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Investigating the components of heterotypic immunity associated with protection against coronavirus related disease

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Dissertation

INVESTIGATING THE COMPONENTS OF HETEROTYPIC IMMUNITY ASSOCIATED WITH PROTECTION AGAINST CORONAVIRUS RELATED DISEASE

by

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DEDICATION

I would like to dedicate this work to my parents and my loving wife, Emily,

for always believing in me.

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INVESTIGATING THE COMPONENTS OF HETEROTYPIC IMMUNITY ASSOCIATED WITH PROTECTION AGAINST CORONAVIRUS RELATED DISEASE

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ABSTRACT

A few highly pathogenic coronaviruses (CoVs) have emerged in the human population in the past twenty-five years and have impacted the world on a global scale. Several demographic and clinical factors are associated with protection against severe disease from these pathogenic human CoV infections. In the context of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a recent infection with a "common cold" causing human endemic CoV (eCoV) is associated with protection against severe coronavirus disease 2019 (COVID-19). Yet, the cross-reactive immune components providing this heterotypic immunity between heterologous coronaviruses have not been fully elucidated. We hypothesize that heterotypic immune protection is not mediated by neutralizing antibodies (nAbs) because of the diversity in spike protein receptor binding domains within the CoV family, but by alternative adaptive immune factors that target more conserved CoV regions.

To test this hypothesis, we identified individuals from Boston Medical Center (BMC) with prior infections or vaccinations to SARS-CoV-2 or the eCoVs (HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43) and measured both homologous and

heterologous CoV directed immune responses. First, in a study of risk factors of SARS-CoV-2 reinfection, SARS-CoV-2 antibody responses were similar between those with or without a SARS-CoV-2 reinfection and nAbs were not associated with long-term homotypic CoV protection. To explore heterotypic immunity, we classified individuals with or without a presumed or documented recent eCoV infection in a cohort of SARS-CoV-2 naïve individuals. Cross-reactive T cell and nAb responses against SARS-CoV-2 were similar between individuals regardless of recent eCoV infection history. Meanwhile, individuals with a presumed or documented recent eCoV infection had higher and correlative levels of Fc receptor (FcR) binding antibodies against eCoV spikes (S) and SARS-CoV-2 S2 subunit. Lastly to investigate the extent of coronavirus heterotypic immunity, we investigated immune components associated with SARS-CoV-2 mediated protection against subsequent symptomatic eCoV infections. Cross-reactive replication and transcription complex (RTC)-specific $CD8^+$ T cells were associated with protection against symptomatic eCoV infections in individuals with a previous SARS-CoV-2 infection, while other eCoV-responsive T cells and nAbs were not predictive of heterotypic immune protection. In aggregate, nAbs were not associated with long-term protection against homologous or heterologous CoV infections and associated disease, but instead, antibody Fc effector functions and $CD8^+$ T cells were associated with protective roles. These findings indicate that eliciting diverse immune functions along with nAbs in a future pan-CoV vaccine will be important to protect against disease from current and novel human CoVs.

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LIST OF ABBREVIATIONS

ACE2..... Angiotensin-Converting Enzyme 2

ADAR .....Adenosine Deaminases

ADCC .....Antibody-Dependent Cellular Cytotoxicity

ADCD ..... Antibody-Dependent Complement Deposition

ADCP .....Antibody-Dependent Cellular Phagocytosis

ADE .....Antibody-Dependent Enhancement

AEB..... Average Number of Enzymes per Bead

aHR .....Adjusted Hazard Ratio

AIM..... Activation-Induced Marker

AM ..... Alveolar Macrophage

APC.....Antigen-Presenting Cell

APN ..... Aminopeptidase N

APOBEC. Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide-Like Proteins

ARDS.....Acute Respiratory Distress Syndrome

ATCC.....American Type Culture Collection

AUC .....Area Under the Curve

BMC..... Boston Medical Center

bnAb..... Broadly Neutralizing Antibody

CDC .....Centers for Disease Control and Prevention

CEF .....CMV, Epstein-Barr Virus, and Influenza

CI..... Confidence Interval

CKD ..... Chronic Kidney Disease

CMV ..... Cytomegalovirus

CoV ..... Coronavirus

COVID-19 ..... Severe Coronavirus Disease 2019

CRP-PCR ..... Comprehensive Respiratory Panel-Polymerase Chain Reaction

cT_{fh} ..... Circulating $CD4^+$ T Follicular Helper

DC ..... Dendritic Cell

DDP4 ..... Dipeptidyl-Peptidase 4

DM ..... Diabetes Mellitus

DMEM ..... Dulbecco's Modified Eagle Medium

DMSO ..... Dimethyl Sulfoxide

dsRNA ..... Double Stranded Ribonucleic Acid

E ..... Envelope

eCoV ..... Endemic Coronavirus

EF ..... Extrafollicular

ELISA ..... Enzyme-Linked Immunosorbent Assay

EMR ..... Electronic Medical Records

ERGIC ..... Endoplasmic Reticulum-Golgi Intermediate Compartment

ExoN ..... Exoribonuclease

FACS ..... Fluorescence-Activated Cell Sorting

FBS ..... Fetal Bovine Serum

Fc.....Fragment Crystallizable

FcR..... Fragment Crystallizable Receptor

FcγR .....Fragment Crystallizable Gamma Receptor

FDC.....Follicular Dendritic Cell

FEASI .Fucose-Sensitive ELISA-Based Method of Antigen-Specific IgG Fc Fucosylation

FP ..... Fusion Peptide

G..... Vesicular Stomatitis Virus G Protein

GC .....Germinal Center

HCoV .....Human Coronavirus

HEK ..... Human Epithelial Kidney

HIV ..... Human Immunodeficiency Virus

HLA .....Human Leukocyte Antigen

HR..... Hazard Ratio

HR.....Heptad Repeat

HRP..... Horseradish Peroxidase

IBV.....Infectious Bronchitis Virus

ICU..... Intensive Care Unit

IEDB ..... Immune Epitope Database and Analysis Resource

IFN ..... Interferon

IG ..... Immunoglobulin

IL.....Interleukin

IQR..... Interquartile Range

IRF ..... Interferon Regulatory Factor

ISG ..... Interferon-Stimulated Gene

KM ..... Kaplan Meier

L .....Liter

M ..... Membrane

M .....Molar

MAIT ..... Mucosal-Associated Invariant T

MAVS ..... Mitochondrial Antiviral-Signaling Protein

MDM ..... Monocyte-Derived Macrophage

MERS-CoV ..... Middle East Respiratory Syndrome Coronavirus

MHC ..... Major Histocompatibility Complex

MHV ..... Mouse Hepatitis Virus

MIC ..... Major Histocompatibility Complex Class I Polypeptide-Related Sequence

mL ..... Milliliter

mM ..... Millimolar

MyD88 ..... Myeloid Differentiation Primary Response 88

N.....Nucleocapsid

nAb..... Neutralizing Antibody

NP ..... Nucleocapsid Protein

NET.....Neutrophil Extracellular Trap

NF-κB ..... Nuclear Factor Kappa B

NIH ..... National Institutes of Health

NK ..... Natural Killer

NKT ..... Natural Killer T

nm ..... Nanometer

NSP ..... Non-Structural Protein

NTD ..... N-Terminal Domain

OAS1 ..... 2'-5'-Oligodenylate Synthetase

OD ..... Optical Density

ORF ..... Open-Reading Frame

PASC ..... Post-Acute Sequelae of COVID-19

PBMC ..... Peripheral Blood Mononuclear Cell

PBS ..... Phosphate Buffered Saline

PBST ..... Phosphate Buffered Saline Containing 0.05% Tween 20

PCR ..... Polymerase Chain Reaction

PEDV ..... Porcine Epidemic Diarrhea Virus

PP ..... Polyprotein

PRR ..... Pattern Recognition Receptor

RBD ..... Receptor Binding Domain

RdRp ..... RNA-Dependent RNA Polymerase

RIG-I ..... Retinoic Acid-Inducible Gene I

RLR ..... Retinoic Acid-Inducible Gene I-Like Receptor

RNA ..... Ribonucleic Acid

RNase L ..... Ribonuclease L

RNP.....Ribonucleoprotein

RPMI-1640 ..... Roswell Park Memorial Institute 1640 Medium

RT-PCR ..... Reverse Transcription-Polymerase Chain Reaction

RTC.....Replication and Transcription Complex

S ..... Spike

SI.....Stimulation Index

SP .....Spike Protein

SARS-CoV..... Severe Acute Respiratory Syndrome Coronavirus

SARS-CoV-2 ..... Severe Acute Respiratory Syndrome Coronavirus 2

sgRNA ..... Subgenomic Ribonucleic Acid

Simoa .....Single Molecule Array

STAT..... Signal Transducer and Activator of Transcription

TCR.....T Cell Receptor

TLR.....Toll-Like Receptor

TMB.....3,3',5,5' Tetramethylbenzidine

TMPRSS2 .....Transmembrane Protease Serine 2

TNF-α ..... Tumor Necrosis Factor-α

T_{reg}..... Regulatory T Cell

TRIF ..... TIR-Domain-Containing Adaptor-Inducing Interferon-β

TRS .....Transcription Regulatory Sequence

U..... Unit

UTR..... Untranslated Region

VLP ..... Virus-Like Particle

VOC ..... Variant of Concern

VSV ..... Vesicular Stomatitis Virus

x ..... Times

xg ..... G-Force

μg ..... Microgram

°C ..... Degrees Celsius

4PL ..... Four-Parameter Logistic

CHAPTER ONE: INTRODUCTION

Emergence and Pathogenesis of Human Coronaviruses

Coronaviruses (CoVs) are a group of positive sense RNA viruses that make up the subfamily *Orthocoronavirinae* within the *Coronaviridae* family. This family of viruses were first discovered in the 1930s, when infectious bronchitis virus (IBV) (later renamed avian coronavirus) was identified as the agent causing severe respiratory disease in chickens (Schalk and Hawn 1931; Beach and Schalm 1936). A decade later, similarly shaped viruses were identified in mice causing a range of symptoms from encephalitis to hepatitis, which led to the naming of mouse hepatitis virus (MHV) (later renamed murine coronavirus) (Cheever and Daniels 1949; Gledhill and Andrewes 1951). It was not until the 1960s that these novel viruses were grouped together and named "coronaviruses" after scientists identified the signature halo of spike proteins protruding from these viruses through the use of electron microscopy ("Virology: Coronaviruses" 1968). Since the initial discovery of these CoVs, dozens of CoVs have been identified in a large range of bird and mammalian hosts. The majority of known CoVs have been isolated and identified in small mammals, such as bats and rodents, which have been speculated as a viral reservoir and source of viral spillover into the human population (Cui, Li, and Shi 2019). To date, there have been seven CoVs that have crossed the inter-species barrier and persisted in the human population (Illustration 1.1).

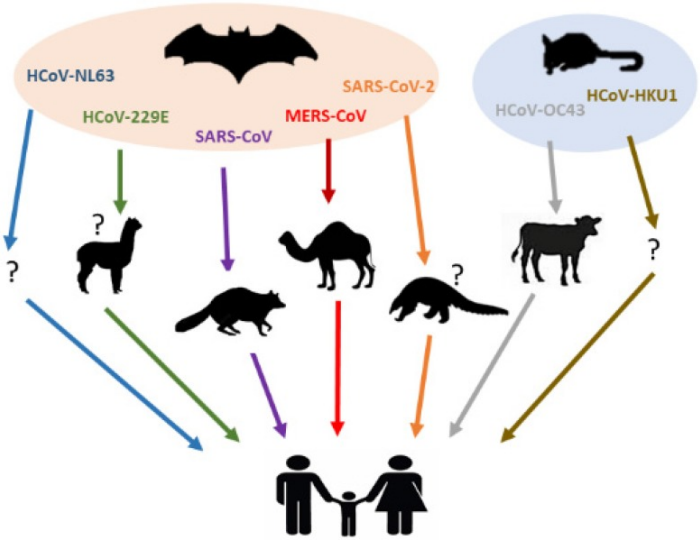


Illustration 1.1. Zoonotic origins of human CoVs. Animals suspected to be the primary (bats, rodents) or intermediatory hosts (camelids, civets, pangolins, bovine) of human CoVs. Image adapted from (Ye et al. 2020).

