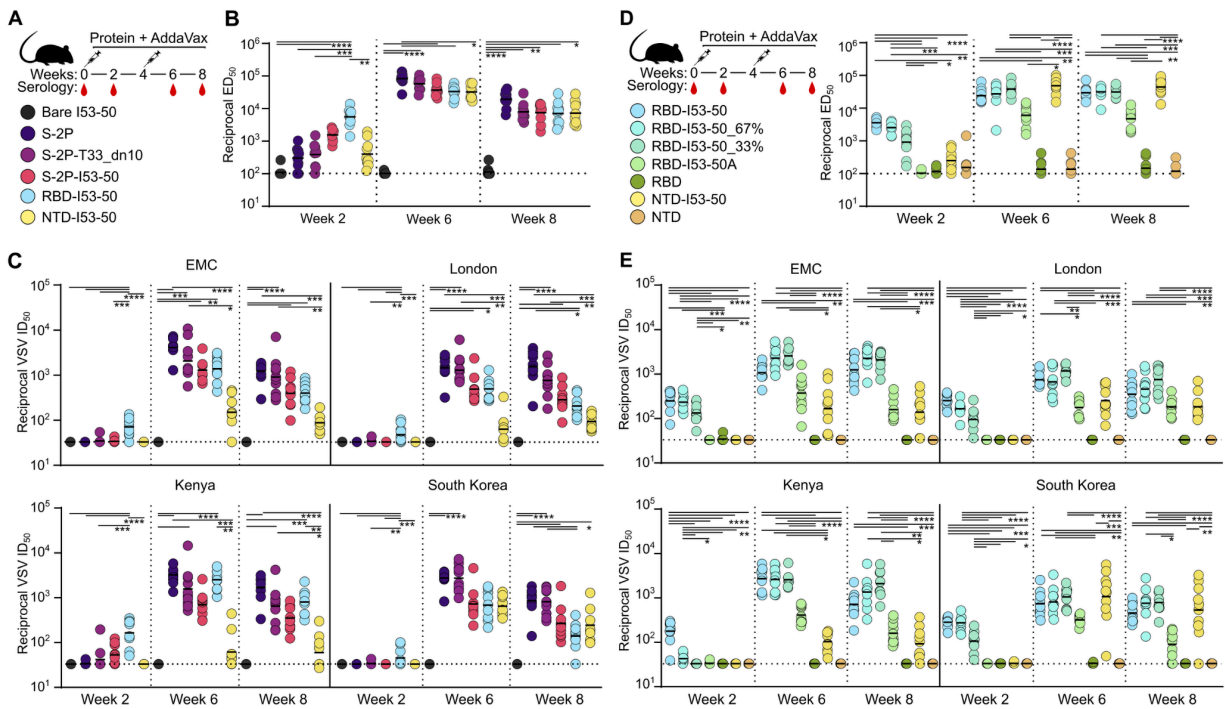


Figure 2. Antibody responses elicited by MERS-CoV nanoparticle immunogens in mice

(A) Study design and groups. Groups of 10 BALB/c mice were immunized at weeks 0 and 4, and serum was obtained at weeks 0, 2, 6, and 8.

(B) Serum antibody binding titers against vaccine-matched (EMC) MERS-CoV S-2P,

measured by ELISA.

(C) Vaccine-elicited neutralizing activity against VSV pseudotyped with closely related MERS-CoV EMC, London variant, Kenya variant, or South Korea variant spikes. The mouse immunization study was performed twice, and representative data from one study are shown.

(D) *Left*, Domain-based antigen study design and groups. Groups of 10 BALB/c mice were immunized at weeks 0 and 4, and bled at weeks 0, 2, 6, and 8. *Right*, Serum antibody binding titers against vaccine-matched (EMC) MERS-CoV S-2P, measured by ELISA.

(E) Vaccine-elicited neutralizing activity against VSV pseudotyped with closely related MERS-CoV EMC, London variant, Kenya variant, or South Korea variant spikes. Groups were compared using Kruskal-Wallis followed by Dunn's multiple comparisons. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$. Only significant differences are shown; see Supplemental Data for all statistical comparisons.

Serum neutralizing activity against a panel of VSV pseudotypes bearing closely related MERS-CoV Spikes (EMC, London, Kenya, and South Korea; the latter three have 99% amino acid identity to the vaccine strain; **Table S1**) largely mirrored the binding antibody titers. Specifically, RBD-I53-50 was the only immunogen that elicited appreciable neutralizing activity post-prime (EMC strain GMT 0.7×10^2 and Kenya strain GMT 1.6×10^2), while potent neutralization was observed post-boost for all groups at week 6, with slight subsequent waning by week 8 (**Figures 2C** and **S3**). Slightly more separation between the groups was observed in the post-boost neutralization data than in the antigen-specific antibody titers. Specifically, the soluble S-2P trimer and S-2P-T33_dn10 generally induced the highest titers, although these were not significantly different from those induced by S-2P-I53-50. Nevertheless, given that several previous studies have found that immunogenicity tends to increase with increasing antigen valency,^{65,77,188} and that we had previously observed that a SARS-CoV-2 RBD-I53-50 nanoparticle elicited more potent neutralizing activity than the corresponding S-2P trimer,¹⁸⁰ we investigated potential causes for the lower than expected immunogenicity of S-2P-I53-50 relative to the S-2P trimer and S-2P-T33_dn10. First, we compared the thermal stabilities of the SARS-CoV-2 and MERS-CoV S-2P trimers, as previous studies have demonstrated that more stable antigens give rise to enhanced immunogenicity.^{128,131,189,190} However, we did not find substantial differences in stability (**Figure S4A**), suggesting that other mechanisms likely

account for the improved performance of the MERS-CoV S-2P trimer relative to RBD-I53-50 compared to the analogous SARS-CoV-2 immunogens. When analyzing the S-2P-I53-50 nanoparticle, we noticed increasing amounts of unassembled components in electron micrographs prepared from the later fractions of the broad SEC peak obtained after *in vitro* assembly (**Figure S4B**). We also found that S-2P-I53-50 aggregated upon heating, whereas the S-2P-T33_dn10 nanoparticles did not aggregate at temperatures up to 95°C (**Figure S4C**). These data indicated heterogeneity and instability in the S-2P-I53-50 nanoparticles, which is atypical for I53-50-based immunogens. The levels of neutralization after boosting with the RBD-I53-50 nanoparticle often trended slightly lower than the S-2P-based immunogens, although this only reached significance in a couple cases (i.e., against the London and South Korea variants). By contrast, sera from mice that received the NTD-I53-50 nanoparticle consistently had significantly lower neutralizing activity than the other immunogens with the exception of neutralization of the South Korea variant, which contains an H91Y mutation in the sialic acid bind site of the NTD that may sensitize it to neutralization.^{53,83}

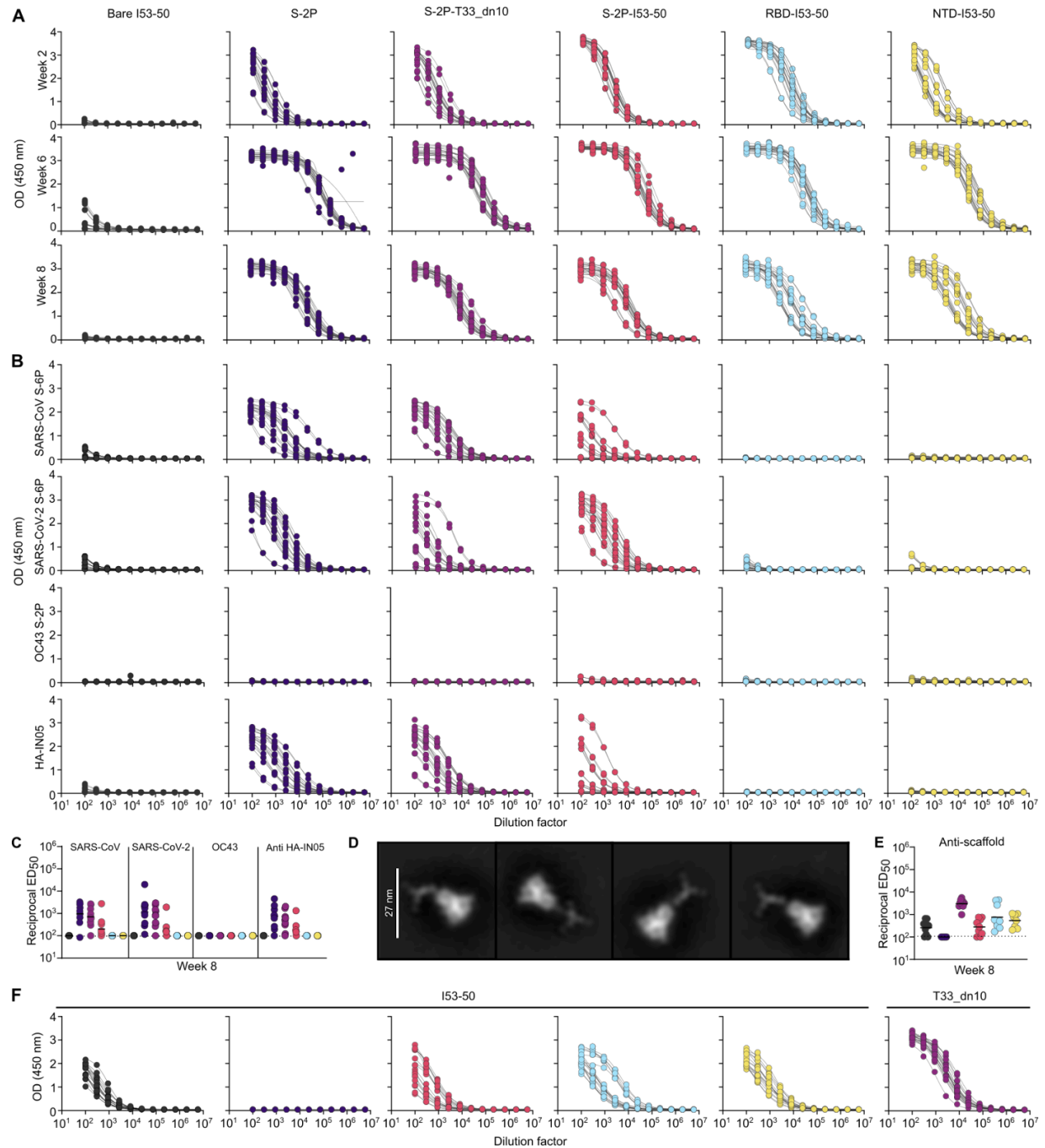


Figure S2. Antigen-, foldon-, and scaffold-specific vaccine-elicited antibodies, related to Figure 2.

(A) Raw week 2, 6, and 8 serum ELISA data against vaccine-matched (EMC) MERS-CoV S-2P.

(B) Week 8 serum ELISA data against SARS-CoV HexaPro (S-6P)-foldon, SARS-CoV-2 HexaPro (S-6P)-foldon, OC43 S-2P-GCN4 and influenza hemagglutinin-foldon (HA-IN05).

(C) Serum antibody titers determined from the data in **(B)**.

(D) nsEM 2D class averages of MERS S-2P vaccine-elicited Fabs complexed with SARS-CoV S-2P, revealing that anti-foldon antibodies predominate.
(E) Titers of vaccine-elicited antibodies against the I53-50 and T33_dn10 nanoparticle scaffolds determined from the data in (F).
(F) Week 8 serum ELISA data against bare I53-50 and T33_dn10 nanoparticles. Groups are colored as in (A) and (B).

Previous work from our group and others has shown that displaying domain-based antigens on nanoparticles significantly improves their immunogenicity.^{167,180,191–196} To evaluate the effect of multimerization on the immunogenicity of the MERS-CoV RBD and NTD, we immunized groups of 10 BALB/c mice twice with 1 µg of RBD-I53-50, the trimeric RBD-I53-50A component alone, NTD-I53-50, monomeric RBD, or monomeric NTD, each formulated with AddaVax (**Figure 2D**). We also included groups of mice that received partial valency RBD-I53-50 nanoparticles in which only 67% or 33% of the trimeric components comprised the RBD as a genetic fusion. Similar to our first study, RBD-I53-50 at 100% valency elicited high levels of S-2P-directed antibody responses after the primary immunization (GMT 3.5×10^3), while NTD-I53-50 yielded lower titers (GMT $< 10^3$) (**Figures 2D** and **S5**). Among the three RBD nanoparticles tested, there appeared to be a slight decrease in immunogenicity with decreasing RBD valency, although the differences between groups were not statistically significant. By contrast, the trimeric RBD-I53-50A and RBD and NTD monomer groups all had minimal responses (GMT $< 10^3$). Two weeks post-boost, all nanoparticle groups displayed comparably high antibody titers (2.4×10^4 – 4.8×10^4), the RBD-I53-50A group was somewhat lower (6.0×10^3), and the monomeric RBD and NTD still failed to induce robust antibody responses. These antigen-specific antibody titers were essentially unchanged in sera obtained at week 8.