The first human CoV (HCoV) was identified in 1961, when researchers in England collected throat swab samples from children with common colds (Kendall, Bynoe, and Tyrrell 1962). They identified a novel infectious virus, B814, that could not be cultured using the common methods at the time and was resistant to antibiotic and antibody treatments. In a similar study in the United States in 1962, researchers isolated a novel virus, HCoV-229E, from the respiratory tract of a medical student (Hamre and Procknow 1966; Hamre, Kindig, and Mann 1967). These two viruses were found to be morphologically similar as both IBV and murine coronavirus and determined to be the first HCoVs (Almeida and Tyrrell 1967). Although B814 has since been lost due to the inability to reliably culture the virus *ex vivo*, HCoV-229E has persisted in the human population for decades. Closely related sequences of HCoV-229E were later discovered in both bats and camels, identifying animal reservoirs and potential sources of zoonotic

spillover events into the human population (Illustration 1.1) (Pfefferle et al. 2009; Corman et al. 2016). This zoonotic transmission to humans may have occurred as early as the 1880s based off of molecular dating of most recent common ancestor between HCoV-229E and bat CoV strains (S. K. P. Lau et al. 2021).

Soon after the discovery of HCoV-229E, another common cold-causing endemic HCoV (eCoV), HCoV-OC43, was discovered in individuals with acute upper respiratory disease by culturing nasopharyngeal samples in tracheal organ cultures (McIntosh et al. 1967). Molecular clock analyses of HCoV-OC43 and bovine coronavirus genomes dated their most recent common ancestor to be from the late 19th century, suggesting a timeline of the zoonotic transmission event (Vijgen et al. 2006). Additional evolutionary analyses of mutation and recombination rates of various HCoV-OC43 genotypes validated HCoV-OC43's relatively recent adaptation to human hosts (S. K. P. Lau et al. 2011). Since the discovery of HCoV-229E and HCoV-OC43, two additional eCoVs were identified. In 2004, HCoV-NL63 was originally isolated from a child suffering from bronchiolitis and subsequently found circulating in the human population (van der Hoek et al. 2004). HCoV-NL43 grouped more closely to HCoV-229E in the *Alphacoronavirus* genera and likely originated from bats based on phylogenetic analyses (Illustration 1.2) (Huynh et al. 2012; Tao et al. 2017). Then in 2005, HCoV-HKU1 was identified in an individual with pneumonia (Woo, Lau, Chu, et al. 2005). HCoV-HKU1 was determined to be a betacoronavirus, like HCoV-OC43, potentially transmitted from murine hosts (S. K. P. Lau et al. 2015).

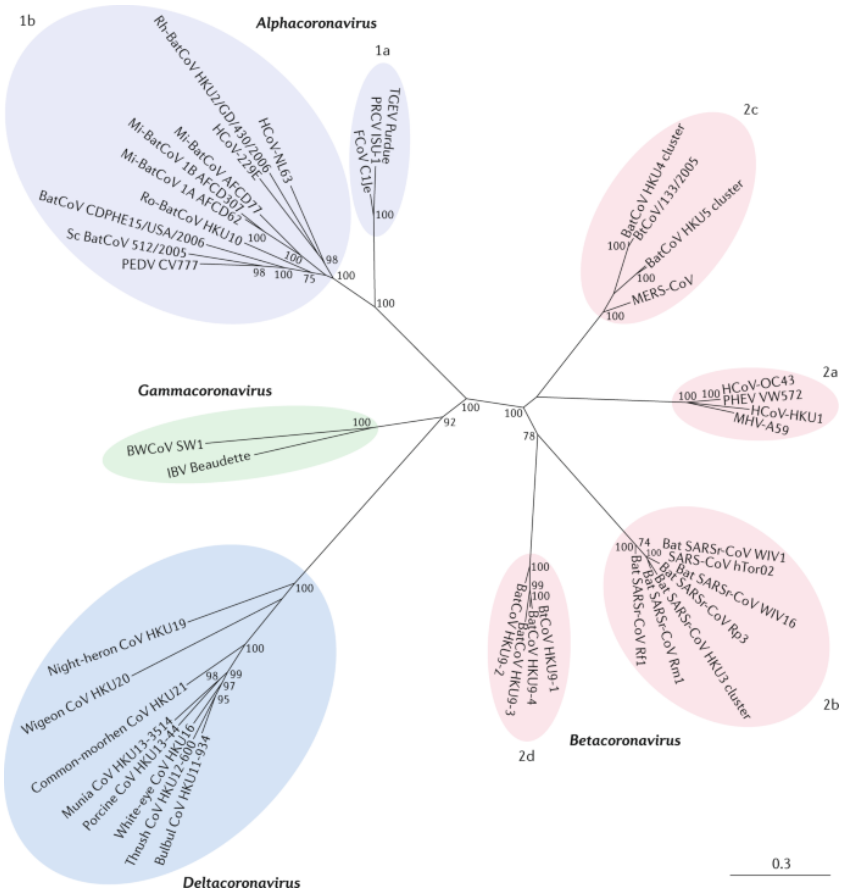


Illustration 1.2. Phylogenetic relationship between human and animal CoVs. Known human and animal CoVs are grouped together based on genetic similarities. Of note, HCoV-229E and HCoV-NL63 are in the *Alphacoronavirus* genera, while HCoV-HKU1, HCoV-OC43, MERS-CoV, SARS-CoV, and SARS-CoV-2 are in the *Betacoronavirus* genera. Image adapted from (Cui, Li, and Shi 2019).

Although genetically distinct and separately emerging in the human population, these four eCoVs (HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43) cause similar clinical symptoms. These eCoVs primarily infect the upper respiratory tract and cause between 10-30% of common colds, often associated with mild symptoms, such as a fever, headaches, and coughing (Gaunt et al. 2010; Walsh, Shin, and Falsey 2013; Galanti

et al. 2019). Infection of the lower respiratory tract by these eCoVs can cause more severe symptoms, such as pneumonia and acute respiratory distress syndrome (ARDS) (Woo, Lau, Tsoi, et al. 2005; W. Sun et al. 2021), and even death in a small percentage of cases (W.-I. Choi et al. 2021; Veiga et al. 2021). The severe cases of eCoV infections are often associated with comorbidities, such as age and immunosuppression (Pene et al. 2003). Although these fatal cases of eCoV infections are rare, highly pathogenic HCoVs emerged in the human population in the early 21st century.

Severe acute respiratory syndrome coronavirus (SARS-CoV) was determined to be the novel coronavirus causing the epidemic in 2003 (Zhong et al. 2003; Drosten et al. 2003). SARS-CoV likely originated in horseshoe bats (S. K. P. Lau et al. 2005; W. Li et al. 2005) and then spilled over into civets as an intermediate host before being transmitted to humans (Illustration 1.1) (Guan et al. 2003; Kan et al. 2005). As alluded to by its name, SARS-CoV caused severe acute respiratory distress in patients leading to a high percentage of infected patients requiring oxygen and ventilator support (Peiris et al. 2003). SARS-CoV led to an overall fatality rate around 10%, but was much higher, around 40-50%, for individuals aged 60 years or older (Donnelly et al. 2003). Since 2004, there have not been any reported cases of SARS-CoV infections.

In 2012, a new human coronavirus was first isolated from an individual with pneumonia and renal failure in Saudi Arabia (Zaki et al. 2012). This novel coronavirus, later named Middle East respiratory syndrome coronavirus (MERS-CoV), quickly spread throughout the Middle East and to parts of Africa, Asia, and Europe (de Groot et al. 2013). Similar to SARS-CoV, MERS-CoV often caused fever, dyspnea, and cough in the

majority of patients, though it led to more cases of severe pneumonia requiring intensive care (Saad et al. 2014). The increased severity of MERS-CoV infections led to an overall case fatality rate of 36% ("MERS Situation Update - December 2023" 2023). Unlike the SARS-CoV outbreak, MERS-CoV continues to cause outbreaks, with the most recent cases occurring as recently as 2023 (World Health Organization (29 August 2023) 2023). Despite the continued prevalence of MERS-CoV, the number of cases and deaths has decreased over time due to improved surveillance and outbreak mitigation efforts (Donnelly et al. 2019). Based off of phylogenetic comparisons, MERS-CoV likely originated in bats due to its similarity with bat coronavirus sequences (Illustration 1.2) (van Boheemen et al. 2012; Cotten et al. 2013). Both neutralizing antibodies and highly similar viral sequences to MERS-CoV have been isolated from dromedary camels, suggesting these camels may serve as an intermediate host before transmission to humans (Illustration 1.1) (Reusken et al. 2013; Raj et al. 2014).

The most recent CoV to emerge in the human population was in late 2019, with the rise of SARS-CoV-2 and its related coronavirus disease (COVID-19). SARS-CoV-2 first emerged in China and was quickly identified due to its similarity to SARS-CoV and bat coronaviruses (P. Zhou et al. 2020). Although controversy surrounds the origin of SARS-CoV-2, the virus was potentially transmitted to humans from an intermediate host in the Huanan Seafood Wholesale Market (Illustration 1.1) (Worobey et al. 2022; Pekar et al. 2022). From these first human cases, SARS-CoV-2 quickly spread across the globe, leading to a worldwide pandemic (J. Li et al. 2021). By the end of 2021, there were more than 280 million cases and around 5.4 million deaths of COVID-19 reported to the World

Health Organization, with estimates closer to 14.83 million excess deaths globally during the first two years of the pandemic (World Health Organization 2023 data.who.int 2023; Msemburi et al. 2023). Since 2021, SARS-CoV-2 has persisted in the human population as new viral variants continuously arise (Carabelli et al. 2023). More recent statistics of COVID-19 cases and deaths are unreliable because of widespread underreporting (Park et al. 2023). Additionally, many cases are undiagnosed as up to 40% of infections are asymptomatic (Ma et al. 2021). For those individuals with COVID-19, most cases are mild with symptoms similar to a common cold (Burke 2020). Severe cases of COVID-19 resemble the diseases caused by SARS-CoV and MERS-CoV, with symptoms ranging from dyspnea, hypoxemia, and ARDS (Berlin, Gulick, and Martinez 2020). As with the other HCoVs, COVID-19 was more severe in elderly and those with underlying medical conditions (L. Kim et al. 2021). Unlike other HCoV infections, COVID-19 symptoms can last months to years after the initial infection in 6-10% of individuals (C. E. Hastie et al. 2023). These persistent infections and post-acute sequelae of COVID-19 (PASC) in various organs throughout the body has led to an increased burden of cardiovascular, neurological, and gastrointestinal disorders (Davis et al. 2021; Al-Aly, Xie, and Bowe 2021). Despite the persistence of SARS-CoV-2 and the potential for long-term adverse health effects, the severity of COVID-19 has generally decreased due to improved medical interventions and widespread vaccination strategies (Bai et al. 2024; Moghadas et al. 2021).

With the emergence of a few highly pathogenic HCoVs in the beginning of the 21st century, there is increasing concern about the next novel coronavirus outbreak. The

threat of new zoonotic transmission events rises as the barrier between human and wildlife communities grows thin (Rulli et al. 2017). The rapid global spread of SARS-CoV-2 and other respiratory viruses highlights the ease of viral transmission across geographical borders with the current transportation methods (Belderok et al. 2013). A better understanding of how humans respond to the CoV family of viruses will potentially help to develop strategies to mitigate the severe consequences from a future CoV outbreak.