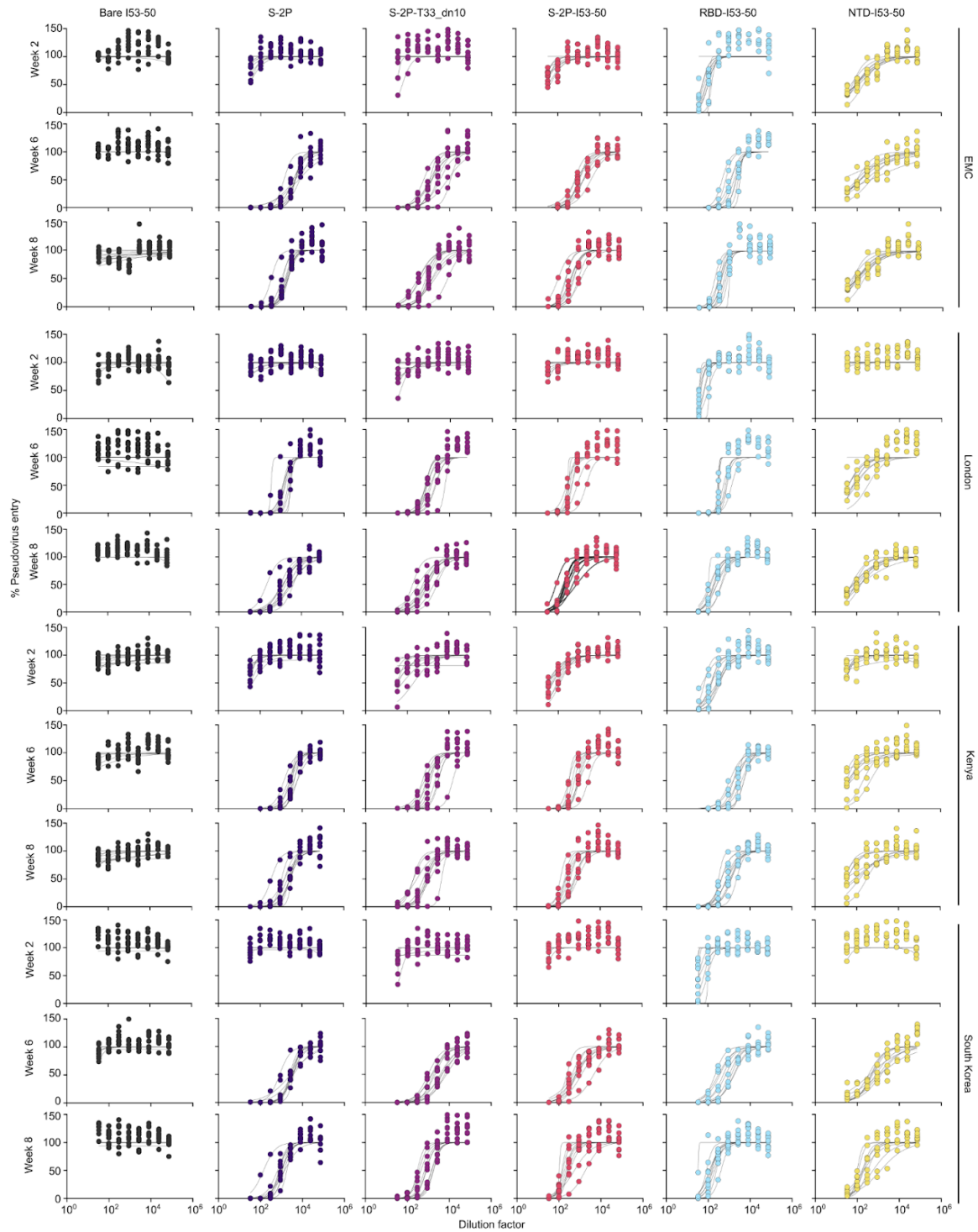


Figure S3. Dose-response curves of MERS-CoV, pseudovirus neutralization, related to Figure 2.

Serum neutralizing activity from weeks 2, 6, and 8 using pseudoviruses bearing the MERS-CoV EMC, London, Kenya, and South Korea variant spikes.

Vaccine-elicited neutralizing activity largely mirrored these observations except that the NTD-I53-50 nanoparticle generally elicited significantly lower levels of neutralization than RBD-I53-50 (**Figures 2E** and **S6**). Notably, all three of the RBD-I53-50 nanoparticles induced detectable neutralizing activity after the primary immunization, while the RBD-I53-50A trimer and the RBD and NTD monomers failed to do so. No change was observed after a second immunization with the monomeric RBD and NTD, while sera from the RBD-I53-50A group yielded detectable neutralization at week 6 and 8 that was lower than the RBD-I53-50 nanoparticle groups, although not always significantly so. These data, in contrast to the S-2P-based immunogens, clearly demonstrate that multimerization significantly enhances the immunogenicity of the domain-based RBD and NTD antigens.

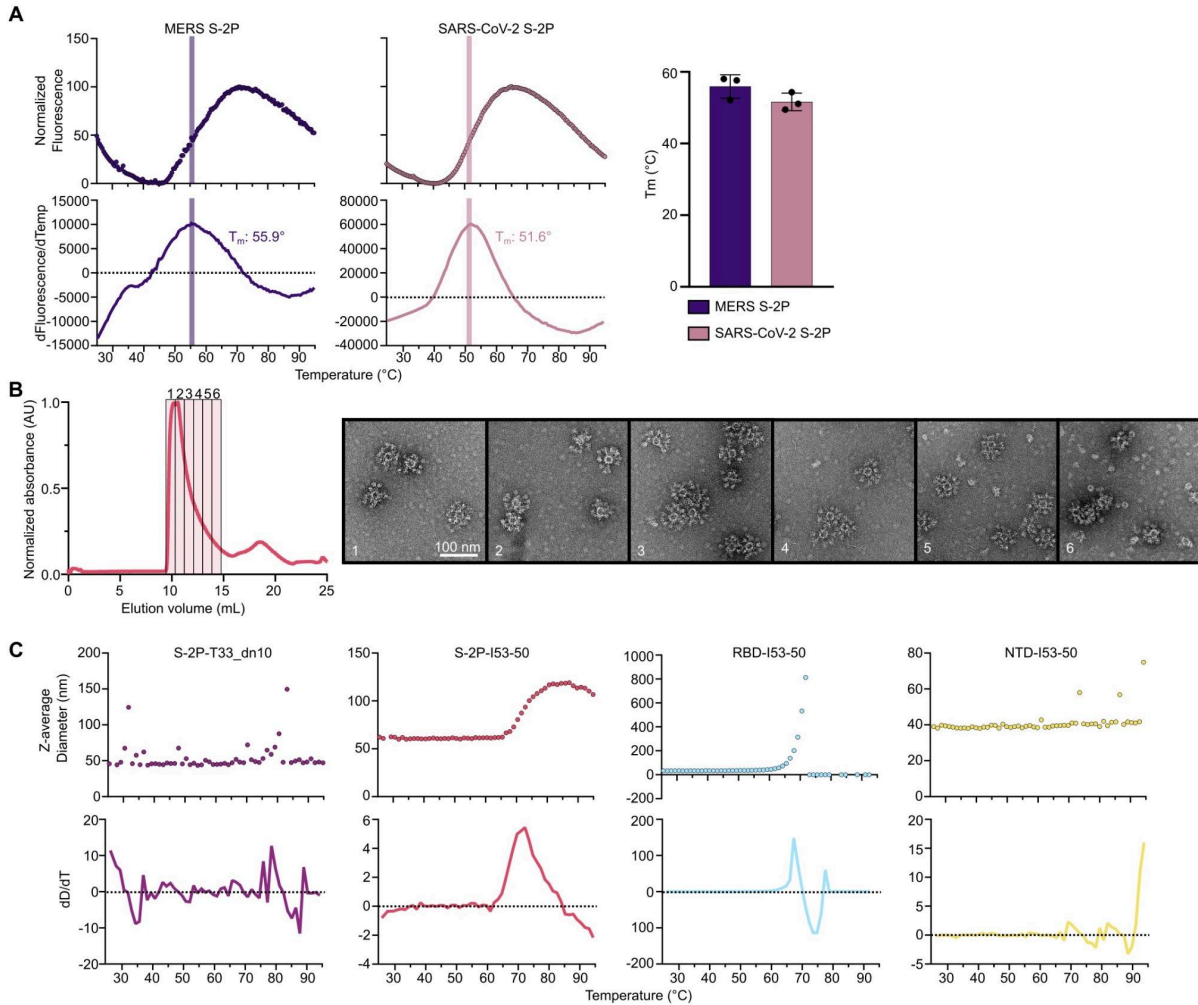


Figure S4. Characterization of MERS-CoV S-2P and nanoparticle immunogen stability, related to Figure 2.

(A) Differential scanning fluorimetry (DSF) of MERS S-2P and SARS-COV-2 S-2P in the presence of SYPRO orange measured from 25–95°C. *Top*, Normalized fluorescence intensity and *bottom*, the first derivative are shown. *Right*, Average melting temperatures (T_m) calculated from three technical replicates are indicated with bars.

(B) Fractions 1–6 of an SEC purification of S-2P-I53-50 nanoparticles were evaluated with ns-EM.

(C) *Top*, DLS measurements of each nanoparticle immunogen from 25–95°C along with *bottom*, corresponding first derivatives.

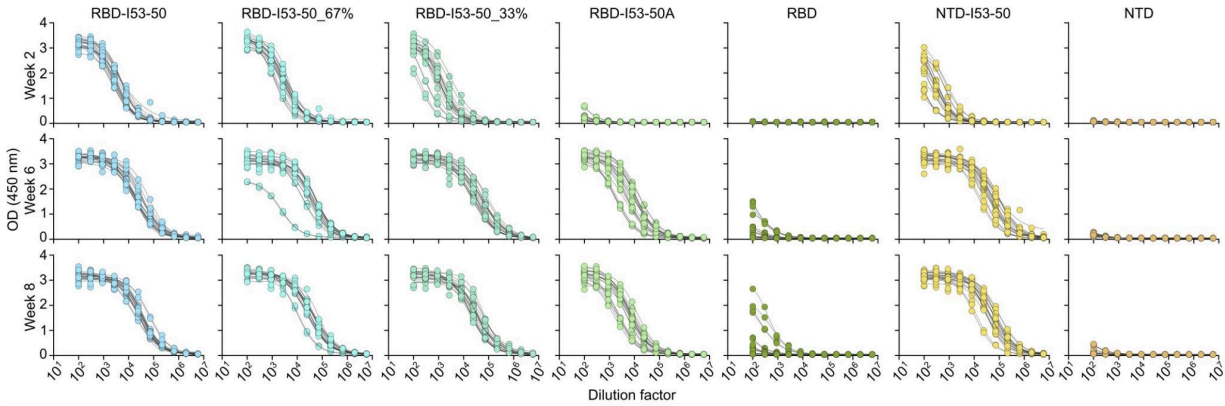


Figure S5. Serum antibody binding to MERS-CoV S-2P from mice immunized with domain-based immunogens, related to Figure 2.

Raw week 2, 6, and 8 serum ELISA data against vaccine-matched (EMC) MERS-CoV S-2P.

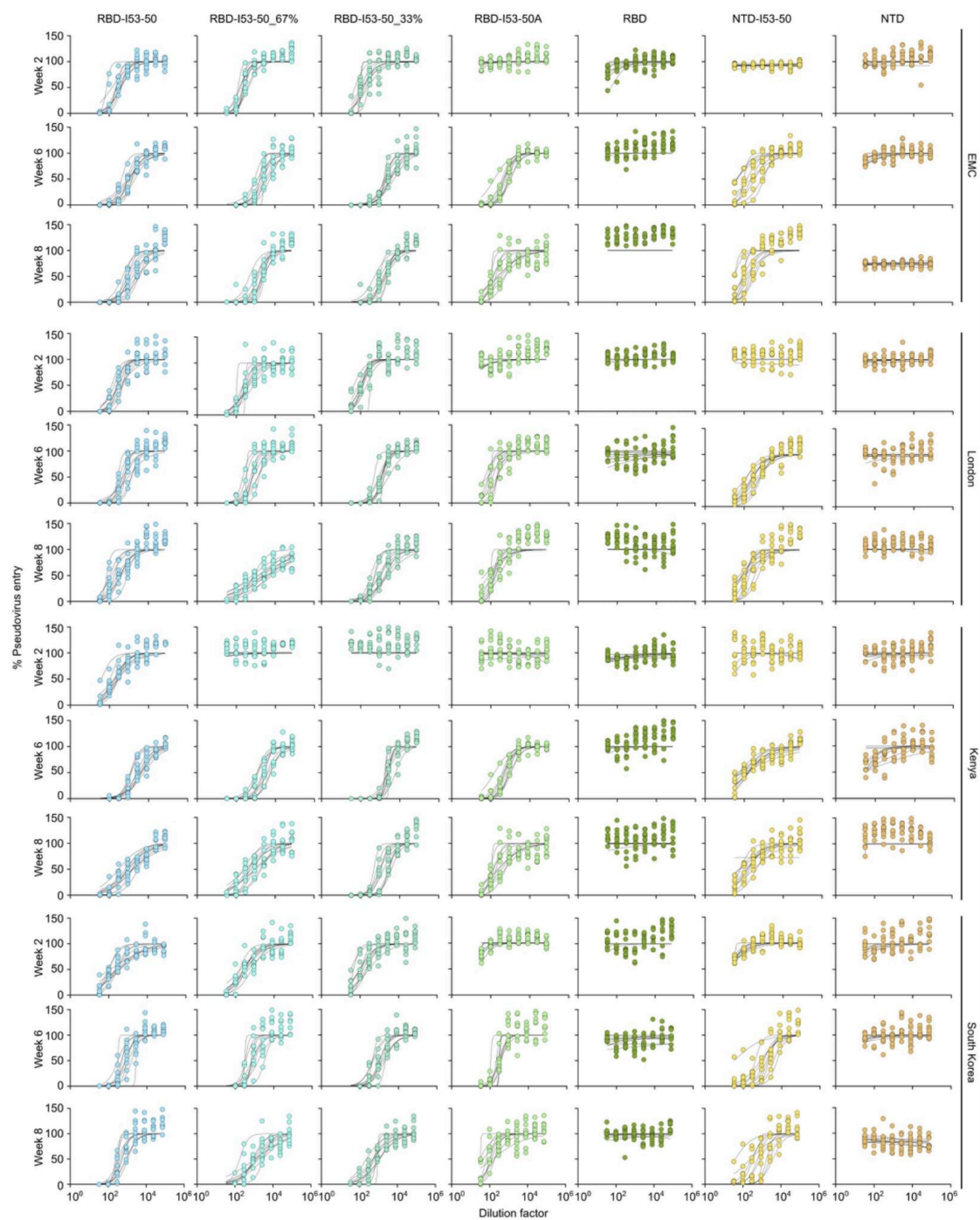


Figure S6. Dose-response curves of MERS-CoV pseudovirus neutralization, related to Figure 2.