Structure and Function of the Coronavirus Proteome

Coronaviruses are single-stranded, positive-sense ribonucleic acid (RNA) viruses (V'kovski et al. 2021). Their genomes are relatively large compared to other RNA viruses, ranging from 29 to 32 kilobases (R. Lu et al. 2020). The CoV genomes share a similar genomic structure (Illustration 1.3), with the first 5' two-thirds of the genome encoding non-structural proteins important for viral transcription and replication, while the 3' one-third contains genes for the structural and accessory proteins (Brian and Baric 2005). Upon entry into a cell, the first proteins translated by the host machinery are the non-structural polyproteins proteins, pp1a and pp1ab (Illustration 1.4) (Finkel et al. 2021). The production of pp1ab is a result of a ribosomal frameshift between the overlap of open-reading frames ORF1a and ORF1b (Irigoyen et al. 2016). The polyproteins, pp1a and pp1ab, are then self-cleaved by two proteases in nsp3 (papain-like protease, PL^{pro}) and nsp5 (3C-like protease, $3CL^{pro}$, or main protease, M^{pro}), resulting in sixteen non-structural proteins (Meyer et al. 2021). Some non-structural proteins indirectly contribute to viral replication by modulating the cellular host, through inhibition of the host's

interferon response (nsp3, nsp5), interference with translation of host mRNA (nsp1), or evasion of the host's sensing of foreign RNA through the formation of double-membrane vesicles (nsp3, nsp4, nsp6) (Thoms et al. 2020; Minkoff and tenOever 2023). The other non-structural proteins comprise the viral replication and transcription complex (RTC). The core of the RTC contains non-structural proteins nsp12-16, with enzymatic activities such as RNA synthesis (nsp12, RNA-dependent RNA polymerase or RdRp), RNA proofreading (nsp14), and RNA capping (nsp13, nsp14, nsp16) (Snijder, Decroly, and Ziebuhr 2016). Additionally, non-structural proteins, nsp7-8 and nsp10, act as cofactors to aid in the activity of the RdRp and RNA capping machinery, respectively (Kirchdoerfer and Ward 2019; Krafcikova et al. 2020).

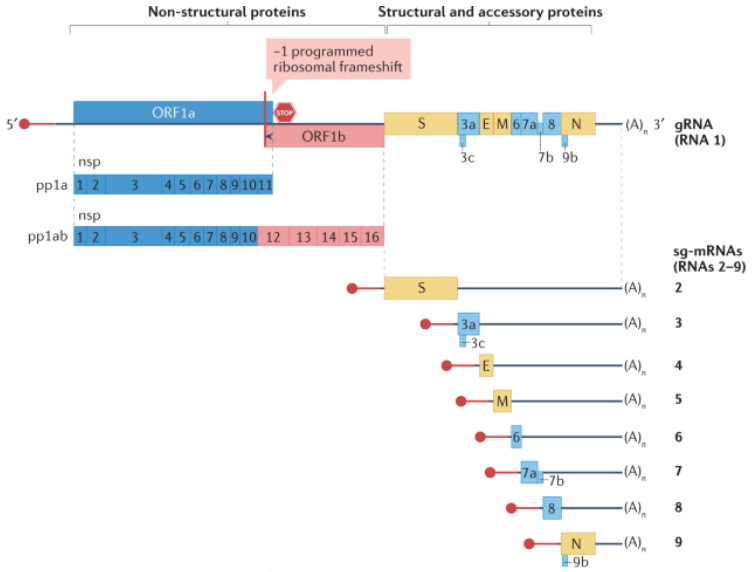


Illustration 1.3. SARS-CoV-2 genome organization. SARS-CoV-2 genome and its corresponding RNA transcripts as a representative example of the genome organization of the family of CoVs. The pp1a and pp1ab RNA transcripts are used to produce the non-structural proteins, while subgenomic (sg) RNA encodes the structural and accessory proteins. Image adapted from (Malone et al. 2022).

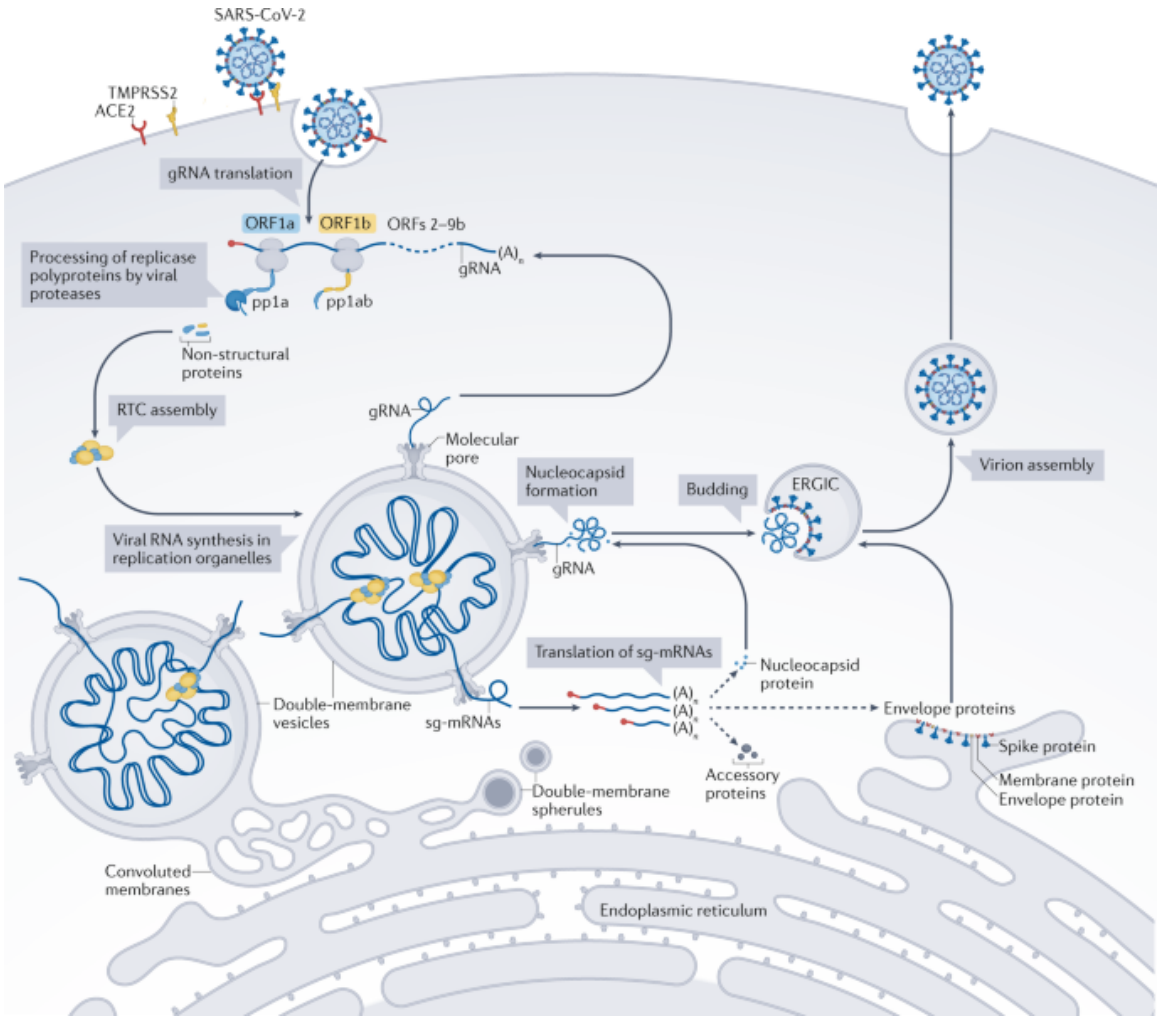


Illustration 1.4. Viral replication cycle of SARS-CoV-2. Schematic depicting SARS-CoV-2 entry, transcription, replication, and assembly. The viral life cycle of SARS-CoV-2 is representative of other CoVs. Image adapted from (Malone et al. 2022).

After the initial translation of the RTC complex, the viral replication machinery then makes copies of the viral RNA through negative-strand and positive-strand RNA synthesis (Illustration 1.5). These new viral RNAs are initially used to produce more non-structural proteins to help further evade cellular detection and increase the rate of viral replication. The transcription of the structural and accessory genes on the 3' end of the genome occurs from the production of subgenomic RNAs (sgRNAs) during negative-

strand RNA synthesis (D. Kim et al. 2020). This discontinuous transcription process results from transcription interruption and re-initiation at transcription regulatory sequences (TRS) (Pasternak et al. 2001). The nested sgRNAs then provide the templates for the translation of the structural and accessory proteins (Illustration 1.3). The major structural proteins include spike (S), envelope (E), membrane (M), and nucleocapsid (N).

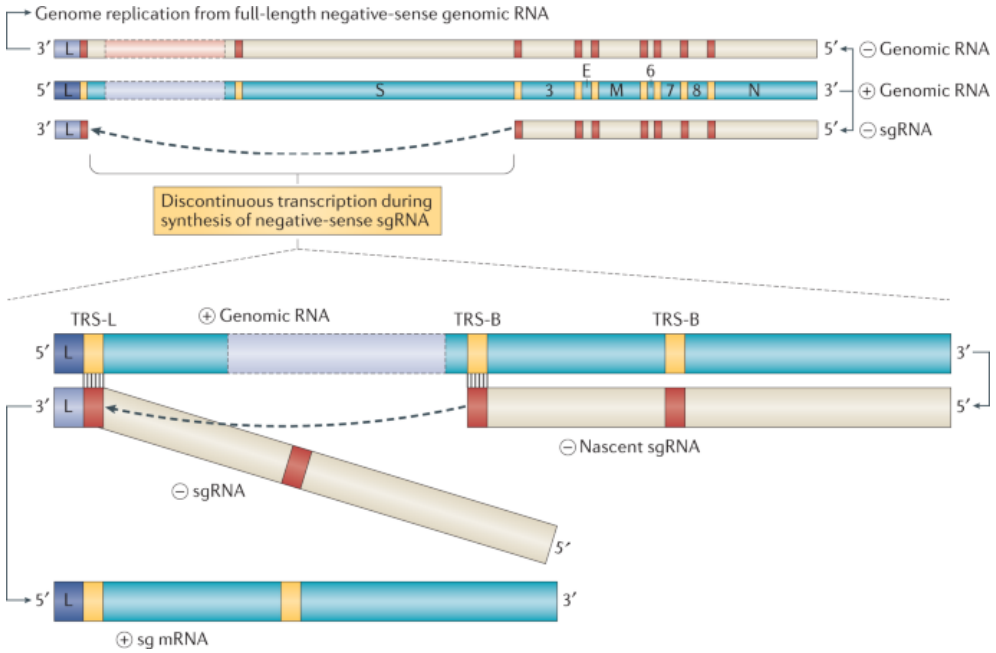


Illustration 1.5. CoV RNA transcription and replication. CoV synthesis of negative-sense and positive-sense viral RNA strands. Sub-genomic RNAs are a result of discontinuous transcription and re-initiation at the transcription regulatory sequences (TRS). Image adapted from (V'kovski et al. 2021).

The spike protein is responsible for the binding to the host receptor and initiating viral entry by fusing the viral and host membranes (F. Li 2016). The HCoVs recognize and utilize a range of host receptors: SARS-CoV, SARS-CoV-2, and HCoV-NL63 primarily use angiotensin-converting enzyme 2 (ACE2), MERS-CoV uses dipeptidyl-

peptidase 4 (DDP4), HCoV-229E uses aminopeptidase N (APN), HCoV-HKU1 uses transmembrane protease serine 2 (TMPRSS2), and HCoV-OC43 recognize 9-*O*-acetylsialic acids, though the primary receptor is largely unknown (Shang et al. 2020; Lan et al. 2020; Hofmann et al. 2005; G. Lu et al. 2013; Yeager et al. 1992; Saunders et al. 2023; Hulswit et al. 2019). Additional molecules have been suggested to be attachment factors to improve viral entry efficiency for each of these HCoVs. For example, SARS-CoV and SARS-CoV-2 has been noted to bind to various lectins and phosphatidylserine receptors, but the entry mechanisms are virus non-specific (Marzi et al. 2004; Jemielity et al. 2013). Upon binding of CoV spike to its respective host receptor, the spike protein requires two proteolytic cleavage events before membrane fusion (Illustration 1.6). The first cleavage event occurs between the spike S1 and S2 subunit, and the second cleavage event occurs at the S2' site within S2 (C. B. Jackson et al. 2022). The S1-S2 cleavage event allows the shedding of the S1 subunit, exposing the S2' site for cleavage by serine proteases and cathepsins, and triggering viral-host membrane fusion (Belouzard, Chu, and Whittaker 2009; Millet and Whittaker 2014). Depending on the CoV spike sequences and presence of host proteases, the cleavage events can occur during trafficking from the producer cell by furin-like enzymes, at the cell surface during attachment of the target cell by serine proteases such as TMPRSS2, or during endosomal trafficking by cathepsin proteases (Peacock et al. 2021; Matsuyama et al. 2010; Simmons et al. 2005). The S2' cleavage event induces a conformational change in the heptad repeat 1 (HR1) domain of S2, injecting the fusion peptide (FP) into the host membrane (Walls et al. 2017; Cai et al. 2020). Upon interaction between the HR1 and

HR2 domains, the host and viral membranes are moved close enough to each other for membrane fusion to occur (Xia et al. 2020; Fan et al. 2020).

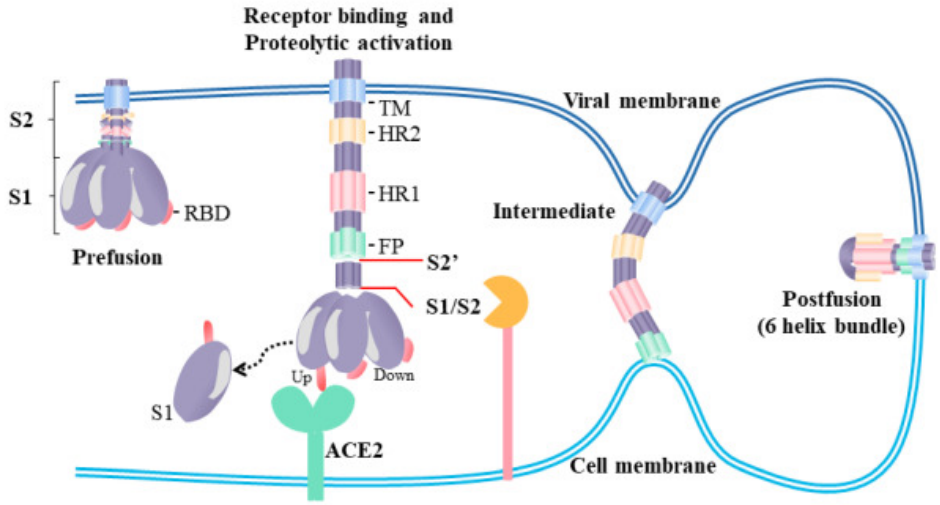


Illustration 1.6. Conformational changes of SARS-CoV-2 spike mediating viral entry. Upon binding to the ACE2 receptor and cleavage of the spike S1 subunit, the S2' cleavage site is exposed. Enzymatic cleavage at the S2' site induces a conformational change in the spike S2 subunit, which injects into the host cell and fuses the host and viral membranes together. Image adapted from (S. Yu et al. 2023).

The other major structural proteins of CoVs primarily help with the formation and stability of the virions (Illustration 1.4). Although the viral RNA packing signals and the order of events required for viral assembly is not well defined and differs amongst the genera of CoVs, the interaction of membrane (M) and nucleocapsid (N) protein with viral genomic RNA is critical for the production of mature virions (Masters 2019). The primary role of the N protein is the organization and protection of viral RNA within the virion, but the N protein also has many other functions, including a role in the efficient transcription of viral RNA (Zúñiga et al. 2010). Viral assembly requires the oligomerization of N proteins (He et al. 2004; Cong et al. 2017) and the binding of N

protein to viral genomic RNA to form a ribonucleoprotein complex (RNP) (Gui et al. 2017). The M protein, localized to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) of infected cells, binds the N protein and recruits the RNP complex closer to the membrane (Narayanan et al. 2000; Zhikuan Zhang et al. 2022). The M protein, along with the envelope (E) protein, define the shape of mature virions and virus-like particles (VLP) into its proper morphology (Vennema et al. 1996; Fischer et al. 1998). Even though only a small proportion of E protein is actually incorporated into mature virions (Nieto-Torres et al. 2011; Venkatagopalan et al. 2015), the E protein induces membrane curvature and promotes M-M homotypic protein interactions during viral assembly (Raamsman et al. 2000; de Haan, Vennema, and Rottier 2000). Additionally, both the M and E proteins keep spike proteins in the ERGIC through direct interaction and modulation of the secretary pathway, respectively (Boson et al. 2021). This direct protein interaction between M protein and spike incorporates the spike protein into the viral envelope of the budding virion (Opstelten et al. 1995; Godeke et al. 2000).

In addition to the non-structural and structural proteins, coronaviruses also contain open-reading frames (ORF) encoding a variety of accessory proteins, which are not well conserved between the genera. For example, SARS-CoV-2 has nine accessory proteins (Gordon et al. 2020), while HCoV-229E only contains a single accessory protein between spike and envelope (Dijkman et al. 2006). In general, the accessory proteins are not required for viral replication, but they play important roles *in vivo* (de Haan et al. 2002). These accessory proteins contribute to the success of the virus through immune evasion and modulation of the host's immune response. For both SARS-CoV and SARS-

CoV-2, ORF9b interferes with the mitochondrial antiviral-signaling protein (MAVS) signaling pathway through a variety of ways, preventing a strong type-I interferon (IFN) response (Shi et al. 2014; J. Wu et al. 2021). Additionally, the type-I IFN response can be antagonized through the downregulation of the type-I IFN receptor and the inhibition of signal transducer and activator of transcription (STAT) signaling by SARS-CoV ORF3a and SARS-CoV/SARS-CoV-2 ORF6 respectively (Minakshi et al. 2009; Miorin et al. 2020). Coronaviruses have been shown to modulate the cytokine response of the host through a variety of pathways. For example, SARS-CoV ORF3a/ORF7a activates nuclear factor kappa B (NF-κB) leading to a pro-inflammatory response (Kanzawa et al. 2006), while porcine epidemic diarrhea virus (PEDV) ORF3 inhibits the interleukin-6 (IL-6) and IL-8 pro-inflammatory response (Z. Wu et al. 2020). These contradictory functions on NF-κB signaling highlights the diverse range of CoV accessory proteins and their respective functions. The function and roles of many CoV accessory proteins remain unknown and continues to be a focus of future research efforts.

Conservation of Coronavirus Proteins and Diversity of Viral Variants

The levels of conservation between homologous proteins from different CoVs is dependent on the importance of protein function within the CoV family. The critical transcription and replication processes are similar between the various CoVs, and thus the non-structural proteins are some of the most conserved proteins amongst the CoV family (Illustration 1.7). Some of the most conserved proteins include the RdRp (nsp12), helicase (nsp13), and RTC cofactor proteins (nsp7-10), while higher diversity is found within proteins such as nsp2, nsp3, spike, and the accessory proteins (Jungreis, Sealfon,

and Kellis 2021). Higher degrees of conservation can be found within a specific genus of CoVs. For example amongst betacoronaviruses, the proteins with the highest sequence identity are the 3' and 5' untranslated regions (UTR), E protein, ORF10, and nsp10 (Chan, Choi, and Schork 2020). Protein diversity and conservation can vary greatly even within a single protein. Within the spike protein, the S2 domain is more conserved than S1, and even within the S1 domain, the receptor binding domain (RBD) is more diverse than the N-terminal domain (NTD) (K. C. Lei and Zhang 2020). Protein domain specific conservation can be observed even within more genetically similar proteins, like those involved with viral replication. A high degree of sequence and structural conservation in the catalytic site of the RdRp has been observed between alpha- and betacoronaviruses (Yazdani et al. 2021) and even between the polymerases of distantly related viruses (Mönttinen, Ravantti, and Poranen 2021). This conservation amongst viral RdRps has led to development of broad-spectrum antiviral drugs, such as Remdesivir (GS-5734), that have been tested as viral inhibitors against a wide range of viral infections, from Ebola virus to pathogenic HCoVs (Warren et al. 2016; Sheahan et al. 2017; Beigel et al. 2020).

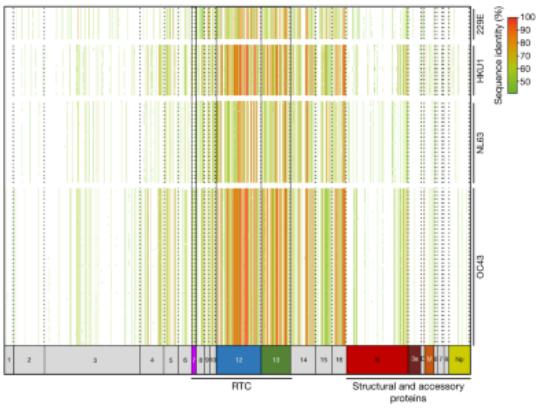


Illustration 1.7. Amino acid sequence homology of SARS-CoV-2 to eCoV protein sequences. Sequence identity between SARS-CoV-2 and eCoV (HCoV-229E, HCoV-

HKU1, HCoV-NL63, HCoV-OC43 protein sequences. Higher levels of conservation are in the non-structural proteins compared to the structural proteins. Image adapted from **(**Swadling et al. 2022).

The levels of protein diversity can also vary greatly between different strains or genotypes of the same virus. The eCoVs, which have been circulating in the human population for potentially hundred(s) of years, each have several different genotypes. HCoV-OC43 has at least 9 different genotypes with less than 0.005 substitutions/site differences between them (Zhaoyong Zhang et al. 2022). Similarly, HCoV-229E has at least 6 different genotypes, with most of the differences between genotypes attributed to positive selection in the spike region (S. K. P. Lau et al. 2021). MERS-CoV has also evolved over time, primarily through recombination events (Sabir et al. 2016), which has led to improved replicative fitness of the virus (Schroeder et al. 2021). This diversity in MERS-CoV varies based on geography as circulating clades differ between the Arabian Peninsula and Africa (D. K. W. Chu et al. 2018). SARS-CoV primarily displayed diversity within two proteins, ORF8 and spike, though both proteins stabilized towards a dominant strain by the end of the epidemic (The Chinese SARS Molecular Epidemiology Consortium 2004).