Serum neutralizing activity elicited by domain-based immunogens at weeks 2, 6, and 8 was evaluated using pseudoviruses bearing the MERS-CoV EMC, London, Kenya, and South Korea variant spikes.

Epitope mapping of vaccine-elicited antibodies

We used serum competition assays to map the specificity of the antibody responses elicited by the S-2P trimer and nanoparticle immunogens during the immunogenicity study depicted in **Figure 2A**. We measured the week 8 serum dilutions required to compete with hDPP4 and G2 binding to examine receptor-blocking and NTD-directed responses, respectively (**Figures 3A and S7A–S7C**). We observed comparable hDPP4 competition across the S-2P, S-2P-T33_dn10, S-2P-I53-50, and RBD-I53-50 groups, suggesting that all of these vaccines induced similar levels of receptor-blocking antibodies (**Figure S7D**). The bare I53-50 nanoparticle control group did not elicit detectable hDPP4- or G2-competing antibodies as expected. We observed more variation among immunogens in the competition of our polyclonal sera with G2 (**Figure S7E**). Although none of the sera completely blocked G2 binding under the conditions tested, they could be stratified into three groups. Sera from mice that received NTD-I53-50 and S-2P-T33_dn10 most potently competed with G2 binding, followed by the S-2P trimer and S-2P-I53-50, followed by the RBD-I53-50 nanoparticle. The competition from RBD-I53-50-induced antibodies, albeit weak, was consistent across the sera from all mice. We interpret these data to indicate that some RBD-directed antibodies sterically compete with the binding of NTD-directed antibodies, a phenomenon that is similar to blockade of DPP4 binding by some NTD-directed antibodies.^{125,126}

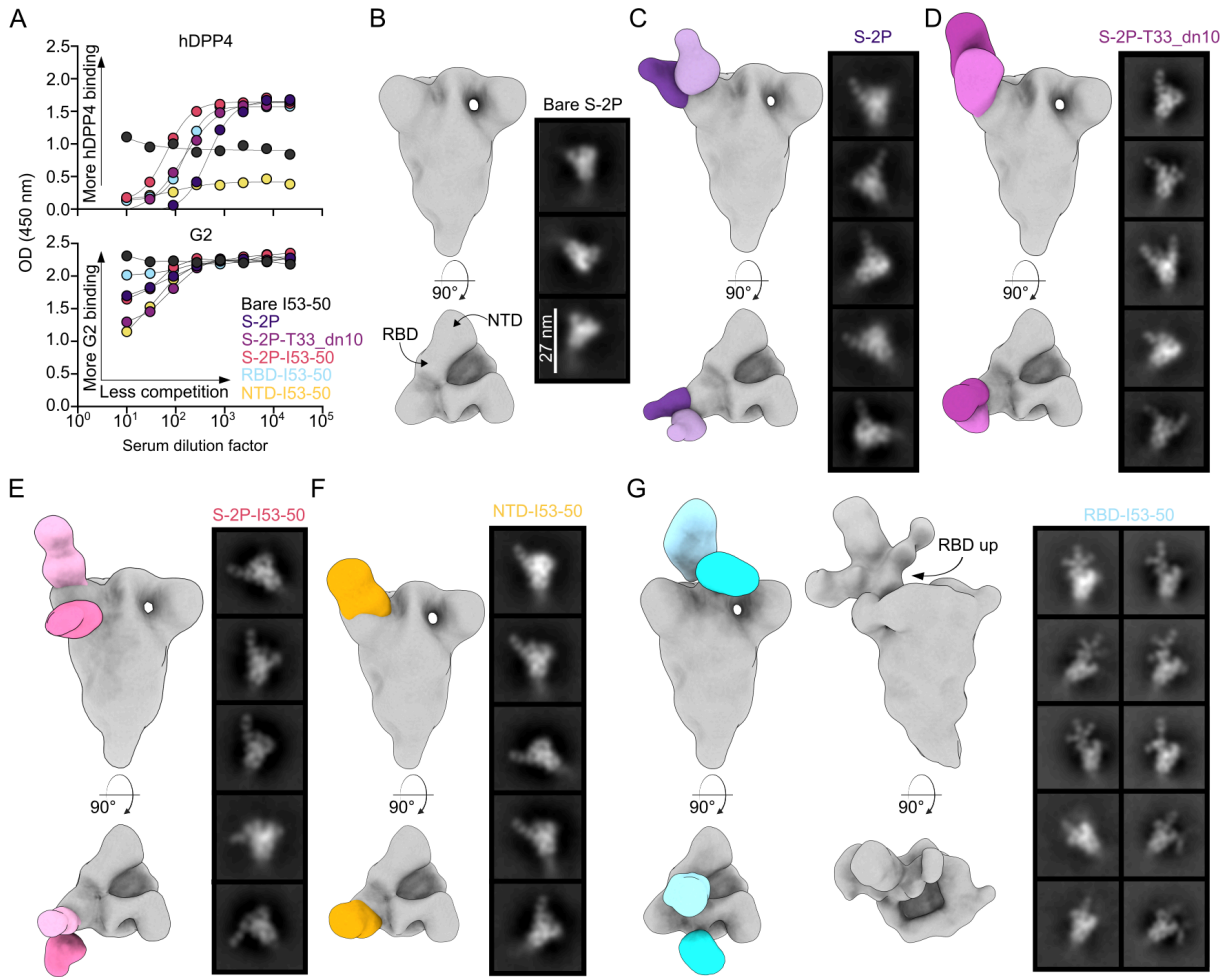


Figure 3. Epitope mapping of vaccine-elicited antibodies

(A) Serum competition of study groups with hDPP4 (top) and G2 (bottom) against MERS-CoV S-2P.

(B) Composite 3D model of MERS S-2P with no bound Fabs and representative 2D class averages. Scale bar, 27 nm.

(C to G) Composite 3D models and representative 2D class averages of ns-EMPEM analysis depicting Fab-bound MERS S-2P spikes from week 8 sera from mice immunized with S-2P **(C)**, S-2P-T33_dn10 **(D)** S-2P-I53-50 **(E)**, NTD-I53-50 **(F)**, and RBD-I53-50 **(G)**. In **(G)**, a composite 3D model is shown to visualize Fabs bound to a "three-down" MERS-CoV Spike, while a separate 3D reconstruction is shown to visualize Fabs bound to an RBD in the "up" conformation.

We next visualized the dominant serum antibody specificities elicited by the S-2P trimer and the three I53-50 nanoparticle immunogens using ns-EMPEM. To do this, we first generated a

low-resolution reconstruction of the MERS-CoV S-2P trimer, which was consistent with structures obtained in previous reports (**Figure 3B**).^{83,158,187} We then pooled and processed mouse sera from week 10 to generate polyclonal Fab complexes with MERS-CoV S-2P and mapped the densities obtained from ns-EMPEM onto the S-2P trimer reconstruction. In sera from mice immunized with soluble S-2P, we observed two slightly distinct classes of NTD-directed Fabs that bind the antigenic site targeted by G2 and many NTD-directed neutralizing antibodies against SARS-CoV-2 (**Figure 3C**).^{125,197–199} We did not detect RBD-directed Fab classes using these sera, possibly due to spike triggering and unfolding.^{111,200} In sera from mice immunized with S-2P-T33_dn10 we observed three classes of Fabs, two of which appeared to be NTD-directed and resembled those seen in the S-2P group, while the third could be visualized in 2D class averages but could not be resolved during 3D reconstruction (**Figure 3D**). We also only observed NTD-directed Fab classes in the sera of mice that received S-2P-I53-50 (**Figure 3E**). One of the two dominant classes targeted the same antigenic site at the apex of the NTD, while the second class bound the side of the NTD with an almost perpendicular angle of approach and was similar to the unresolved density observed in sera from mice immunized with S-2P-T33_dn10. Interestingly, these two classes recapitulate the specificities of cross-reactive antibodies against MERS-CoV S-2P identified in a recent study after immunization with a two-component nanoparticle immunogen displaying the SARS-CoV S-2P trimer.⁷³

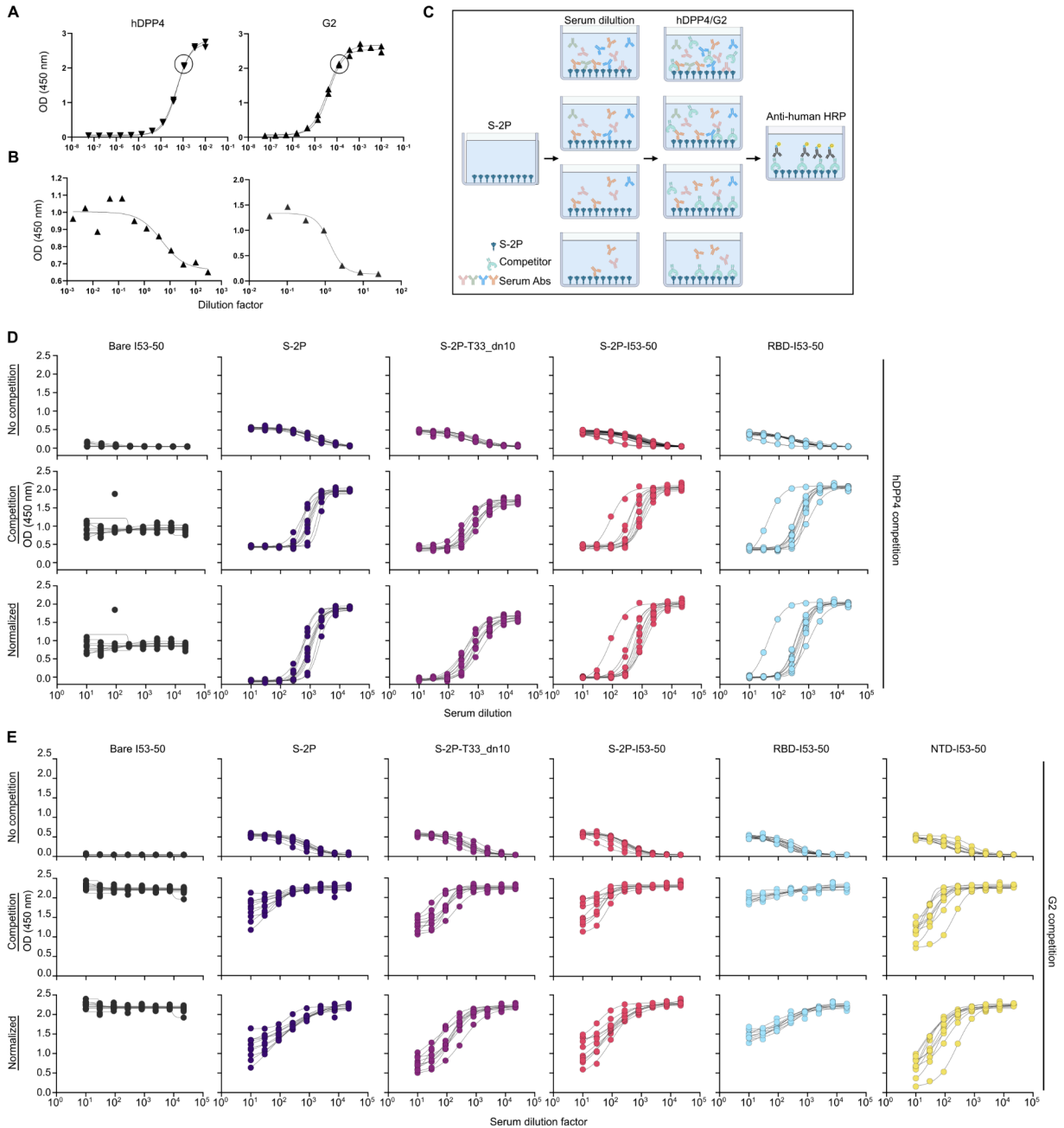


Figure S7. Serum competition against hDPP4 and G2, related to Figure 3.

(A) Anti-S-2P ELISA with hDPP4-Fc and G2 to determine concentration of 80% maximum binding (circled).

(B) Self-competition with biotinylated hDPP4-Fc and G2 against S-2P.

(C) Schematic of serum competition ELISA assay. Serum dilutions were incubated on plates coated with S-2P, followed by a fixed concentration of the competitor. Levels of bound competitors were measured as the final readout.

(D) Per-mouse serum competition from week 8 against hDPP4-Fc binding to S-2P. Background serum reactivity to top, secondary antibody; middle, competition between hDPP4-Fc and serum; and bottom, normalized data obtained by subtracting background secondary antibody signal from competition. The arithmetic mean of the normalized data points at each dilution are shown in Figure 3A.

(E) Per-mouse serum competition from week 8 against G2 binding to S-2P, processed as in **(D)**.

As expected, the domain-based NTD- and RBD-I53-50 nanoparticle immunogens elicited antibodies that bind each respective domain. All of the Fab-antigen complexes observed in the anti-NTD-I53-50 sera target the antigenic site at the apex of the NTD; we did not see 2D classes showing Fabs bound to the side of the NTD as we did for S-2P-T33_dn10 and S-2P-I53-50 (**Figure 3F**). By contrast, in sera from mice immunized with RBD-I53-50 we observed a number of distinct classes of antibodies targeting various epitopes, including those bound to both “up” and “down” RBDs (**Figure 3G**). Strikingly, several 2D class averages clearly showed multiple Fabs simultaneously bound to the same RBD, indicating that they bind non-overlapping epitopes.