Despite SARS-CoV-2 being the most recent CoV to emerge in the human population, the virus has diversified into several different variants each with their own lineages in a relatively short period of time (Carabelli et al. 2023). Interestingly, SARS-CoV-2 has a mutation rate between $1x10^{-6}$ to $2x10^{-6}$ per nucleotide substitutions per replication cycle, which is similar to that of other coronaviruses, but significantly less

than other RNA viruses (Amicone et al. 2022; Sanjuán et al. 2010). This relatively low mutation rate of CoVs is due to the proofreading and exoribonuclease (ExoN) activity of nsp14 (Minskaia et al. 2006). For the first several months of the COVID-19 pandemic, SARS-CoV-2 had a modest substitution rate of around two new mutations in the genome per month (Duchene et al. 2020). At this time, the fixed spike protein D614G mutation was the most significant mutation, which improved the infectivity and viral fitness of the virus (Korber et al. 2020; Plante et al. 2021). By late 2020, new viral variants of concern (VOC) independently arose around the world and were associated with unexpectedly high rates of mutations compared to the original Wuhan-Hu-1 strain. The Alpha (B.1.1.7), Beta (B.1.351), and Gamma (P.1) variants were first discovered in the United Kingdom, South Africa, and Brazil respectively (Rambaut et al. 2020; Tegally et al. 2021; Faria et al. 2021). The Alpha, Beta, and Gamma VOCs were defined by dozens of non-synonymous mutations and deletions, most of which occurred within the spike protein (O'Toole et al. 2022). These spike mutations generally led to increases in the infectivity and immune escape of the VOCs. The Alpha mutations, Q493N and Q498Y, and Beta and Gamma shared mutations, K417N, E484K, and N501Y, all increased the affinity of their respective spike proteins to bind to the host cell receptor ACE2 (Starr et al. 2020; D. Zhou et al. 2021). While the Δ69-70 deletion of Alpha and the shared RBD mutations of Beta and Gamma, K417N, E484K, and N501Y, all helped the viruses escape antibody detection (Kemp et al. 2021; Chang Liu et al. 2022). The rise of these mutations in the VOCs was driven by an episodic 4-fold increase in the substation rate of SARS-CoV-2 (Tay et al. 2022). This increased substitution rate was potentially a result

of ongoing viral evolution during a chronic infection of immunocompromised hosts (Hill et al. 2022; Ghafari et al. 2022). Ultimately, the combination of these beneficial mutations to SARS-CoV-2 led to higher rates of transmission (Volz et al. 2021; Jassat et al. 2021).

The SARS-CoV-2 Delta variant (B.1.617.2) was first discovered in India in late 2020, but by June 2021 Delta was the dominant variant throughout the globe (Cherian et al. 2021; McCrone et al. 2022). The Delta variant was defined by 9 key mutations and deletions in spike, several of which directly enhanced the transmissibility of the virus (Mlcochova et al. 2021). The P681R mutation of the Delta variant improved the fusogenicity of the virus by facilitating the cleavage of spike protein for more efficient viral entry (Saito et al. 2022). Other mutations in spike, like the L452R mutation, decreased the sensitivity of the virus to antibody neutralization by patient serum and monoclonal antibodies (Starr et al. 2021; Planas et al. 2021). These mutations allowed the SARS-CoV-2 Delta variant to be 40-80% more transmissible than other VOCs in numerous regions of the world (Campbell et al. 2021; Earnest et al. 2022). Despite the increased number of cases and higher viral burden associated with an infection of the Delta variant (Huai Luo et al. 2021), the symptoms of COVID-19 (fever, cough, headache) did not last as long compared to the symptoms associated with an Alpha variant infection (Kläser et al. 2022).

The Delta wave of the COVID-19 pandemic lasted until late 2021, when the Omicron variant emerged and swept across the world. The Omicron variant (B.1.1.529) emerged in Africa and was identified by a high level of mutations within the spike

protein (37 mutations), with 15 mutations in the RBD alone (Z. Zhao et al. 2022). The original Omicron variant quickly diverged into several different lineages (BA.1, BA.2, BA.3, BA.4, BA.5) before becoming the most common variant across the world by early 2022 (Viana et al. 2022; Tegally et al. 2022). Since 2022, the Omicron lineages continued to evolve and its sub-lineages, BA.2.286 and JN.1, were the most common SARS-CoV-2 variants circulating throughout the world as of 2024 ("Tracking SARS-CoV-2 Variants" 2024). The hypermutated spikes of the Omicron variants allowed the virus to efficiently evade antibody responses (Planas et al. 2022; Iketani et al. 2022). Despite the increased transmissibility of Omicron variants compared to Delta and previous VOCs (Madewell et al. 2022), the Omicron variants were comparatively less virulent (Halfmann et al. 2022; Lewnard et al. 2022). This attenuation of Omicron variants was driven by a decrease in fusogenicity and pathogenicity of the viruses, due to mutations in spike and nsp6 and a change in cell tropism away from TMPRSS2 usage (Suzuki et al. 2022; D.-Y. Chen et al. 2023; Meng et al. 2022). As the Omicron variant evolved to the recent JN.1 sub-lineages, antibody evasion continuously increased as the replicative fitness of the sub-lineages remained similar (Planas et al. 2024). The persistent evolution of SARS-CoV-2 highlights the constant battle between the virus and the host's immune response.

Host Immune Response to Human Coronaviruses

Models to Study Human Coronavirus Immunity

Since the earliest discovery of CoVs, understanding the host's immune response to CoV infections has been critical in the development of CoV vaccines and therapies. *In vitro* and *ex vivo* models, ranging from immortalized cell lines to tissue organoids, have

provided valuable insight to the mechanisms of HCoV cellular tropism and cell intrinsic innate immunity (S Banach et al. 2009; Hui et al. 2020; L. Yang et al. 2020). Yet, these *in vitro* and *ex vivo* models are limited in their representation of the host's immune response. *In vivo* models are necessary to understand how the innate and adaptive immune system work in concert and provide important information on disease pathogenesis. Studies exploring mouse immunity to murine coronavirus provided initial insight into the role of both innate and adaptive immune responses to CoV infections (Daniel and Talbot 1990; Khanolkar et al. 2009). Unfortunately, mice are not naturally susceptible to the pathogenic HCoVs, which makes studying immunity to HCoV more complicated. Researchers have circumvented this issue by either sensitizing mice to wild-type HCoVs or by adapting HCoVs to infect wild-type mice (H. Chu, Chan, and Yuen 2022). Transgenic mice are made to express HCoV human receptors, such as human ACE2 for SARS-CoV and SARS-CoV-2 research (McCray et al. 2007). Alternatively, HCoVs can be serially passaged in mice until the virus adapts mutations for efficient infection of murine cells (Gu et al. 2020). A combination of the two methods was used for a mouse model of MERS-CoV, where wild-type virus was passaged in genetically modified mice (Cockrell et al. 2016). Although these mouse models recapitulate HCoV disease pathogenesis in many organs, including the lungs, the CoV related disease in these mouse models is often milder with less of a range of clinical symptoms compared to humans (S.-H. Sun et al. 2020). Humanized mouse models offer a more realistic representation of human CoV related disease (Kenney et al. 2022), but human

reconstitution of the mouse immune system may be immature and impacted by donor to donor variability (Garcia-Beltran, Claiborne, et al. 2021).

Some animals are naturally susceptible to the HCoVs and thus are also used for research purposes. Some animal models of natural SARS-CoV and SARS-CoV-2 infections, including ferrets (*Mustela putorius furo*) and golden (Syrian) hamsters (*Mesocricetus auratus*), are often used for transmission studies but disease is mild and non-lethal (Y.-I. Kim et al. 2020; Sia et al. 2020). Non-human primates, including Old World (cynomolgus (*Macaca fascicularis*), rhesus (*Macaca mulatta*) macaques), and New World (common marmosets (*Callithrix jacchus*)) monkeys, have varying degrees of susceptibility and clinical manifestations to HCoV infections (Rockx et al. 2020; Munster et al. 2020; S. Lu et al. 2020). Non-human primates may better represent human's response to HCoV infections, but these studies are limited by their cost and ethical considerations (Estes, Wong, and Brenchley 2018). A precedent has been set for human challenge models to HCoV infections, but these studies are rare and not useful to study novel CoVs with unknown clinical outcomes (Killingley et al. 2022; Lindeboom et al. 2024). Instead, most studies focus on understanding differences in HCoV immunity between different human cohorts. These human cohort and longitudinal studies measure various aspects of HCoV immunity and correlate these changes or differences in immunity to clinical outcomes. However, these studies only find associations and not causations, so follow up studies in *in vivo* models are required to determine mechanisms and validate the findings. Despite the limitation, these association studies in humans

contribute a large percentage of our understanding of HCoV-directed immunity and will be a primary methodology in my dissertation.

Cell Intrinsic Immunity

Cell intrinsic immunity is the first line of defense against a cellular infection by a pathogen, such as the HCoVs. As CoV viral replication begins, the viral RNA is recognized by pattern recognition receptors (PRRs), such as retinoic acid-inducible gene I (RIG-I) -like receptors (RLRs) and Toll-like receptors (TLRs) (Jensen and Thomsen 2012). These receptors recognize various forms of viral proteins and viral RNA, such as short and long double stranded RNA (dsRNA). In the context of SARS-CoV-2, TLR2 senses the viral E protein and melanoma differentiation-associated protein-5 (MDA5) recognizes long dsRNA in lung epithelial cells (Zheng et al. 2021; Yin et al. 2021). Additional methods of viral RNA detection involve 2'-5'-oligodenylate synthetase (OAS1), which coupled with ribonuclease L (RNase L) can detect and cleave dsRNA (Y. Li et al. 2016). High levels of post-translationally modified OAS1 are associated with protection against severe COVID-19 (S. Zhou et al. 2021; Wickenhagen et al. 2021). To combat these cell intrinsic pathways, the HCoVs have adapted methods to avoid pathogen recognition by compartmentalizing viral RNA in replication organelles and by inhibiting the activation of these PRRs and their degradation pathways. MERS-CoV, SARS-CoV, and SARS-CoV-2 proteins nsp3, nsp4, and nsp6 generate and tether double-membrane vesicles to the endoplasmic reticulum and physically separates viral RNA from PRRs (Oudshoorn et al. 2017; Ricciardi et al. 2022). Meanwhile, MERS-CoV and related bat CoVs use the phosphodiesterase activity of protein ns4b to degrade the

activators of RNase L (Thornbrough et al. 2016) and the N protein of both SARS-CoV and MERS-CoV inhibits the activation of RIG-I (Hu et al. 2017).

Upon recognition of a viral pathogen, the PRRs signal through a series of cellular pathways to activate the expression of IFN genes. The RLRs signal through the activity of MAVS and downstream kinases, while the TLRs recruit myeloid differentiation primary response 88 (MyD88) or TIR-domain-containing adaptor-inducing interferon-β (TRIF); both eventually leading to the activation of the transcription factors, NF-κB and/or interferon regulatory factors (IRFs) (Diamond and Kanneganti 2022). These transcription factors translocate into the nucleus and initiate the type-I and -III IFN responses, which leads to the upregulation and expression of interferon-stimulated genes (ISGs) and induction of antiviral activity (Lazear, Schoggins, and Diamond 2019). A hallmark of severe COVID-19 is a delayed or impaired type-I IFN response (Galani et al. 2021; Hadjadj et al. 2020). An early type-I IFN response is associated with improved clinical outcomes to both MERS-CoV and SASRS-CoV-2 (Channappanavar et al. 2019; Masood et al. 2021). Timing of this innate immune response is critical in the prevention against severe CoV-related disease (Illustration 1.8).

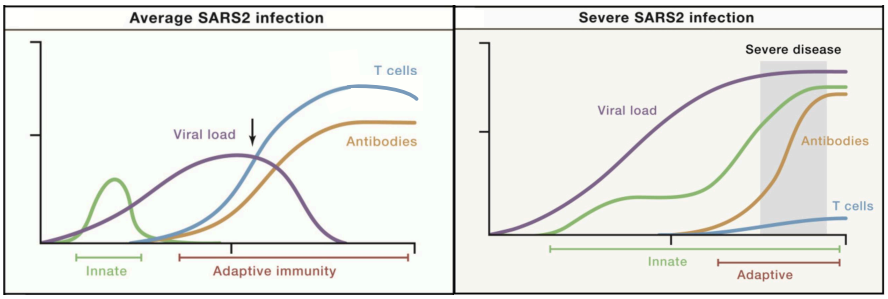


Illustration 1.8. Model of SARS-CoV-2 immunity and COVID-19 severity. Proposed model depicting the relationship between the timing of the immune response to SARS-

CoV-2 and COVID-19 severity. Average or mild SARS-CoV-2 infection is a result of the rapid induction of the host immune response and decreases in viral load. Severe SARS-CoV-2 infections are a result of a delayed or impaired immune response, which results in increased inflammation and higher viral loads. Image adapted from **(**Sette and Crotty 2021).

SARS-CoV-2 and the other HCoVs encode many different proteins to interfere with the various steps along the IFN response pathway. Early in the signaling cascade, both the M and N proteins of SARS-CoV-2 are capable of preventing the aggregation and signaling of MAVS (Fu et al. 2021; S. Wang et al. 2021). As SARS-CoV-2 has evolved into its VOCs, mutations in ORF9b allow for more efficient suppression of MAVS activation (Thorne et al. 2022). Downstream of MAVS, ORF3b and ORF6 of SARS-CoV and ORF9b of SARS-CoV-2 are all associated with antagonizing transcription factor signaling (Kopecky-Bromberg et al. 2007; J. Wu et al. 2021). On the ISG end of the IFN response, the downregulation of the type-I IFN receptor by nsp14 is conserved between SARS-CoV, SARS-CoV-2, and the bat CoV, RaTG13-CoV (Hayn et al. 2021). Additionally, ORF6 of SARS-CoV and SARS-CoV-2 blocks the nuclear translocation of STAT1 and prevents the expression of ISGs (Frieman et al. 2007; Miorin et al. 2020). SARS-CoV-2 Omicron subvariants have evolved over time and induce higher expression of ORF6, which increasingly suppresses STAT1 and also IRF3 activity and antagonizes the innate response even more (Reuschl et al. 2024). Ultimately, poor induction of ISGs in the nasopharynx is associated with worse clinical outcomes to COVID-19 (Ziegler et al. 2021).

Hosts have evolved other cellular intrinsic pathways to combat against intracellular pathogens, including viral nucleic acid editing and cellular degradation mechanisms. Editing of viral RNA and DNA in mammalian hosts is performed by adenosine deaminases (ADARs) and apolipoprotein B mRNA editing enzyme catalytic polypeptide-like proteins (APOBECs) (Eisenberg and Levanon 2018; Harris and Dudley 2015). Evidence of RNA editing by both ADARs and APOBECs is found in SARS-CoV-2, SARS-CoV, and MER-CoV transcripts (Di Giorgio et al. 2020). Interestingly, some studies suggest that the overabundance of cytosine to uracil changes induced by APOBECs in SARS-CoV-2 may contribute to its evolution (K. Kim et al. 2022). Autophagy, often induced by PRR activation, allows the cell to digest its cytoplasmic components in order to eliminate microbes and control inflammation (Deretic, Saitoh, and Akira 2013). Although this mechanism is used against many pathogens, SARS-CoV-2 reduces autophagy by modulating cellular metabolites and inhibiting the function of autophagosomes (Gassen et al. 2021; Hayn et al. 2021). Though SARS-CoV-2 and the other pathogenic HCoVs have evolved to overcome cell intrinsic obstacles, the human immune system has other ways to fight the infection.

Innate Immune Cells

The first cells likely to be infected by SARS-CoV-2 are multiciliated cells in the nasal passage, and the virus is capable of infecting cells in the lower respiratory tract, such as alveolar type 2 cells (Hou et al. 2020). These initial cell infections induce a pro-inflammatory response (Neufeldt et al. 2022). After the initial infection, bystander lung epithelial cells respond to the IFN signals or become infected themselves, amplifying the

pro-inflammatory response (Mulay et al. 2021; H. Chen et al. 2021). This pro-inflammatory response directs where immune cells go and how to respond to the infection. Resident alveolar macrophages (AMs) are one of the first immune cells to respond to the SARS-CoV-2 infection and they attempt to minimize the cell damage in the lung with an anti-inflammatory response (Wauters et al. 2021). In severe COVID-19 though, AMs are depleted in the lung or have impaired antigen presentation signatures (S. T. Chen et al. 2022), while hyperinflammatory monocyte-derived macrophages (MDMs) are more abundant in the lung (Liao et al. 2020; Melms et al. 2021). The combination of damaged lung epithelial cells and hyperinflammatory macrophages leads to elevated levels of pro-inflammatory cytokines, such as IL-6, IL-8, IL-17, tumor necrosis factor-α (TNF-α), and IFNγ, which are a hallmark of severe COVID-19 and the associated "cytokine storm" (Del Valle et al. 2020; C. Huang et al. 2024). SARS-CoV-2 infection is often restricted and aborted in these recruited macrophages, but the macrophage infection does contribute to the pro-inflammatory response (Jalloh et al. 2022). The pro-inflammatory state in the lungs during severe COVID-19 is further exacerbated by the hyperinflammatory macrophages response to the epithelial cell damage through the expression of chemokines, such as CCL2, CCL3, CXCL3, and CXCL10 (Chua et al. 2020). These chemokines recruit additional immune cells to the lungs, which can lead to additional cell and tissue damage.

Other than macrophages, additional myeloid cells are recruited to the site of an infection by cytokines and chemokines. Monocytes are often recruited from the bloodstream into the tissues and can differentiate into macrophages and dendritic cells

(DCs) (Jakubzick, Randolph, and Henson 2017). For SARS-CoV-2, mild cases of COVID-19 are associated with elevated levels of human leukocyte antigen (HLA)-$DR^{hi}CD11c^{hi}$ inflammatory monocytes (Schulte-Schrepping et al. 2020). In contrast, the disappearance of non-classical $CD14^{low}CD16^{hi}$ monocytes and increase in HLA-DR^{low} monocytes is associated with severe COVID-19 (Silvin et al. 2020). Dendritic cells also play a pivotal in the anti-viral response to HCoVs by secreting excessive type-I and -III IFNs and acting as antigen-presenting cells (Scheuplein et al. 2015; Saichi et al. 2021). Deficiencies in DCs are associated with worse clinical outcomes to COVID-19 and these defects in the myeloid compartment can last months after infection (Pérez-Gómez et al. 2021). Neutrophils quickly respond to a SARS-CoV-2 infection and infiltrate the lung (Rendeiro et al. 2021). The pathogenesis and severity of COVID-19 can depend on the functionality of these neutrophils. The accumulation of either hyperactivated or immature neutrophils are both associated with severe COVID-19 (P. Wu et al. 2021; Schulte-Schrepping et al. 2020). Excessive activation of the neutrophils can induce neutrophil extracellular trap (NET) production and mediate lung epithelial cell death and tissue damage (Veras et al. 2020). The combination of SARS-CoV-2 induced cell death and hyperactivation of the myeloid compartment is a driving force of the lung tissue damage associated with ARDS and severe COVID-19 (D'Agnillo et al. 2021; Chua et al. 2020).

Natural killer (NK) cells are an innate lymphoid cells with cytotoxic abilities to control both infected and stressed cell targets (Björkström, Strunz, and Ljunggren 2022). The expression of chemokine receptors, CXCR3, CXCR6, and CCR5, on NK cells aid NK cell homing through their respective ligands to the site of SARS-CoV-2 infection in

the lungs (Brownlie et al. 2022). The infiltration of NK cells into SARS-CoV-2 infected lungs leads to a decrease in circulating NK cells during acute infection (Maucourant et al. 2020). Impairment or dysfunction of this NK cells response is associated with worse clinical symptoms (Osman et al. 2020), which seems to be related to cytokine specific activation of the NK cells. Stimulation with either IL-15 or IFN-α are associated with impaired NK cell function and severe COVID-19, while activation with TNF is associated with moderate COVID-19 cases (Can Liu et al. 2021; Krämer et al. 2021). At the same time, SARS-CoV-2 and other sarbecoviruses use the conserved ORF6 to evade the NK cell response by promoting the shedding of major histocompatibility complex (MHC) class I polypeptide-related sequence (MIC)-A/B (Hartmann et al. 2024). NK cells and the other innate immune cells are just the first response to the HCoV infection, until the host develops a more specific response against the invading pathogen.

T Cell Immunity

One of the primary functions of innate immune cells, like dendritic cells and macrophages, is the presentation of antigens on their cellular surface. These antigen-presenting cells (APCs) will process viral antigens and display these viral peptides on the outside of the cell by the MHC (Pishesha, Harmand, and Ploegh 2022). Adaptive immune cells will recognize these MHC-peptide complexes by their specific receptors, such as the T cell receptor (TCR) for T cells, and through a signaling cascade, the adaptive immune cell will activate and differentiate into their effector functions (Hwang et al. 2020). T cells have a variety of functions that are important for the clearance of a HCoV infection. Cytotoxic $CD8^+$ T cells are capable of killing virally infected cells through the secretion

of granzymes and perforins, which is important for improved clinical outcomes to COVID-19 (Y. Su et al. 2020). Early induction and peak of the $CD8^+$ T response within the first two weeks is critical for improved clinical outcomes as a delayed $CD8^+$ T response is associated with severe COVID-19 (Bergamaschi et al. 2021). $CD8^+$ T cells have also been implicated in improved clinical outcomes to both MERS-CoV and SARS-CoV in humans and in animal models (Jingxian Zhao et al. 2017; Jincun Zhao, Zhao, and Perlman 2010). $CD4^+$ T cells largely contribute to the protection against severe disease from a HCoV infection by coordinating and stimulating the immune response. Airway memory $CD4^+$ T cells help protect against both SARS-CoV and MERS-CoV in mouse models by coordinating an early $CD8^+$ T response (Jincun Zhao et al. 2016). Circulating $CD4^+$ T follicular helper (cT_{fh}) cells stimulate the humoral immune response against SARS-CoV-2 and are correlated with elevated antibody neutralization titers (Boppana et al. 2021). Delays in the generation of cT_{fh} cells decreases SARS-CoV-2 antibody and neutralization titers, and is associated with more severe COVID-19 (M. Yu et al. 2023). Regulatory T cells (T_{reg}) are immunosuppressive and play an important role in modulating the effector T cell response. Low levels of T_{reg} cells compared to cytotoxic T cells is associated with worse COVID-19 clinical outcomes (Meckiff et al. 2020).

The combination of both $CD4^+$ T and $CD8^+$ T is crucial for viral clearance as decreases in T cell counts is associated with severe SARS-CoV-2 infections (Kuri-Cervantes et al. 2020). Lymphopenia is also commonly found in SARS-CoV infections (N. Lee et al. 2003). T cells do recover after these HCoV infections. Robust memory T cell responses against SARS-CoV-2 persist for months (Zuo et al. 2021; Jung et al. 2021)

and can even be detected many years after a SARS-CoV infection (O.-W. Ng et al. 2016; Le Bert et al. 2020). It is estimated that the half-life of SARS-CoV-2 specific T cells is around 200 days (Cohen et al. 2021). The HCoV specific T cells are likely to decline with age, as observed with the loss of eCoV-specific T cells in older adults (Saletti et al. 2020). Aging is also associated with a decrease in naïve T cells and a decline in $CD4^+$ T and $CD8^+$ T coordination, which ultimately correlates with severity of COVID-19 (Rydyznski Moderbacher et al. 2020). The coordination of strong $CD4^+$ T and $CD8^+$ T cell responses is critical during a HCoV infection.

The breadth of the $CD4^+$ T and $CD8^+$ T cell response covers the entire proteome of SARS-CoV-2 (Grifoni et al. 2020). The most immunodominant antigenic regions of SARS-CoV-2 are in the highly expressed structural proteins, like spike, nucleocapsid, and membrane, but the immunodominant regions differ between $CD4^+$ T and $CD8^+$ T cells (Tarke, Sidney, Kidd, et al. 2021). $CD8^+$ T cells also strongly recognize peptides from non-structural proteins that are expressed early during viral replication (Weingarten-Gabbay et al. 2021). T cell responses against these non-structural proteins, such as those involved with viral replication in the RTC, are commonly found in asymptomatic and abortive SARS-CoV-2 infection cases (Swadling et al. 2022). Additionally, T cells are able to recognize SARS-CoV-2 peptides derived from out-of-frame ORFs as well (Nagler et al. 2021; Weingarten-Gabbay et al. 2021). It is estimated that each individual recognizes about 19 and 17 SARS-CoV-2 epitopes specific for $CD4^+$ T and $CD8^+$ T cells respectively (Tarke, Sidney, Kidd, et al. 2021). Despite SARS-CoV-2 evolving into the VOCs, less than 10% of common T cell epitopes are affected by the new mutations

(Tarke, Sidney, Methot, et al. 2021). T cell responses are similar against the SARS-CoV-2 VOCs, suggesting minimal T cell escape (GeurtsvanKessel et al. 2022). However, SARS-CoV-2, MERS-CoV, and the other HCoVs avoid T cell responses by inhibiting or downregulating MHC-I expression (Yoo et al. 2021; Menachery et al. 2018).