Our inability to resolve RBD-directed densities from the soluble S-2P and S-2P-I53-50 groups was surprising given the apparent immunodominance of the RBD in MERS-CoV and other coronaviruses.^{110,124,201,202} Furthermore, our hDPP4 competition data clearly indicate the presence of high levels of RBD-directed antibodies in these sera (**Figure 3A**). We note that we had to optimize our experimental conditions to prevent destabilization of the Spike protein²⁰³ by shortening the polyclonal Fab-antigen incubation time and using a lower molar ratio of polyclonal Fabs to antigen (see Methods). It is likely that these conditions prevented us from observing all Fab classes prevalent in the sera. As a result, we only provide qualitative interpretations of our ns-EMPEM data. Nevertheless, taken together our epitope mapping data suggest that the RBD and NTD of the MERS-CoV spike are immunodominant during vaccination, as was observed in many studies of antibody responses to SARS-CoV-2^{108,110,204–206}

and a limited number of studies of other betacoronaviruses.^{187,202,205,206} Our results further suggest that a single antigenic site is immunodominant in the NTD, whereas several antigenic sites in the RBD are highly immunogenic.

Protection against challenge with mouse-adapted MERS-CoV

Finally, we evaluated the protection afforded by each immunogen in a mouse immunogenicity and challenge study. We immunized 288/330^{+/+} mice that express a chimeric DPP4 receptor in the C57BL/6J genetic background with two doses of our S-2P or RBD-based immunogens and subsequently challenged them at study week 15 with 1×10^5 pfu of mouse-adapted MERS-CoV (maM35c4) (**Figure 4A**). This model has previously been shown to recapitulate many aspects of the acute respiratory distress syndrome that humans experience upon MERS-CoV infection.^{207,208} The levels of S-2P-specific antibodies in sera collected two weeks post-prime and post-boost were generally similar to those observed in our earlier study in BALB/c mice, except that the post-prime titers were slightly higher for the S-2P-based immunogens in this study (**Figures 4B** and **S8A**). Similar results were obtained in pseudovirus neutralization assays: the data mirrored our earlier BALB/c immunogenicity study except that the S-2P-displaying nanoparticles induced detectable neutralizing activity at week 2 in addition to the RBD-I53-50 nanoparticle (**Figure 4C** and **S8B**). Overall, the trends in antigen-specific and neutralizing antibody titers between the 288/330^{+/+} and BALB/c mice were comparable, showing slightly higher responses from the nanoparticle groups after priming and similar responses after boosting.

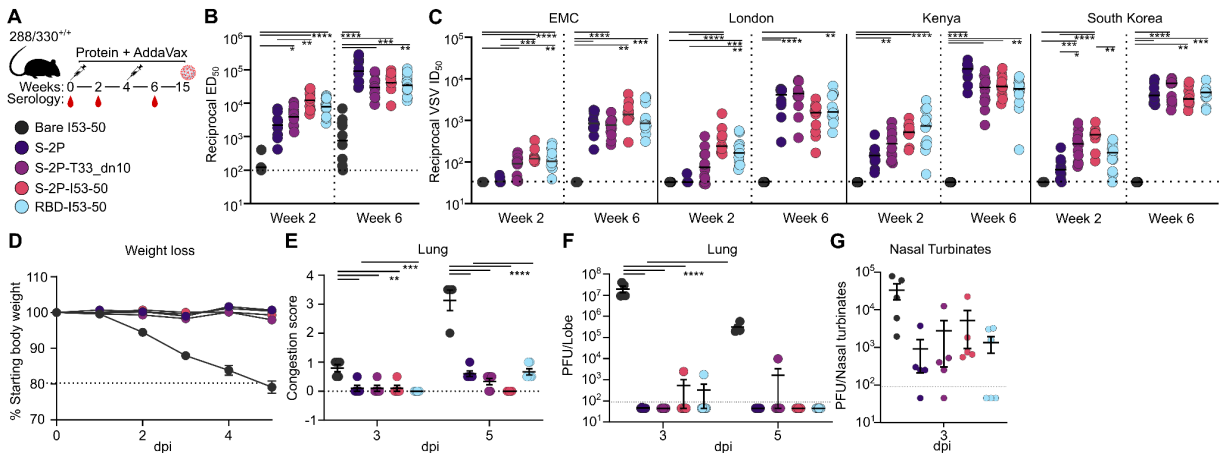


Figure 4. Protection against challenge with mouse-adapted MERS-CoV

(A) Challenge study design and groups. Groups of 288/330^{+/+} mice were immunized at weeks 0 and 4, serum was obtained at weeks 0, 2, and 6, and the mice were challenged at week 15. **(B)** Serum antibody binding titers against vaccine-matched (EMC) MERS-CoV S-2P, measured by ELISA.

(C) Vaccine-elicited neutralizing activity against VSV pseudotyped with closely related MERS-CoV EMC, London variant, Kenya variant, or South Korea variant spikes. Groups were compared using Kruskal-Wallis followed by Dunn's multiple comparisons. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

(D) Changes in body weight after maM35c4 challenge. Data are represented as mean $\pm$ SEM.

(E) Lung congestion score. Data analyzed with two-way ANOVA or mixed model followed by Sidak's multiple comparison. ****, $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$. Data are represented as mean $\pm$ SEM.

(F) Viral titers in the lungs of challenged mice from (3 dpi: $n = 5$ for bare I53-50, S-2P, S-2P-T33_dn10, and S-2P-I53-50; $n = 6$ for RBD-I53-50. 5 dpi: $n = 4$ for bare I53-50; $n = 5$ for S-2P and S-2P-I53-50; $n = 6$ for S-2P-T33_dn10 and RBD-I53-50. Data were analyzed with two-way ANOVA or mixed model followed by Dunnett's multiple comparison. ****, $p < 0.0001$. Data are represented as mean $\pm$ SEM.

(G) Viral titers in nasal turbinates of challenged mice. $n = 5$ for bare I53-50, S-2P, S-2P-T33_dn10, and S-2P-I53-50; $n = 6$ for RBD-I53-50. Data analyzed with one-way ANOVA with Geisser-Greenhouse correction followed by Dunnett's multiple comparison. In panels **(B)**, **(C)**, **(E)**, **(F)**, and **(G)**, only significant differences are shown; see Supplemental Data for all statistical comparisons. Data are represented as mean $\pm$ SEM.

maM35c4 challenge at week 15 allowed us to evaluate the efficacy of our immunogens against the development of severe disease. All 11 control mice immunized with two doses of bare I53-50 lost 20% of their starting weight by day 5 post-infection and had to be euthanized (**Figure 4D**). By contrast, all mice given two doses of MERS-CoV immunogens were protected from

severe disease, showing no appreciable weight loss after infection. Furthermore, the mice receiving MERS immunogens displayed undetectable to mild lung damage 3 and 5 days post-infection, while those receiving the bare I53-50 control immunogen showed considerable damage at day 5 (**Figure 4E**). While viral titers in the lung were below the limit of detection in nearly all mice immunized with antigen-bearing immunogens throughout the duration of the experiment, viral titers in nasal turbinates were reduced ≥ 1 –2 logs relative to those in the bare I53-50 control group at 3 days post-infection (**Figures 4F and 4G**). Together, these data show that immunization with prefusion-stabilized S-2P and a series of two-component nanoparticle immunogens elicited potent neutralizing antibody responses and protected 288/330^{+/+} mice from developing severe disease when challenged with a lethal dose of maM35c4.

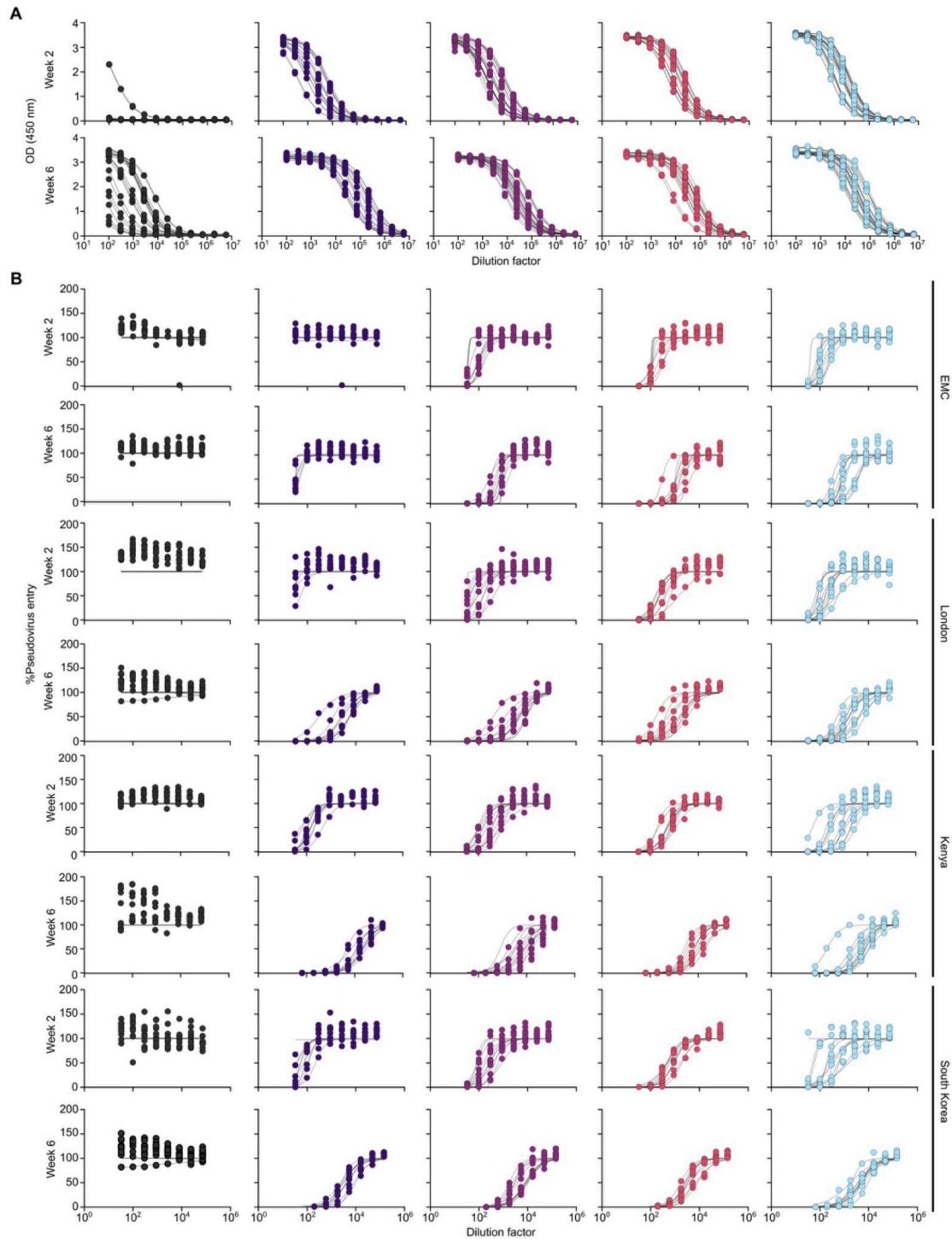


Figure S8. Serum antibody binding to MERS-CoV S-2P and pseudovirus neutralization from immunized 288/330^{+/+} mice, related to Figure 4.

(A) Raw week 2 and 6 serum ELISA data against vaccine-matched (EMC) MERS-CoV S-2P.

(B) Raw neutralization data from weeks 2 and 6 using pseudoviruses bearing the MERS-CoV EMC, London, Kenya, and South Korea variant spikes.

DISCUSSION

Here we demonstrated that two-component nanoparticle vaccines elicit potent neutralizing activity and protective responses against a Merbecovirus. The successful application of two-component nanoparticle vaccines to a second subgenus of betacoronaviruses suggests that their utility may extend even further across the *Coronaviridae*. Although several other protein nanoparticle technologies were evaluated as SARS-CoV-2 vaccines^{82,112,209–214} and some have begun to be applied to other betacoronaviruses,^{81,215,216} to our knowledge NVX-CoV2373 from NovaVax and the RBD-I53-50 nanoparticle vaccine SKYCovione™ are the only two that have obtained regulatory approval. Two-component protein nanoparticle vaccine candidates for RSV and RSV/HMPV have also been found to be safe and immunogenic in clinical trials,^{77,217–219} and a two-component mosaic nanoparticle vaccine for influenza virus is currently in Phase I clinical trials (NCT04896086 and NCT05968989).⁷⁴ These precedents combine with the data presented here to establish two-component protein nanoparticles as a platform for pandemic preparedness vaccine development.

The SARS-CoV-2 pandemic led to a deep understanding of the mechanisms of antibody-mediated protection against a betacoronavirus. For SARS-CoV-2, although effector functions from non-neutralizing antibodies can also protect,^{152,220–224} the most potently neutralizing antibodies target the RBD and a single immunodominant antigenic site in the NTD.^{110,197,199,225,226} Our data extend those observations to Merbecoviruses. The immunogens we tested can be separated into three categories based on the antigen they comprise: the prefusion-stabilized S-2P trimer, the RBD, or the NTD. We found that all of the vaccine candidates tested, including those based on the S-2P trimer or nanoparticles displaying

domain-based antigens, elicited robust neutralizing activity. The RBD-I53-50 nanoparticle elicited the most potent antibody responses after a single immunization, mirroring results we previously obtained for SARS-CoV-2.¹⁸⁰ However, unlike that study, the MERS-CoV S-2P-based immunogens tended to induce higher neutralizing activity post-boost, though most of the differences were not statistically significant. Of the three antigens, nanoparticles displaying the NTD elicited the lowest neutralizing activity. Although the NTD has been less explored as a vaccine antigen, a recent report highlighted that most of the MERS-CoV neutralizing activity in the sera of infected humans targets the RBD and NTD,¹²⁴ and our results obtained with NTD-I53-50 clearly establish the neutralizing activity of vaccine-elicited antibodies against the NTD. These antibodies, like several previously described mAbs,^{103,126,147,227,228} appear to primarily target a dominant antigenic site near the α -2,3-linked sialoside binding site.^{83,125} The role of sialoside binding in MERS-CoV attachment to host cells suggests a potential mechanism for neutralization by NTD-directed antibodies beyond blocking DPP4 engagement sterically.⁵³ Nevertheless, given the lower neutralizing activity elicited by NTD-I53-50, the rapid evolution of the SARS-CoV-2 NTD during the pandemic,^{199,229–231} and the high sequence diversity in Merbecovirus NTDs, it is not clear that an NTD-based immunogen would be preferred for further development.

Comparing a series of MERS-CoV nanoparticle immunogens to corresponding soluble antigens led to several interesting findings. First, multivalent display of the prefusion S-2P trimer did not enhance immunogenicity beyond that of the soluble S-2P trimer. It must be noted that the immunogenicity of the S-2P-I53-50 nanoparticles may be compromised by their heterogeneity and instability. Given that we have previously shown that incomplete assembly can result in I53-50 nanoparticles with reduced physical stability,²³² we suspect that the prefusion Spike trimer is large enough to hinder display at full valency on I53-50, and that some fraction of the preparations consisted of incomplete assemblies. If such incomplete assemblies also have

compromised stability *in vivo*, this may reduce their immunogenicity,²³³ a hypothesis that can be tested in future studies. However, the S-2P-T33_dn10 nanoparticles were highly monodisperse and showed no evidence of instability, yet were also not more immunogenic than the soluble S-2P trimer. Several previous studies that compared nanoparticles displaying prefusion-stabilized Spike trimers from SARS-CoV, SARS-CoV-2, and MERS-CoV to their soluble counterparts made similar observations.^{73,178,183,234} Alongside these studies, our results suggest that large, glycosylated antigens like prefusion Spike trimers do not significantly benefit from nanoparticle display. Previous work has shown that glycosylated nanoparticle immunogens traffic more efficiently to lymph nodes and B cell follicles *in vivo* compared to non-glycosylated or soluble antigens, increasing their immunogenicity.^{174,175,235} However, the large size of the prefusion Spike trimer and its extensive glycosylation may intrinsically confer this benefit and reduce the enhancement typically afforded by multimerization.

By contrast, we found that multivalent display of the RBD and NTD—both domain-based antigens—significantly improved their immunogenicity. Immunization with monomeric RBD or NTD failed to elicit neutralizing activity and barely resulted in detectable antigen-specific antibody in serum. While a trimerized version of the RBD performed better than the monomer, particularly after boosting, it was still substantially less immunogenic than the RBD-I53-50 nanoparticle. Together with the data on our Spike-based immunogens, these results suggest that enhancing immunogenicity via nanoparticle display may be most effective for smaller antigens. Consistent with this, I53-50 nanoparticles displaying the RBD at different densities did not elicit significantly different levels of antigen-specific or neutralizing antibodies. This result may indicate that nanoparticle display enhances the immunogenicity of the RBD mainly by increasing its size and therefore presumably its trafficking *in vivo*, although we did not directly test that hypothesis here. Overall, our comparisons between particulate and soluble antigens

simultaneously provide guidance on how to best apply nanoparticle display in vaccine design and raise interesting mechanistic questions that can be addressed in future studies.

In summary, we found that the prefusion-stabilized MERS-CoV Spike and several two-component nanoparticle immunogens induce robust and protective immune responses against MERS-CoV in vaccinated mice. The high performance of the S-2P Spike and the RBD-I53-50 nanoparticle, and the use of the same platform as the licensed SARS-CoV-2 vaccine SKYCovione™ by the latter, motivate further development of these vaccine candidates. More generally, our data highlight two-component protein nanoparticle immunogens as a platform technology that may be broadly useful for designing vaccines against betacoronaviruses and other virus families.

LIMITATIONS OF THE STUDY

One limitation of our study is that we were unable to resolve all vaccine-elicited antibody specificities by nsEMPEM. This was highlighted by our inability to visualize RBD-bound Fabs in the groups administered immunogens based on the S-2P spike, despite our observation of hDPP4-competing antibodies by ELISA. We suggest that this was due to polyclonal antibodies binding to multiple epitopes in the spike and triggering its disassembly, a phenomenon that has been observed for other class I viral fusion proteins.^{236–238} Another limitation is that we did not evaluate the protective capacity of the NTD-I53-50 nanoparticle immunogen, leaving this as an open question for future studies. In addition, the challenge studies we did perform were limited to a mouse model. Although this model has been shown to reflect the acute respiratory distress syndrome observed in MERS-CoV-infected humans,^{207,208} its capacity to predict vaccine performance in higher mammals is currently unknown. Finally, additional studies will be required to measure the breadth of antibody responses elicited by these MERS-CoV immunogens against other Merbecoviruses. We note that the phylogenetic diversity of known Merbecoviruses

is growing, which may increase the number of vaccine antigens required to develop broadly protective Merbecovirus vaccines.^{26,46,239}

ACKNOWLEDGEMENTS

This study was supported by the National Institute of Allergy and Infectious Diseases (P01AI167966 to R.S.B., D.V., and N.P.K.; 75N93022C00036 to D.V.), an Investigators in the Pathogenesis of Infectious Disease Awards from the Burroughs Wellcome Fund (D.V.), the National Science Foundation Graduate Research Fellowships Program Award (DGE-2140004 to C.W.C.), the University of Washington Arnold and Mabel Beckman cryoEM center and the National Institute of Health grant S10OD032290 (D.V.), and the Audacious Project at the Institute for Protein Design (N.P.K.). D.V. is an Investigator of the Howard Hughes Medical Institute and the Hans Neurath Endowed Chair in Biochemistry at the University of Washington. The authors thank Sebastian Ols, Cyrus Haas, Marti Tooley, Kenneth Carr, and Andrew Borst for helpful discussions.

AUTHOR CONTRIBUTIONS

C.W.C., M.C.M., A.C.W., D.V., and N.P.K. designed the study. C.W.C., M.C.M., and A.C.W. designed and characterized immunogens. C.W.C. and K.R.S. performed *in vitro* experiments, and N.J.C. performed challenge experiments. C.W.C., M.C.M., A.A., C.S., J.T.B., A.D., A.V., R.R., and G.G.H. produced proteins and provided reagents. C.T., I.W., E.M.L., and M.H., J.M., and B.S. conducted and performed animal immunizations. M.A., C.D., and A.H. provided and maintained cells. C.W.C. and K.R.S. analyzed the data. A.T.M., A.S., and R.S.B. provided oversight of immunization and challenge studies. C.W.C., N.P.K., D.V., K.R.S., E.M.L., and A.S. wrote the manuscript with input from all authors.

DECLARATION OF INTEREST

M.C.M., G.G.H., A.C.W., N.P.K., and D.V. are named as inventors on patent applications filed by the University of Washington related to coronavirus vaccines. The King lab has received unrelated sponsored research agreements from Pfizer and GSK. All other authors declare no competing interests.

EXPERIMENTAL MODELS AND PARTICIPANT DETAILS

Cell lines

Expi293F cells are derived from the HEK293F cell line (Life Technologies). Cells were grown in Expi293 Expression Medium (Life Technologies), cultured at 36.5°C with 8% CO₂ and shaking at 150 rpm. Huh7 cells cultured in DMEM with 10% FBS (Hyclone), 1% PenStrep (100 I.U./mL penicillin and 100 µg streptomycin) and cultured at 37°C with 5% CO₂. Vero81 cells were cultured in DMEM (Gibco: 11995065 + 5% FBS (VWR: 76237-678) + 1% Pen/Strep (Gibco; 15140122) at 37°C with 5% CO₂.

Mice

For immunogenicity studies, female BALB/cAnNHsd were purchased from Envigo (order code 047) at 7 weeks of age. Mice were housed in a specific-pathogen free facility within the Department of Comparative Medicine at the University of Washington, Seattle, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal studies were conducted in accordance with the University of Washington's Institutional Animal Care and Use Committee.

For *in vivo* protection studies, 288/330-hDPP4 transgenic mice were used, which were bred and maintained at the University of North Carolina at Chapel Hill (Animal Welfare Assurance #A3410-01). The study was performed using protocols (#23-085) approved by the UNC

Institutional Animal Care and Use Committee (IACUC) and carried out in accordance with the recommendations for care and use of animals by the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health, and the IACUC. Immunizations were performed in a BSL2 facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

METHOD DETAILS

Protein purification and characterization

All constructs contained a C-terminal histidine affinity tag and were codon optimized by GenScript for mammalian cell expression. Expi293F cells were transiently transfected using PEI MAX and cultured for three days 37°C. Cells were harvested, centrifuged, and filtered to obtain clarified supernatants. Clarified cell supernatants were buffered with 5 mL of 5M NaCl and 7 mL of Tris pH 8.0 per 100 mL of supernatant and batch bound with Ni Sepharose Excel resin while shaking for 30 minutes to an hour at room temperature. Resin were collected in a gravity column, washed with five column volumes of wash buffer containing 25 mM Tris pH 8.0, 150 mM NaCl, and 30 mM imidazole, and eluted with three column volumes of elution buffer comprised of 25 mM Tris pH 8.0, 150 mM NaCl, and 300 mM imidazole. Eluted component constructs were further purified using SEC on either a Superose 6 Increase 10/300 gel filtration column if they were spike-bearing, or a Superdex 200 Increase 10/300 gel filtration column if they were domain-bearing constructs. *In vitro* assembly of nanoparticles was conducted using a 1:1 molar ratio of each component and incubated at room temperature for 30 minutes with rocking. Following nanoparticle assembly, all particles were purified one last time through a Superose 6 Increase 10/300 column. All spike and NTD protein components and assembled nanoparticles were purified into 50 mM Tris pH 8.0, 150 mM NaCl, 0.25% w/v Histidine, and 5% glycerol. The RBD component and nanoparticle were purified in 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% Glycerol, and 0.75% CHAPS.

Bio-Layer interferometry (BLI)

All samples were diluted in kinetics buffer (0.05% bovine serum albumin and 0.01% Tween 20 in PBS) and measurements were carried out using an Octet Red 96 system at 25°C shaking at 1000 rpm. Antibodies were diluted to a final concentration of 20 µg/mL before being immobilized on ProteinA tips (Sartorius) for 600 s. Unassembled and nanoparticle proteins were diluted to 250 nM and associations were measured for 600 s, followed by dissociation for 900 s in kinetics buffer.

Nano differential scanning fluorimetry

The thermal stabilities of the MERS-CoV and SARS-CoV-2 S-2P trimers were measured at 0.40 mg/mL in the presence of the hydrophobic dye 20× SYPRO Orange using an UNcle (UNchained Labs). Changes in fluorescence emission were used to evaluate protein unfolding as readings were measured from 25–95°C with an increasing ramp rate of 1°C/minute.

Aggregation temperature (T_{agg}) determination

Aggregation of nanoparticle immunogens at 0.20 mg/mL was evaluated using an UNcle (UNchained Labs). DLS was monitored from 25–95°C with an increasing ramp rate of 0.15°C/minute and an acquisition time of 10 seconds to measure changes in particle size or aggregation state as a function of temperature.

Immunogenicity studies

For each immunization, low-endotoxin nanoparticles were diluted to 20 µg/mL in buffer and mixed with 1:1 v/v AddaVax adjuvant (InvivoGen vac-adx-10) to obtain a final dose of 1 µg of immunogen per animal per injection (**Figure 2 and S2-3, S5-7**). At 8 weeks of age, 10 mice per group were injected subcutaneously in the inguinal region with 100 µL of immunogen at weeks 0

and 4. Animals were bled using the submental route at weeks 0, 2, and 6. A terminal bleed was collected at week 8 via cardiac puncture. Whole blood was collected in serum separator tubes (BD #365967) and rested for 30 min at room temperature for coagulation. Tubes were then centrifuged for 10 min at 2,000 *g* and serum was collected and stored at -80°C until use.

288/330-hDDP4 transgenic mouse vaccinations and virus challenge experiments

The subsequent challenge study was conducted in a BSL3 facility at the University of North Carolina. Briefly, groups of 288/330-hDPP4 transgenic mice (n=5/6 per condition and time point, mixed sexes, ~19–23 weeks of age) were vaccinated subcutaneously in the inguinal region with 100 μ L of immunogen with 1 μ g of nanoparticle immunogen at weeks 0 and 4 (**Figure 4 and S8**). Animals were bled via submandibular route at -1, 2, and 6 weeks. Mice were then moved into the BSL3 and acclimated for a few days. For infection, mice were anesthetized with an intraperitoneal delivery of xylazine and ketamine and intranasally inoculated with 1×10^5 PFU of MERS-CoV maM35c4. Mice were monitored daily for weight loss and mortality. On 3 dpi and 5 dpi, groups of mice were euthanized with isoflurane and nasal turbinates and the caudal lobe of the right lung were collected to determine viral load by plaque assay. For plaque assay, harvested tissues were homogenized in $1 \times$ PBS, and the resulting homogenate was serial-diluted onto a confluent monolayer of Vero E81 cells, followed by agarose overlay. Plaques were then visualized with an overlay of Neutral Red on 3 dpi.