Unconventional T cells also contribute to the immune response against HCoV infections, though they are less commonly studied. Circulating effector γδ T cell populations decrease upon mild and severe COVID-19, likely indicative of γδ T cell recruitment to the lungs (L. Lei et al. 2020). The loss of circulating γδ T cells, specifically VD2 T cells, in relation to immature neutrophils in the blood is predictive of pneumonia and hypoxia in severe COVID-19 cases (Carissimo et al. 2020). In moderate cases of COVID-19, natural killer T (NKT) cells are expanded (J.-Y. Zhang et al. 2020), but in severe cases invariant NKT cells display an exhausted phenotype (Jouan et al. 2020). Lastly, mucosal-associated invariant T (MAIT) cells may actually contribute to COVID-19 severity due to hyperactivation in the lungs (Parrot et al. 2020; Flament et al. 2021). The role of T cells in the context of HCoV infections continues to be a major research focus.

Humoral Immunity

The humoral immune response is the other arm of adaptive immunity. After the start of a viral infection, APCs, such as follicular dendritic cells (FDCs), present antigen in secondary lymphoid organs (Batista and Harwood 2009). B cells are activated and initially differentiate into short-lived plasmablast cells in the extrafollicular (EF) regions (Elsner and Shlomchik 2020). The antibodies produced by these initial plasmablast cells

have few somatic hypermutations and are mainly the immunoglobulin (Ig) M isotype but can class switch to IgG or IgA isotypes. At the same time, some B cells migrate into B cell follicles, where they form germinal centers (GC). In these GCs, antigen-specific B cells undergo somatic hypermutation and affinity-based selection to give rise to long-lived plasma cells that produce high-affinity antibodies. After the start of a SARS-CoV-2 infection, antibodies become detectable a few days after symptom onset, indicative of antibodies from the EF space (Woodruff et al. 2020). The initial antibody response to SARS-CoV-2 is predominantly IgM, but class-switching to IgG and IgA isotypes happens quickly (Long, Liu, et al. 2020). Plasma antibody titers against SARS-CoV-2 peak 2-4 weeks after the onset of symptoms and decline afterwards, with IgM titers declining faster than IgA and IgG levels (Isho et al. 2020). The plasma IgG antibodies against SARS-CoV-2 spike are more stable and have a half-life of around 200 days (Wei et al. 2021; Cohen et al. 2021). Antibody levels in the mucosa, detected by saliva concentrations, are similar to that of the plasma antibodies (Isho et al. 2020). Anti-SARS-CoV-2 mucosal IgA antibodies in the nasal cavity do remain elevated for months after the start of the infection (Fröberg et al. 2021). Neutralizing antibodies (nAbs) are detected early in the infection, with IgA antibodies contributing most to the early neutralization responses (Sterlin et al. 2021). Class switching to IgG also happens quickly, and IgG isotypes make up the majority of neutralizing antibodies in later phases of the immune response (Suthar et al. 2020). Slightly delayed compared to the antibody titers, nAb levels peak around 4-6 weeks and slowly decline afterwards (K. Wang et al. 2021), but they are still detectable in some patients a year after the start of the infection (Y. Yang et al.

2022). Neutralizing antibody titers and duration of the response varies greatly between individuals. Asymptomatic and mild cases of COVID-19 are associated with lower nAb responses compared to individuals with severe COVID-19 (Robbiani et al. 2020; Long, Tang, et al. 2020). The pattern of COVID-19 severity and correlation with stronger antibody responses is also observed with the other HCoVs. Antibodies can be detected years after an infection with a pathogenic SARS-CoV or MERS-CoV infection (Cao et al. 2010; Choe et al. 2017). Infections with the less pathogenic eCoVs induce short-term antibody responses (Edridge et al. 2020).

Most antibody responses against SARS-CoV-2 are targeted against the spike and nucleocapsid proteins. Antibodies are more common against nucleocapsid than spike early during a SARS-CoV-2 infection (Burbelo et al. 2020), but these nucleocapsid antibodies tend to wane quickly (Fenwick et al. 2021). Anti-spike antibodies become more common as the CoV infection progresses, which is critical for disease prevention; a higher ratio of spike to nucleocapsid antibodies is associated with milder cases of COVID-19 (Röltgen et al. 2020). The antibodies against spike protein generally target the RBD, NTD, and S2 domains, with increasing breadth and potency of antibody responses over time (Moriyama et al. 2021). Most SARS-CoV-2 nAbs target the RBD domain and these antibodies can be further divided into several different classes based on their specific binding regions (K. M. Hastie et al. 2021). These anti-RBD nAbs block spike binding to the ACE2 receptor, though some classes of anti-RBD antibodies inhibit viral membrane fusion (Barnes et al. 2020). Neutralizing antibodies can also be found targeting a supersite in or around the NTD of SARS-CoV-2 spike (Cerutti et al. 2021).

These NTD supersite nAbs interfere with conformational changes in spike critical for viral fusion (McCallum et al. 2021). Antibodies against the SARS-CoV-2 S2 subunit of spike are generally poor at neutralization, though a few S2 nAbs have been isolated that target the stem helix of S2 and inhibit membrane fusion (Pinto et al. 2021). These various sites targeted by nAbs are the primary regions of mutations in SARS-CoV-2 VOCs. Mutations in RBD and NTD domains of spike associated with VOCs are largely resistant to previously derived neutralization responses (P. Wang et al. 2021; L. Liu et al. 2022; Iketani et al. 2022).

Neutralizing antibodies are the primary source of protection against SARS-CoV-2 challenge in non-human primate models (McMahan et al. 2021). In humans, nAbs against SARS-CoV-2 prevents infection of neutralization sensitive strains for several months, but protects against severe disease for longer periods of time (Khoury et al. 2021). Even after infection with neutralization resistant strains, antibodies and possibly cellular responses continue to provide protection against progression to severe disease (Altarawneh et al. 2022). The potency of the neutralization response is one of the strongest predictors for survival among patients with COVID-19 (Garcia-Beltran, Lam, et al. 2021). Neutralizing antibodies may be increased during severe COVID-19, but delays in this nAb response is associated with more severe and fatal COVID-19 cases (Lucas et al. 2021; Kawasuji et al. 2021). The lack of germinal centers in severe SARS-CoV-2 infections may contribute to the delay in nAbs and decline in SARS-CoV-2 antibodies over time (Kaneko et al. 2020). Additionally, depletion of B cells with anti-CD20 therapy is associated with worse COVID-19 clinical outcomes and higher mortality rates (Calderón-Parra et al. 2022).

The fragment crystallizable (Fc) region of antibodies may contribute to either the protection against or exacerbation of severe COVID-19. The Fc region of antibodies can mediate antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), but it can also enhance virus entry into host cells via antibody-dependent enhancement (ADE) (Bournazos, Gupta, and Ravetch 2020). SARS-CoV-2-specific ADCC activity peaks 2-3 weeks following the start of a SARS-CoV-2 infection and remains detectable for over a year (Y. Yu et al. 2021). Antibodies with Fc effector functions against SARS-CoV-2 decline over time, but they persist longer than neutralizing antibodies in the serum (W. S. Lee et al. 2021). Afucosylated antibodies, with higher affinity towards Fc receptors (FcR) and capable of mediating enhanced ADCC activity, are correlated with worse COVID-19 severity (Larsen et al. 2021; Chakraborty et al. 2022). Similarly, ADCC activity is higher amongst individuals with symptomatic versus asymptomatic SARS-CoV-2 infections (Dufloo et al. 2021). Higher levels of afucosylated and ADCC eliciting antibodies may not be worsening COVID-19 severity though. Antibody effector function activity may be increased due to prolonged elevated viral loads during severe SARS-CoV-2 infections, similar to the relationship observed between neutralizing antibodies and COVID-19 severity (Y. Wang et al. 2020). Higher ADCC activity is observed in patients who recover from severe COVID-19 compared to fatal cases, suggesting a potential protective role of ADCC activity (Y. Yu et al. 2021). Other Fc mediated functions may contribute to the pathogenesis of COVID-19 severity. While increased ADCP activity is associated with mild cases of COVID-19, increased activity of antibody-dependent complement deposition (ADCD) is associated

with higher systemic inflammation and more severe disease (Adeniji et al. 2021). ADE has been noted in the antibody-dependent SARS-CoV-2 infection of monocytes and macrophages (Junqueira et al. 2022). Although the SARS-CoV-2 infection of monocytes is aborted, the monocyte infection and subsequent cell death may contribute to inflammation in the lungs. Altogether, the role of the Fc effector function in COVID-19 pathogenesis remains uncertain.

Preventions Against SARS-CoV-2 Infections and COVID-19 Severity

SARS-CoV-2 Infection and Vaccination Immunity

SARS-CoV-2 infections elicit both cellular and humoral immune responses that persist for several months, but wanes over time (Cohen et al. 2021; Dan et al. 2021). This natural immunity against SARS-CoV-2 protects against reinfection for several months (Lumley et al. 2021), but loses its efficacy quicker against the VOCs, especially the Omicron variants (Stein et al. 2023). Despite short-lasting protection against SARS-CoV-2 reinfection, natural immunity protects against severe COVID-19 for longer periods of time (Uusküla et al. 2023). Using natural infections to gain immunity is not recommended because the risk of COVID-19 severity is high in the elderly or with underlying medical conditions (L. Kim et al. 2021). Additionally, many individuals with SARS-CoV-2 infections experience medical issues associated with PASC for months to even years (Groff et al. 2021). The immediate need to prevent new infections and slow the spread of SARS-CoV-2, led to the rapid development of vaccines against SARS-CoV-2 within months of the start of the COVID-19 pandemic (Corbett et al. 2020). The first human clinical trials in the United States for a COVID-19 vaccine began in the

spring of 2020, and by mid-2021, the first COVID-19 vaccine was approved by the U.S. Food and Drug Administration (L. A. Jackson et al. 2020; Mulligan et al. 2020). The first vaccines approved in the United States were mRNA based vaccines, BNT162b1 by Pfizer-BioNTech and mRNA-1273 by Moderna, each with 95% and 94.1% efficacy against COVID-19, respectively (Polack et al. 2020; Baden et al. 2021). Numerous other COVID-19 vaccines were developed throughout the world. Traditional protein based vaccines, such as NVX-CoV2373 by Novavax, provided 90% efficacy against infection and even higher protection against severe COVID-19 (Dunkle et al. 2022). Both AstraZeneca and Johnson & Johnson-Janssen developed adenovirus vector based COVID-19 vaccines (ChADOx1 nCoV-19 and Ad26.COV2.S, respectively) with slightly lower efficacies in the 60-80% range depending on the patient population (Falsey et al. 2021; Sadoff et al. 2022). The inactivated whole virus vaccine BBIBP-CorV by Sinopharm was developed in China and has since been distributed throughout the world, with vaccine efficacy around 80% (Chao Wang et al. 2022). Vaccines against SARS-CoV-2 and its VOCs continue to be developed throughout the world.