Enzyme-linked immunosorbent assay (ELISA)

Costar 96-well high protein binding plates were coated overnight at 4°C with spike proteins at 2 μ g/mL and blocked in 200 μ L of blocking buffer composed of (TBST: 1x Tris-buffered saline with 25 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween 20, and 5% nonfat milk). Plates were then washed 3x with TBST in an automated plate washer (Biotek); all washing steps follow the same protocol. 3-fold serial serum dilutions were made starting at 1:100 and 100 μ L were plated and

incubated for one hour at room temperature shaking at 500 rpm. Plates were washed then 100 μ L of secondary anti-mouse (Cell Signaling Technology) (1:2,000 dilution) or anti-human (SouthernBiotech) (1:5,000 dilution) IgG-HRP were added to each well and incubated shaking at room temperature. Plates were washed before 100 μ L per well of TMB were added and developed for 3 minutes, then quenched with 100 μ L of 1N HCl. Reading at absorbance at 450 nM was carried out with an Epoch plate reader (Biotek).

Competition ELISA

Competition ELISAs were performed in a similar manner as the aforementioned ELISA method with minor adjustments. To determine fixed competitor concentrations for 80% maximal binding against S-2P, a 3-fold serial dilution series with a starting dilution of 1:10 was carried out in blocking buffer and 100 μ L per well of were applied and incubated on plates coated with S-2P for 45 minutes then washed. 100 μ L per well of Goat anti-human IgG-HRP secondary (1:20,000 dilution) was plated for 30 minutes at room temperature. Plates were washed then developed for two minutes with 100 μ L of TMB and quenched with 100 μ L of 1 N HCl. The concentration of competitors at which approximately 80% of maximal binding observed was used for competition assays.

As a positive control for achieving full competition against the competitor, hDPP4-Fc and G2 were biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo Scientific) and 80% maximum binding concentrations against S-2P were determined with biotinylated proteins (hDPP4-Fc-biot and G2-biot). For hDPP4-Fc self-competition, a 3-fold serial dilution was performed starting at 300 μ g/mL of hDPP4-Fc in blocking buffer and 100 μ L per well was plated on S-2P coated plates for 30 minutes. Plates were washed and 100 μ L per well of biotinylated hDPP4-Fc at 8 μ g/mL was added and incubated for 45 minutes. Plates were washed and 100 μ L per well of secondary HRP-conjugated Streptavidin (1:5,000 dilution) (Thermo Scientific) was plated for 30

minutes. Plates were washed one last time then developed for 2 minutes with TMB and quenched with 1 N HCl before reading. For G2 self-competition, a 3-fold serial dilution of G2 starting at 25 µg/mL was carried out and added to S-2P coated plates for 30 minutes and washed before biotinylated G2 was added as the competitor at 0.37 µg/mL for 45 minutes then washed. 100 µL of secondary HRP-Conjugated Streptavidin was added for 30 minutes before plates were washed and developed with TMB for 2 minutes before being quenched with 1 N HCl.

For the serum competition assay, 3-fold serial serum dilutions were performed starting at 1:10 in blocking buffer, transferred to S-2P-coated plates and incubated at room temperature for 30 minutes, then washed. 100 µL per well of hDPP4-Fc or G2 at 1.11 µg/mL or 0.12 µg/mL, respectively, was added and incubated for 45 minutes then washed. Goat anti-human IgG-HRP at a 1:20,000 dilution was added for 30 minutes. Plates were developed for 2 minutes and optical densities were read at 450 nm.

Generation of pseudoviruses

To produce pseudoviruses for entry and neutralization assays, HEK293T cells were seeded in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% Fetal bovine serum (FBS, Hyclone), 1% PenStrep (100 I.U./mL penicillin and 100 µg streptomycin) (Gibco 15140-122) at the appropriate density to yield 80% confluency in polylysine-coated 100 mm cell culture dishes and placed in an incubator at 37°C with 5% CO₂. After 18-22 hr incubation, cells were washed with OPTI-Minimum Essential Media (Opti-MEM, Life Technologies). 24 µg of MERS-CoV-EMC, MERS-CoV-London, MERS-CoV-South Korea, MERS-CoV-Kenya full length S plasmids were prepared in 1.5mL of OPTI-MEM and combined with 60µL of Lipofectamine 2000 (Life Technologies) diluted in 1.5mL of OPTI-MEM and incubated at room temperature for 15-20 min. The mixture was added to the HEK293T cells which were placed for 2 hours in an incubator at

37°C with 5% CO₂ after which 2mL of DMEM enriched with 20% FBS and 2% PenStrep (200 I.U./mL penicillin and 200 µg streptomycin) was added to the transfected cells and incubated overnight. The following day, cells were washed with DMEM and transduced with VSVΔG/Fluc and incubated for 2 hours at 37°C with 5% CO₂. After washing with DMEM, medium supplemented with anti-VSV-G antibody (I1-mouse hybridoma supernatant diluted to 1:25 from CRL-2700, ATCC) was added to the cells to reduce background from the parental virus and an additional incubation at 37°C with 5% CO₂ was performed overnight. The next day, the supernatant from the cells was harvested from the 100 mm dishes, further clarified by centrifugation at 3,000xg for 10 minutes, filtered (0.45µm), and concentrated 10 times by using centrifugal devices with 30 kDa cutoff membranes. Pseudoviruses were then aliquoted and frozen at -80°C until used.

Pseudovirus neutralization assay

Huh7 cells cultured in DMEM with 10% FBS (Hyclone), 1% PenStrep (100 I.U./mL penicillin and 100 µg streptomycin) were subsequently trypsinized, counted and reseeded at ~30,000 cells per well in cell-culture grade 96 well plates and placed in an incubator at 37°C with 5% CO₂ overnight. The next day, after cell health and confluency was evaluated, a half-area 96-well plate was prepared with a 1:3 serial dilution of sera in DMEM in 22 µL final volume. 22 µL of pseudovirus was then added to each well and incubated at room temperature for 30-45 min. The media was removed from Huh7 cells and they were subsequently washed 2-3x and 40 µL of the sera/pseudovirus mixture from the half area plate was added to the cells and incubated for 2 h at 37°C with 5% CO₂ before adding 40 µL of 20% FBS and 2% PenStrep (200 I.U./mL penicillin and 200 µg streptomycin) containing DMEM. Following 18-22 hour incubation, 40 µL of One-GloEX (Promega) was added to the cells and incubated in the dark for 5 min prior to reading on an Agilent BioTek Neo2 plate reader. Relative luciferase units were plotted and

normalized in Prism (GraphPad) using a zero value of cells alone and a 100% value of 1:2 virus alone. Nonlinear regression of log(inhibitor) vs. normalized response was used to determine ID₅₀ values from curve fits. At least two biological replicates with two distinct batches of pseudovirus were conducted for each serum sample.

Negative stain electron microscopy (nsEM)

400-mesh carbon coated grids (Electron Microscopy Sciences) were glow-discharged and 3 μ L of 20 μ g/mL nanoparticles were applied then stained with 2% (w/v) uranyl formate. All nsEM data were collected with a BM-Ceta camera at 57,000x magnification using EPU 2.0 on a 120 kV Talos L120C transmission electron microscope (Thermo Scientific). CTF processing, particle picking, particle extraction, 2D classification, and 3D refinement steps were all performed with CryoSPARC.²⁴⁰

ns-EMPEM

To obtain Fabs for immune complexes, 500 μ L of serum from mice were pooled from the terminal bleed time point to maximize the workable volume for processing. Sera were incubated with PierceTM Protein G Agarose (Thermo Fisher) at pH 5.0 for 12-24 hours at 4°C and polyclonal antibodies were washed with five column volumes of PBS to remove unbound sera and eluted from gravity filtration columns with three column volumes of 0.1 M glycine, pH 2.5 and neutralized with 1 M Tris pH 8.0 to a final diluted concentration of 50 mM Tris pH 8.0. Eluted IgGs were cleaved with papain in 20 mM NaPO₄, pH 6.5, 10 mM EDTA, 20 mM Cysteine at 37°C with shaking for 5-20 hours. Cleaved Fabs were retrieved using gravity filtration columns and dialyzed into PBS before the final step of SEC in a Superdex 200 Increase 10/300 gel filtration column equilibrated with PBS. To prepare immune complexes for ns-EMPEM, purified Fab and MERS S-2P were mixed at a 5:1 ratio and incubated at room temperature with rocking

for 30-45 minutes, then immediately applied onto glow-discharged 400-mesh carbon coated grids for ns-EMPEM. Three rounds of 2D classification were performed, selecting for only spike proteins with clear bound Fab densities. Ab initio 3D reconstruction followed by heterogeneous refinement with no symmetry was used to generate 3D models of immune complexes. Only classes with distinct spike densities that captured a representation of bound Fabs were further processed with 3D refinement. Chimera²⁴¹ and ChimeraX²⁴² were used to make figures.

Quantification and statistical analyses

Group comparisons were conducted using one-way ANOVA with Geisser-Greenhouse correction followed by Dunnett's multiple comparison in Prism 9 (GraphPad). Differences were considered significant when p values were less than 0.1. Data were also analyzed with two-way ANOVA or mixed model followed by Sidak's multiple comparison. Differences were considered statistically significant when p values were less than 0.05.

SUPPLEMENTAL FILES

Table S1. Amino acid sequences of proteins and pseudovirus constructs used in this study, related to STAR Methods.

Table S2. Statistical comparisons of serological analyses performed in this study, related to STAR Methods.

Chapter 3: Exploring a pan-merbecovirus vaccine

ABSTRACT

Middle East Respiratory Syndrome is a severe respiratory infection caused by the MERS coronavirus (MERS-CoV), a member of the Merbecovirus subgenus in the Betacoronavirus genus. There are no licensed vaccines against MERS-CoV, furthermore our understanding of the members within the Merbecovirus subgenus is still within early stages. As global surveillance efforts continue to unveil new members within this subgenus, analysis of receptor utilization and viral tropism reveal the broad use of ACE2 and DPP4 receptor orthologues, some of which are capable of utilizing human receptors for cell entry, demonstrating the need for ongoing viral surveillance to monitor for future outbreak potentials.^{26–29,37,38,46,91,239,243,244} With the success of the licensed protein nanoparticle SARS-CoV-2 vaccine, SKYCovione, we sought to investigate MER-CoV vaccines using this same technology. We previously demonstrated the efficacy of MERS-CoV nanoparticle immunogens as efficacious vaccine candidates capable of eliciting protective responses in transgenic mice challenged with a lab-adapted MERS-CoV virus.⁷⁰ With these promising results, here, we not only aim to evaluate nanoparticle immunogens as vaccine candidates against MERS-CoV, but we also seek to explore the possibility of a pan-Merbecovirus vaccine in non-human primates.

INTRODUCTION

Improved global viral sequencing efforts have uncovered numerous members within the Merbecovirus subgenus with the help of next-generation sequencing and expanded field work. many of which have been revealed to use DPP4 and ACE2 orthologues within bats and other mammals.^{26,27,38,46} Members of this subgenus have also been found to be highly genetically diverse when comparing their S proteins, and further evolutionary analyses reveal signs of independent evolution of receptor recognition modalities.^{27,38} Although many of these new CoVs have not been labeled as immediate threats to human health, close monitoring and broadening our knowledge of host tropism will be necessary to prevent or mitigate future spillovers.

Recent surveys within Central European Russia uncovered a MERS-related CoV circulating within bats near Moscow, named MOW-CoV, that is capable of infecting hedgehogs, and is closely related to another MERS-like CoV found in *Neoromicia* bats (Neo-CoV), and Eri-CoV, a hedgehog CoV.^{46,245,246} Another related Merbecovirus that falls within this same clade is PDF-2180-CoV, which was found within vesper bats in Southwest Uganda.²⁴⁷ Evaluation of host tropism and receptor usage uncovered that members within this clade evolved to use ACE2 receptors in bats and other mammals.³⁷ Phylogenetic analysis of Merbecovirus RBDs places CoVs within these clades of viruses as the most genetically divergent from the MERS-CoV RBD (**Figure 1.3**). A separate clade of Merbecoviruses is HKU5-CoVs, first found from Japanese pipistrelles bats, that, upon more recent analyses, were found to utilize not only bat ACE2 for infection, but also demonstrated the ability to use other mammalian ACE2 receptors.^{27,33} Notably, a new lineage of HKU5-CoV was recently determined capable of using human ACE2 and even broader use of mammalian ACE2 receptors for cell entry *in vitro*, demonstrating the expansion of host tropism.²⁸ HKU25-CoV, identified in Chinese pipistrelles bats, comprise another clade of Merbecovirus that bridges the evolutionary gap between MERS-CoV with other

bat CoVs and were found to also use ACE2 as receptor.^{243,248} Phylogenetic analysis of HKU5- and HKU25-CoV RBDs places these two clades next closest in sequence homology to MERS-CoV RBDs (**Figure 1**). HKU4-CoV Merbecovirus including HKU4-, MjHKU4-, and NL140422-CoV RBDs are the closest relatives to MERS-CoV RBD and are also known to utilize DPP4 as an entry receptor.^{27,29,91,244} Viral isolates within this clade have been identified from Malayan and Chinese pangolins as well as *Tylonycteris* bamboo bats in Eastern and Southeastern parts of Asia.^{91,243,249} Furthermore, recent works unveiled a furin cleavage site in MjHKU4-CoV that may be responsible for expanding the host range of this virus.⁹¹ Characterization of Merbecoviruses and identification of their functional receptors has revealed a broad range of hosts capable of supporting viral infection in multiple parts of the world, spanning from hedgehogs in Poland, bats in Uganda, southern Africa, and Russia, to minks and civets in parts of China, and southern Asia.

The three highly pathogenic zoonotic infections in humans all involved an intermediary reservoir where they likely acquired viral adaptations necessary for human infection. This pattern marks facilities and locations with high densities of known reservoirs, including wet markets, livestock, and farms, as potential sites for cross-species transmission to occur.²⁵⁰ Improving our understanding of barriers for viral emergence of these CoVs is necessary to preclude zoonotic infections and aid the development of therapeutics.

In this project, I sought to take a phylogenetic approach to investigate broadly protective Merbecovirus vaccines by applying similar methods implemented in the development of MERS RBD nanoparticle immunogens towards the production of novel Merbecovirus RBD nanoparticles. In order to produce monovalent RBD nanoparticles, displaying a single type of RBD, I utilized a combinatorial approach using proteinMPNN and deep mutational scanning towards stabilizing Merbecovirus RBDs for nanoparticle display. I hypothesized that monovalent

Merbecovirus RBD nanoparticles would be well-behaved and stable, and facilitate downstream production of mosaic nanoparticles, displaying multiple different RBDs, which could then be utilized to investigate pan-Merbecovirus immunogens.

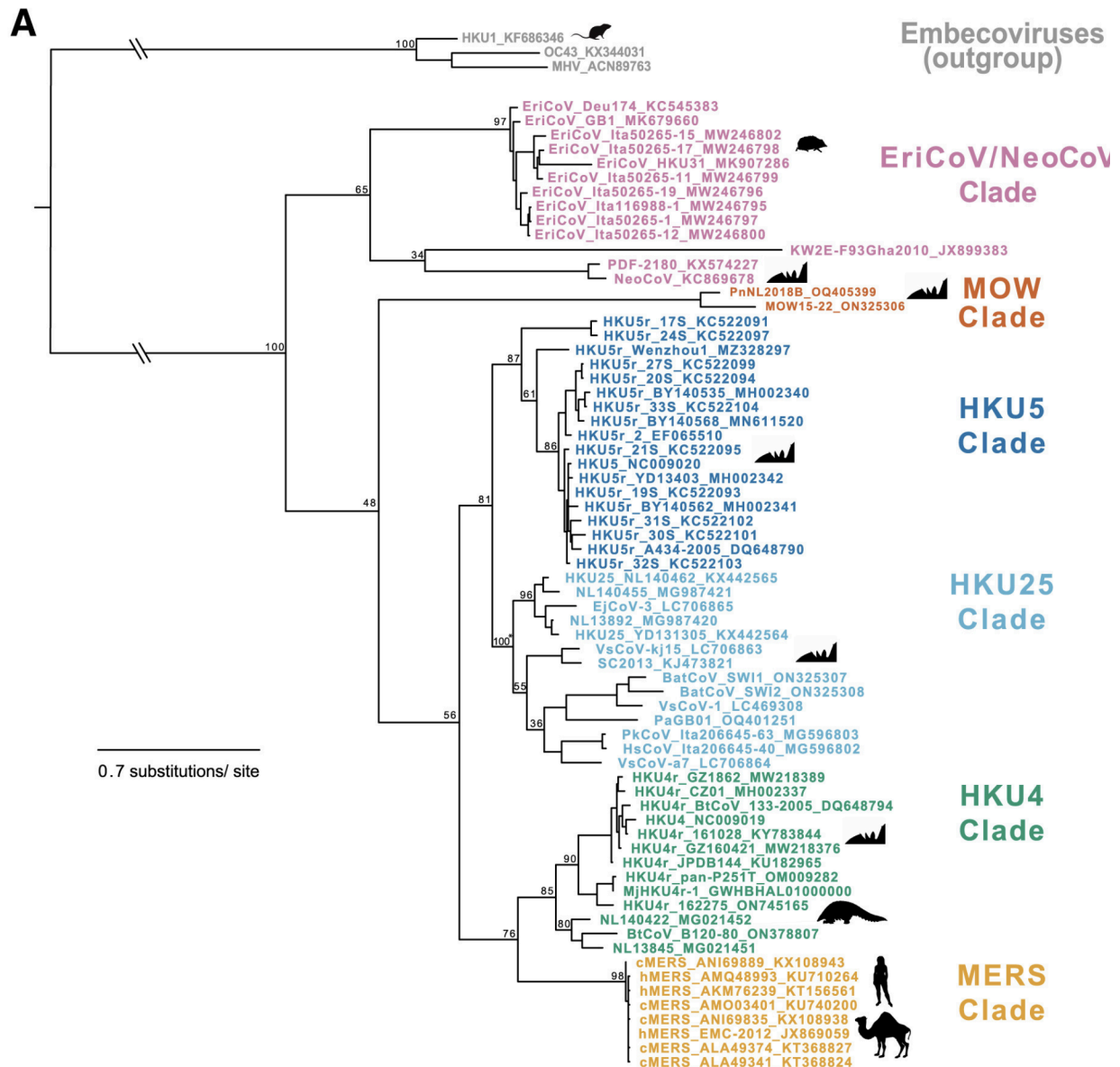


Figure 1. Diversity of Merbecovirus RBDs

A Merbecovirus phylogenetic tree depicting the 6 existing clades within the subgenus and the respective hosts from which isolates were first identified.

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RESULTS

Identification of Rpk mutations to stabilize Merbecovirus RBDs

The COVID-19 pandemic brought on a bounty of knowledge about CoVs that has been instrumental in tackling the development of more broadly protective therapeutics against Sarbecoviruses, expanding beyond SARS-CoV-2. DMS has played a large role in narrowing the gap in understanding the evolutionary history of Sarbecoviruses, which has in turn guided the design of vaccines against them.^{131,251–254} Building a comprehensive DMS library will elucidate Merbecovirus receptor binding capabilities and uncover evolutionary relationships within its clades. This information can further be adapted to inform antigen design for broader Merbecovirus vaccine design strategies. In collaboration with the Starr lab at The University of Utah, we sought to develop a DMS library for Merbecovirus RBDs, measuring RBD expression, receptor and antibody binding, looking at potential cross-reactive responses from polyclonal antibodies or vaccine-elicited sera (**Figure 2A**). Preliminary efforts to establish a yeast surface display library for the Merbecovirus subgenus were hampered by poorly expressed RBDs. Wildtype (WT) MERS-CoV RBD expressed and remained antigenically intact (**Figure 2B**), and were used to develop a DMS expression library for MERS-CoV RBD, highlighting point mutations along the RBD that enhance expression (**Figure 2C**). HKU4 RBD, which comprises the clade with the highest RBD sequence homology to the MERS clade (**Figure 1**), did not express on yeast display. Thus, a design challenge was introduced. DMS datasets provide a wealth of information but further refinement steps are necessary to find and down select combinatorial mutations in appropriate regions of the protein while maintaining functionality and known epitopes intact. To address this, we concurrently developed a semi-automated pipeline to

identify buried or solvent inaccessible residues for proteinMPNN to design leaving surface residues untouched to preserve antigenic epitopes. Log probabilities for each amino to occur at every residue position from proteinMPNN were parsed through and suggested mutations with the highest probability were selected. Two positions to mutate, suggested by proteinMPNN, were L414I and G570V. Both positions are found within the core subdomain of the RBD, of which L414 is tucked inside the linoleic acid binding pocket where the previous Rpk9 mutations were found (**Figure 2D**).¹³¹ Upon comparing proteinMPNN suggested mutations with DMS, the G570V mutational effects were corroborated by the expression data showing great enhancement (**Figure 2D**). L414I did not improve expression but closer analysis of the structure and DMS suggested that L414F would be advantageous (**Figure 2D**). Combining the two mutations, further referred to as Rpk, rescued expression and receptor binding of HKU4 RBD (**Figure 2E**) and MERS RBD (data not shown).

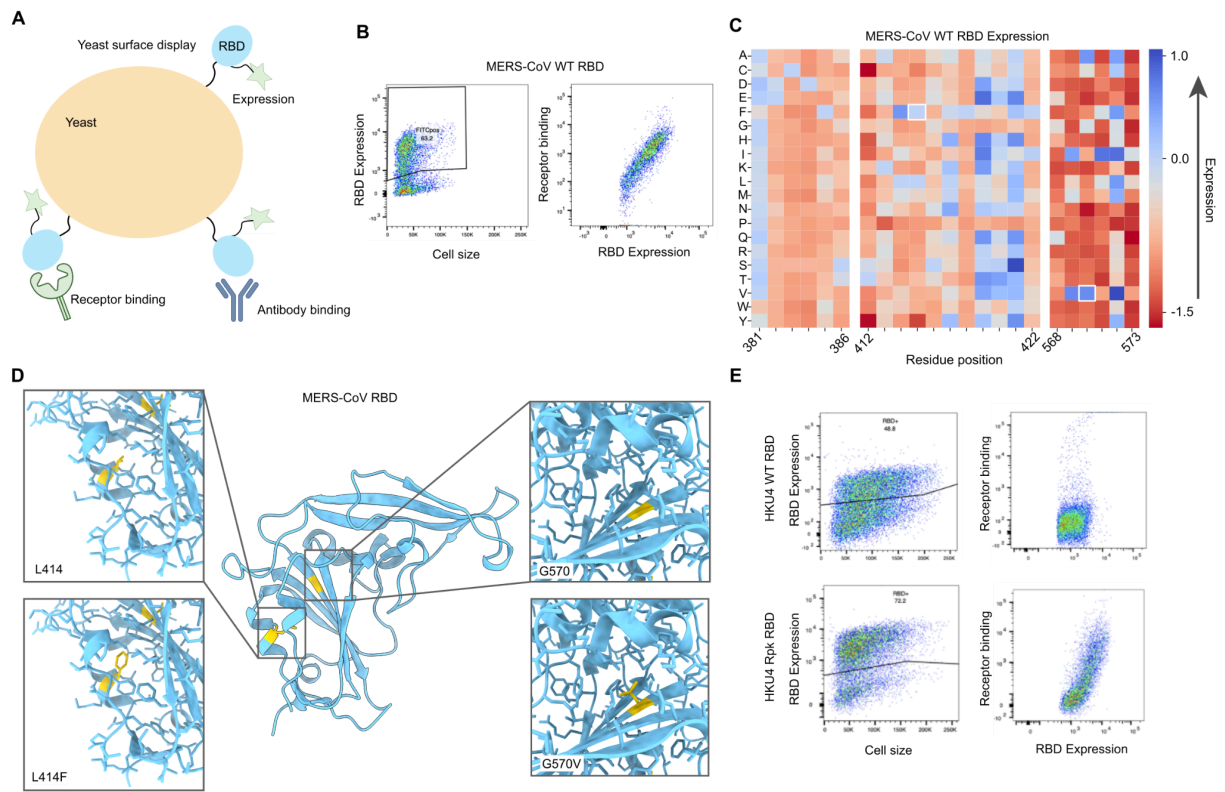


Figure 2. Stabilizing mutations identified through DMS and proteinMPNN.

- (A) Schematic of yeast surface display library of CoV RBDs with expression tags. Surface expression, antibody and receptor binding can be rapidly evaluated.
- (B) WT MERS-CoV RBD expression through yeast display (left) and hDPP4 binding (right).
- (C) DMS of MERS-CoV RBD showing changes in expression from a mutation library. Rpk mutations are indicated in a white box and the scale bar indicates changes in expression (blue is enhanced and red is reduced).
- (D) MERS-CoV RBD (PDB: 6Q04) with Rpk mutations shown in yellow.
- (E) Yeast surface display of WT HKU4-CoV RBD (top row) and Rpk-stabilized HKU4-CoV RBD (bottom row). Surface expression and receptor binding are shown.

Given the success of the Rpk mutations for HKU4, we sought to broadly apply them to other Merbecovirus RBDs genetically fused to the trimeric component of I53-50 for nanoparticle assembly. RBDs from each Merbecovirus clade were selected based on current expression data, known receptor usage and available probes, as well as the state of appropriate animal models for immunogenicity studies.^{26,27,29,37,38,248} Eight RBDs were selected and genetically fused to the N-terminus of I53-50A with a C-terminal 6x-His purification tag. Expi293F cells were transfected and proteins were purified with metal affinity chromatography before the final SEC purification step on a Superdex S200 Increase 10/300 gel filtration column (**Figure 3**). All RBD-50A constructs eluted at the expected volume on an S200 column.¹³¹ Nanoparticle assemblies were then conducted by adding appropriate amounts of the second component, I53-50B, and purified again through SEC on a Superose 6 Increase 10/300 gel filtration column. Assemblies yielded mostly well-behaved and monodisperse nanoparticles confirmed by ns-EM (**Figure 3**). HKU5 and HKU25 displayed insoluble characteristics during assemblies resulting in precipitation of the proteins from solution. Modulating assembly conditions to encourage cooperative and complete assemblies in addition to excipient screens for optimal buffer conditions failed to rescue assembly at full RBD valency.²³² We previously demonstrated through a valency titration of MERS RBD-I53-50 nanoparticles, displaying RBDs at 100%, 67%, and 33% occupancy, behaved similarly in animal studies with slight decreases in immunogenicity with decreasing RBD valency.⁷⁰ WT MERS RBD-I53-50 demonstrated

suboptimal assembly but reducing RBD valency improved nanoparticle behavior and solubility. Thus, we applied this same methodology to HKU5. HKU5 RBD-I53-50 at 67% valency completely rescued particle assembly with no signs of aggregation or precipitation (**Figure 3**).

Production of Merbecovirus nanoparticle immunogens

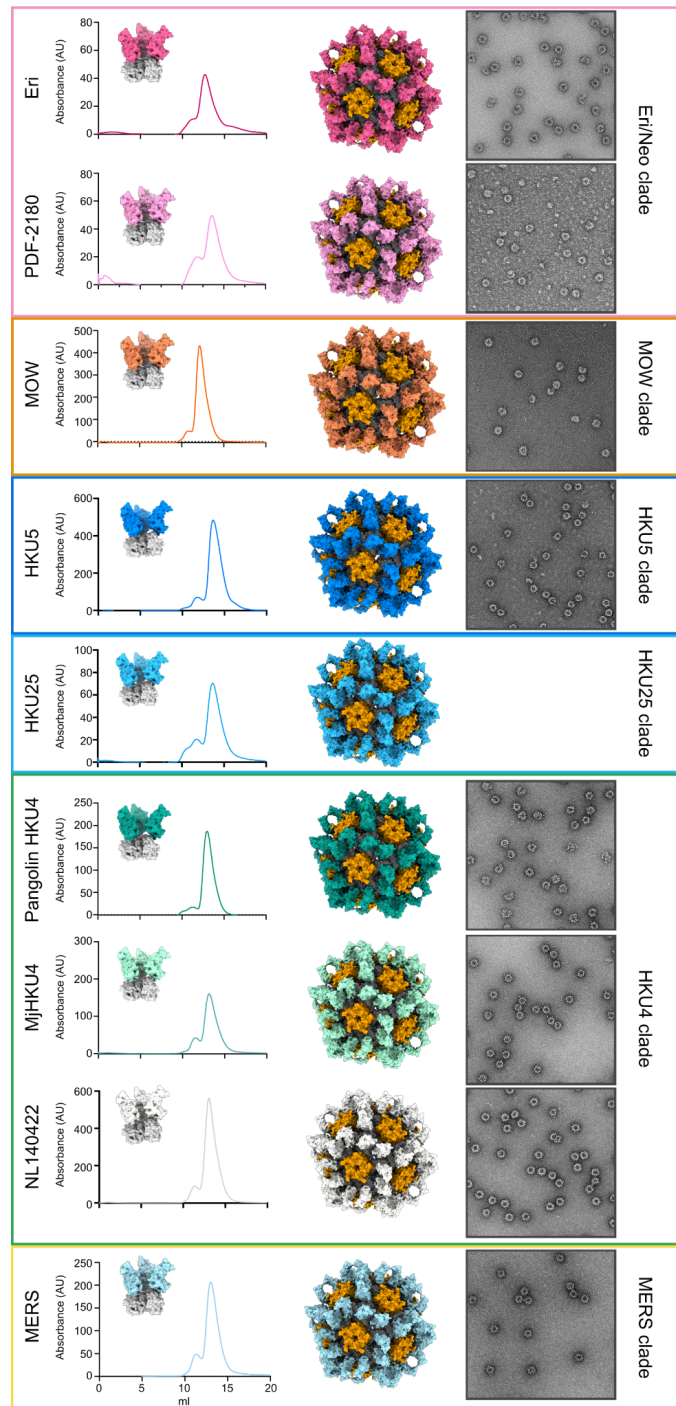


Figure 3. Merbecovirus RBDs express and assemble into nanoparticles

Representative Merbecovirus Rpk RBDs on I53-50a from each clade of the subgenus.

RBD-50a components were purified on a Superdex 200 Increase 10/300 gel filtration column

and assembled into nanoparticles with the addition of I53-50b. Negative stain micrographs of each assembly are shown.

With a handful of well-behaved monovalent Merbecovirus RBD nanoparticles, we next sought to produce a mosaic nanoparticle composed of RBDs from each clade for immunogenicity studies. We selected NL140422 (HKU4 clade), HKU5 (HKU5 clade), MOW (MOW clade), MERS (MERS clade), PDF-2180 and Eri (Eri/Neo clade) as candidate immunogens encompassing the subgenus for the hexavalent mosaic nanoparticle (**Figure 4A**). As the most genetically distant clade from MERS, two representatives were selected from the Eri/Neo clade to encourage more expansive breadth. Furthermore, binding analyses from MERS RBD-I53-50 vaccine-elicited polyclonal serum demonstrated notable cross-reactivity towards RBDs across the HKU5 and HKU25 clades (data not shown), implicating that immunizing with MERS-CoV RBD nanoparticles can elicit antibodies that are reactive across clades. The hexavalent assembly was monodisperse and well-behaved after SEC purification on a Superose 6 Increase 10/300 column, characteristics corroborated with ns-EM and DLS showing well-assembled nanoparticles with no signs of aggregation (**Figure 4B-4D**). BLI of each component immobilized on Ni-NTA tips showed binding of RBDs to their known receptors: NL140422 bound to hDPP4, HKU5 to *P. abramus* ACE2, MOW demonstrated binding to *P. dav* ACE2, MERS to hDPP4, and PDF-2180 bound to *A. pallidus* ACE2 (**Figure 4F**). No receptors were available for Eri-CoV at the time of production. Sandwich BLI of immobilized monoclonal antibodies or Fc-fused receptors loaded with assembled nanoparticles also demonstrated binding to multiple receptors, suggesting nanoparticle mosaicism (data not shown). SDS-PAGE of assembled particles showed bands of each component at their expected molecular weights (approximately 50 kDa) (**Figure 4F**). Immunoprecipitation assays will need to be performed to further validate mosaicism as an orthogonal experiment. Collectively these data demonstrate a well-behaved

and mosaic assembly displaying what we presume to be all of the RBD components, given that not all of the RBDs had probes.

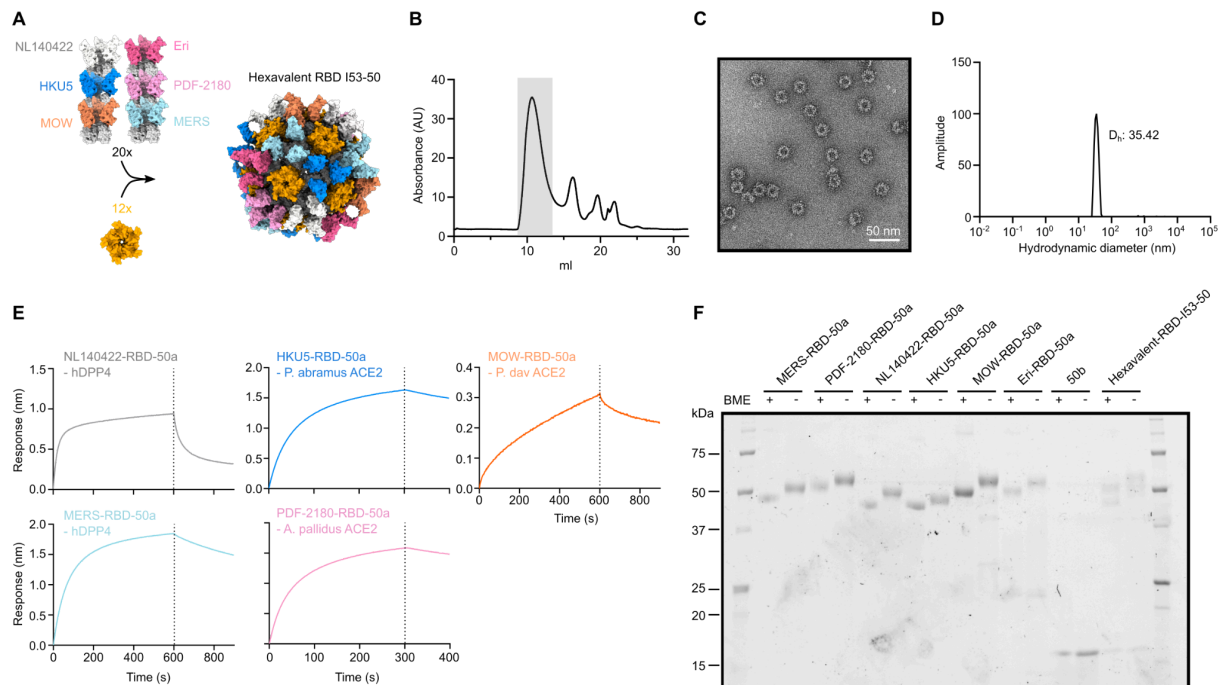


Figure 4. Producing Hexavalent RBD-I53-50 Merbecovirus nanoparticles.

(A) Assembly schematic of the Hexavalent RBD-I53-50 nanoparticle containing RBDs from NL140422-, HKU5-, MOW-, Eri-, PDF-2180- and MERS-CoV stabilized with Rpk mutations. A fully assembled nanoparticle will display 60 RBDs.

(B) SEC of Hexavalent RBD-I53-50 on a Superose 6 Increase 10/300 column.

(C) ns-EM of assembled nanoparticles after SEC in **(B)**. Scale bar, 50 nm.

(D) DLS Hexavalent RBD-I53-50 measured on an UNcle (UNchained Labs). Hydrodynamic diameter (D_h) is indicated.

(E) BLI of nanoparticle components. Ni-NTA tips were used to immobilize His-tag bearing components before binding cognate receptors of each RBD.

(F) SDS-PAGE before and after assembly under reducing and non-reducing conditions. BME, β -mercaptoethanol.

Evaluating MERS-CoV nanoparticle immunogens as vaccine candidates in non-human primates

Our previous work evaluating MERS RBD-I53-50 in transgenic mice challenged with lab adapted MERS-CoV EMC showed protective immune responses after two doses of immunogen

that were comparable to other tested MERS-CoV immunogens.⁷⁰ Given the low barrier to de-risk a MERS-CoV RBD nanoparticle vaccine due to SKYCovione, it is necessary to evaluate MERS RBD-I53-50 in higher order animal models. Murine models play a foundational role in early stages of vaccines and therapeutics development, however, there are many biological differences between mice and humans that may not be accurately depicted in mouse models. Furthermore many common mouse lines used are highly inbred which presents additional disadvantages when looking at immune responses. As a closer model to humans in anatomy, immune response and pathogenesis, non-human primates (NHPs) play an important role in the development of therapeutics preceding human clinical trials.²⁵⁵ In collaboration with the Villinger and Pulendran groups at the University of Louisiana at Lafayette and Stanford University, respectively, we designed an immunogenicity study in NHP with rhesus macaques as our model.

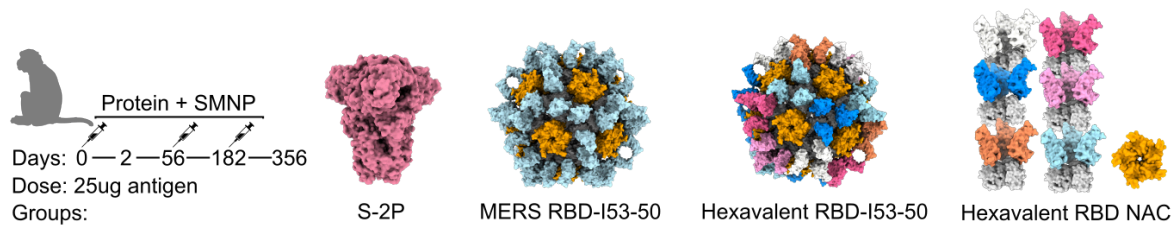


Figure 5. Evaluating Merbecovirus vaccine immunogens in NHPs.

Study design to assess Merbecovirus immunogens within Rhesus Macaques. Three doses (25ug antigen) will be administered at days 0, 56, and 182, with the study lasting 365 days. The four groups are MERS S-2P, MERS RBD-I53-50, Hexavalent RBD-I53-50, and NAC cocktail of the Hexavalent nanoparticle.

The study will consist of four groups (n=5): soluble prefusion stabilized MERS-CoV spike (S-2P), MERS RBD-I53-50, Hexavalent RBD-I53-50, as well as a cocktail of non-assembling control (NAC) comprised of unassembled components in Hexavalent RBD-I53-50 (**Figure 5**). Each group will have a size of n=5 and all groups will receive three 25 ug doses of antigen adjuvanted with SMNP, a immune-stimulating complex comprised of saponin and Toll-like

receptor 4 agonist monophosphoryl lipid A.²⁵⁶ The first boost will be administered two months post prime and the second boost immunization will take place four months post boost, with the study terminating after 365 days (**Figure 5**). Throughout the duration of the study, we will obtain multiple samples including blood, fine needle aspirates, bone marrow, bronchoalveolar lavage, as well as nasal and pharyngeal swabs. Binding and neutralizing antibodies against MERS spike will be determined in addition to T cell responses and cytokine measurements. We will also track germinal center formation and B cell maturation across the duration of the study. Samples from this study will be utilized in collaboration with the Pulendran, Baric, Veelsler, and Starr labs to conduct a series of key readouts to evaluate vaccine efficacy along with antibody discovery and DMS to improve our understanding of Merbecoviruses.

Separate ongoing and future immunogenicity studies evaluating these new Merbecovirus RBD nanoparticles will also unveil more about these prototype pathogens and hopefully provide insights into immune correlates of protection within the subgenus. Additional challenge studies in murine models will be necessary to evaluate the protective efficacy of candidate immunogens largely due to the lack of MERS-CoV and Merbecovirus challenge models in higher order mammals.²⁰⁸ These results will provide insights into whether these new Merbecovirus nanoparticle immunogens can elicit cross-protective immunity.

SUMMARY

The emergence of MERS-CoV in 2012 marked the second highly pathogenic zoonotic coronavirus transmission into humans preceding SARS-CoV-2. With a 35% mortality rate, the virus continues to cause outbreaks within the Middle East where natural reservoirs reside, with the most recent reported case from Saudi Arabia in September 2024. Furthermore, new viruses within the same viral subgenus that are capable of using human receptors (or orthologues) for viral entry are being discovered. With no vaccine strategies against any Merbecovirus, a potential future zoonotic infection stemming from this subgenera poses a threat to global health.

In this thesis, I demonstrate the development of multiple MERS-CoV nanoparticle vaccine candidates that elicit protective immune responses in mouse models. I also unveil regions along the MERS-CoV S protein that are predominantly targeted in polyclonal antibody responses from vaccine-elicited sera, which suggests that immunizing with certain S antigens can potentially skew antibody responses. These findings serve as the groundwork for future immunogenicity studies that will further investigate the MERS RBD-I53-50 nanoparticle as a vaccine candidate. I also jointly utilized deep learning methods and DMS libraries to identify stabilizing mutations for Merbecovirus RBDs. This advancement was applied towards producing a panel of novel self-assembling Merbecovirus RBD nanoparticles to be used as tools to explore other members of the viral subgenus. My work on developing nanoparticle immunogens has opened avenues to investigate and hopefully establish immune correlates of protection for Merbecoviruses. This work can further be applied towards developing vaccines to protect against CoV outbreaks within Betacoronaviruses that pose a risk for future pandemics.

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