Most COVID-19 vaccines, except for inactivated or live-attenuated vaccines, provide protection by inducing immune responses against the SARS-CoV-2 spike protein. These spike-based COVID-19 vaccines are capable of generating both nAbs and T cell responses against SARS-CoV-2 spike (Sahin et al. 2021). Compared to natural infection, the vaccine elicited antibody responses are generally stronger and T cell immunity against SARS-CoV-2 spike protein is similar, but these responses depend on the vaccine platform (Townsend et al. 2022). In general however, infection or vaccine-

elicited SARS-CoV-2 immunity initially provide similar protection against COVID-19 (Franchi et al. 2023), with vaccine-elicited protection waning faster than the protection provided by a previous SARS-CoV-2 infection (Rennert et al. 2022). The duration of COVID-19 vaccine-mediated protection is complicated by the emergence of SARS-CoV-2 VOCs. SARS-CoV-2 VOCs, like Omicron and its subvariants, can evade the vaccine-mediated immunity, though individuals are still protected against severe disease (J. J. Lau et al. 2023). Boosting SARS-CoV-2 immunity through administration of additional vaccines can improve protection against VOCs, but this protection also quickly wanes over time (Andrews, Stowe, et al. 2022). The current COVID-19 vaccines may not provide long-lasting sterilizing immunity, but they reduce both the severity of COVID-19 and the risk of long-term health issues associated with PASC (Lam et al. 2024).

Currently, a combination of infection and vaccination elicited immunity, termed hybrid immunity, provides the best protection against SARS-CoV-2 and severe COVID-19. Compared to individuals with either only a previous SARS-CoV-2 infection or COVID-19 vaccinations, those individuals with hybrid immunity have higher levels of protection for longer periods of time (Goldberg et al. 2022). Hybrid immunity boosts both nAbs and T cells against SARS-CoV-2 spike, even in those individuals with only a single dose of a mRNA vaccine (Reynolds et al. 2021). This increase in neutralization response from hybrid immunity is not dependent on the order of whether infection or vaccination occurs first (Bates et al. 2022). Instead the timing between immune boosting matters, as longer intervals between infection and vaccination increases the potency of the SARS-CoV-2 neutralization response (Bates et al. 2023). Other than increasing the

magnitude and durability of the SARS-CoV-2 immune response, hybrid immunity also increases the breadth of the neutralization response (Z. Wang et al. 2021). The increased breadth of nAbs allows individuals with hybrid immunity better protection against SARS-CoV-2 VOCs (Stamatatos et al. 2021; de Gier et al. 2023). Over time, however, newer SARS-CoV-2 VOCs can escape the immune responses in individuals with hybrid immunity (P. Li et al. 2024).

Heterotypic Immunity

From early in the COVID-19 pandemic, many diverse outcomes of COVID-19 were observed, potentially suggesting some degree of pre-existing immunity to SARS-CoV-2 in some individuals. The existence of pre-existing immunity to a novel human pathogen underscores the concept of heterotypic immunity. Heterotypic, or heterologous, immunity is when immune responses against a previous pathogen cross-react against a different, newly acquired pathogen. The concept and potential benefits of heterotypic immunity was first shown with the first vaccine in the late 18th century, where pre-exposure to cowpox protected against smallpox (Riedel 2005). Since then, heterotypic immunity has been shown to play a protective role against a variety of pathogens, including influenza (Skountzou et al. 2010) and *Mycobacterium tuberculosis* (Castillo-Rodal et al. 2006). Though with dengue virus, heterotypic immunity can have a negative clinical impact and worsen disease outcomes (Katzelnick et al. 2017). In the context of COVID-19, the role of heterotypic immunity is debated, and studies have found positive, negative, or neutral impact on COVID-19 outcomes (Sagar et al. 2021; Guo et al. 2021; Anderson et al. 2021). Despite the conflicting reports, increasing evidence suggests

heterotypic immunity has a positive impact on CoV infections. Several studies have found that a recent infection with an eCoV does not protect against SARS-CoV-2 infections, but does reduce the severity of COVID-19 and improve clinical outcomes (Sagar et al. 2021; Abela et al. 2024). The component(s) of the immune response mediating this heterotypic immune protection against severe COVID-19 remains understudied.

Many studies have noted pre-existing T cells that cross-react against SARS-CoV-2 from human samples taken prior to the start of the COVID-19 pandemic (Le Bert et al. 2020; Mateus et al. 2020; Nelde et al. 2021). Depending on the study and method of T cell stimulation, up to 80% of unexposed individuals have pre-existing T cells that recognize at least one SARS-CoV-2 peptide, with cross-reactive $CD4^+$ T cells being more common than cross-reactive $CD8^+$ T cells (Nelde et al. 2021). These pre-existing T cells recognize a range of SARS-CoV-2 epitopes across the viral proteome, with preference to immunodominant proteins, like spike, and conserved regions in non-structural proteins (Grifoni et al. 2020). Due to the presence of conserved epitopes between SARS-CoV-2 and the eCoVs, the cross-reactive T cells against SARS-CoV-2 are most certainly from prior eCoV infections (Mateus et al. 2020). Even in a highly variable protein like spike, more cross-reactive T cells recognize peptides from the more conserved S2 subunit compared to the more variable regions in S1 (Loyal et al. 2021). Varying levels of conservation exist between the four eCoVs and SARS-CoV-2, which impacts the extent of cross-reactivity (Lineburg et al. 2021). The pre-existing T cells from prior eCoV infections have a lower avidity toward the corresponding SARS-CoV-2 peptides due to

some amino acid differences, and thus it is more difficult to activate these cross-reactive T cells (Dykema et al. 2021). When the cross-reactive T cells do respond, they clonally expand upon SARS-CoV-2 infection (Low et al. 2021).

The role of cross-reactive T cells in the protection against severe COVID-19 is debated, but most studies suggest they are beneficial. In a mouse model, HCoV-OC43 elicited T cells are able to reduce viral burden and lung damage upon SARS-CoV-2 challenge (dos Santos Alves et al. 2024). Additionally, cross-reactive memory $CD4^+$ T cells in the airway of mice are able to coordinate a protective response against SARS-CoV, MERS-CoV, and bat CoVs (Jincun Zhao et al. 2016). In humans, pre-existing T cells with reactivity against the conserved CoV polymerase are associated with sub-clinical infections of SARS-CoV-2 (Swadling et al. 2022). Another indication of the clinical importance of cross-reactive T cells can be interpreted from the disparity in COVID-19 severity between children and older adults. Older adults, who lack eCoV directed T cells (Saletti et al. 2020), are more at risk of severe COVID-19, while children, who have twice as many eCoV directed T cells as adults (Dowell et al. 2022), generally have mild cases of COVID-19. Yet, cross-reactive T cells may not play a protective role in all SARS-CoV-2 infection scenarios. The clonal expansion of cross-reactive T cells with low avidity and limited functionality is associated with more severe cases of COVID-19 (Bacher et al. 2020). T cells may be changing the course of CoV infections in other ways. Cross-reactive memory $CD4^+$ T cells are able to enhance the antibody response against SARS-CoV-2 after either a natural infection or vaccination (Loyal et al.

2021; Mateus et al. 2021). Likely, the protective role of heterotypic immunity is a combination of cross-reactive T cell and antibody responses.

Pre-existing antibodies against SARS-CoV-2 have also been detected in samples collected before the emergence of SARS-CoV-2 in the human population (K. W. Ng et al. 2020; Shrock et al. 2020; Yuen et al. 2021). Similarly, cross-reactive memory B cells against SARS-CoV-2 spike have also been isolated from SARS-CoV-2 naïve individuals (Song et al. 2021; Teng et al. 2024). These pre-existing antibodies consist of many antibody classes, such as IgM, IgG, and IgA, and they target a range of CoV antigens, including spike and nucleocapsid (Yuen et al. 2021). Within spike, these cross-reactive antibodies primarily bind the S2 subunit, likely due to its conservation between the eCoVs (Elko et al. 2022). These pre-existing antibodies are most commonly a result from prior exposure to the eCoVs due to their high prevalence and genomic similarity to SARS-Cov-2 (Majdoubi et al. 2021), though individuals with prior SARS-CoV infections also have increased cross-reactive antibody levels against SARS-CoV-2 antigens (Zhu et al. 2020). Upon SARS-CoV-2 infection or vaccination, these cross-reactive antibodies are elevated against the eCoVs, particularly to beta eCoVs, like HCoV-OC43 and HCoV-HKU1 (Anderson et al. 2021; Geanes et al. 2022). A similar boost in beta eCoV directed antibodies is observed after infection with SARS-CoV (Che et al. 2005). Antibodies against other pathogenic HCoVs, SARS-CoV and MERS-CoV, are also increased after SARS-CoV-2 infection or vaccination (Grobben et al. 2021).

The functionality of these cross-reactive antibodies is heavily debated. Pre-existing nAbs against SARS-CoV-2 have been identified in both children and adults (K.

W. Ng et al. 2020), but children and younger adults tend to elicit stronger and more potent antibody responses (Garrido et al. 2021). Several cross-reactive antibodies targeting the spike S2 subunit have been isolated with the ability to broadly cross-neutralize both pathogenic and endemic HCoVs (Chunyan Wang et al. 2021; Song et al. 2021). Increased levels of cross-reactive IgA and IgG antibodies are more prevalent in asymptomatic cases of COVID-19 (Ortega et al. 2021). The elevated cross-reactive antibody levels in the blood of asymptomatic individuals with a SARS-CoV-2 infection mirror the response of cross-reactive antibodies in the saliva, suggesting a role of cross-reactive mucosal immunity in the protection against symptomatic COVID-19 (Abela et al. 2024). This heterotypic immune protection between CoVs largely depends on the similarity between the prior and subsequent infecting CoV (Bean and Sagar 2021). Higher similarity between the prior and subsequent CoV, for example between SARS-CoV and SARS-CoV-2, provides better immune protection against the subsequent virus, while less immune protection is elicited between more distantly related CoVs, like between SARS-CoV-2 and HCoV-OC43 (Dangi et al. 2021). This suggests cross-reactivity between CoVs does not always provide protection against subsequent heterologous CoV infection or disease. Indeed, pre-existing nAbs are absent in many pre-pandemic samples (Ercanoglu et al. 2022). While SARS-CoV-2 does elicit an eCoV memory response, these cross-reactive antibodies target non-neutralizing epitopes (Dugan et al. 2021; Miyara et al. 2022). Even between more closely related CoVs, like SARS-CoV and SARS-CoV-2, non-neutralizing antibodies are more prevalent than nAbs (Lv et al. 2020). This boost in non-neutralizing cross-reactive antibodies is not associated

with protection against severe COVID-19 (Anderson et al. 2021). Instead, a higher proportion of cross-reactive memory responses compared to de-novo neutralizing responses to SARS-CoV-2 correlates with increased COVID-19 severity (Aguilar-Bretones et al. 2021).

The role of heterotypic immunity in the protection against HCoVs is complicated. Pre-existing SARS-CoV-2 antibodies and T cells reactive are commonly detected in most individuals early in the immune response and are generally less effective than *de novo* immunity. The clinical impact of these cross-reactive immune responses against COVID-19 is more often associated with improved clinical outcomes but varies from study to study or is often not directly tested. Many of these COVID-19 heterotypic immune studies only correlate immune responses with disease outcomes, so it is difficult to determine causation. Despite the conflicting reports and many unknowns, understanding heterotypic immunity and its role in preventing CoV-related disease can provide valuable insight into potential mechanisms of immune protection. Additional research needs to further address which components of the immune system are crucial for the broad protection against CoVs in the hope of developing a pan-CoV vaccine.

Scientific Proposal and Hypothesis

In aggregate, prior CoV research suggests that heterotypic immunity primarily protects against CoV-related disease severity, but not against subsequent CoV infections (Sagar et al. 2021). The lack of protection against subsequent CoV infections implies that nAbs against the initial infecting CoV provide limited cross-protection against a heterologous CoV (Aguilar-Bretones et al. 2021). Most cross-reactive CoV antibodies

target the spike S2 subunit because of its conservation amongst the diverse CoVs (Elko et al. 2022), but neutralizing S2-directed antibodies are rare and instead typically target non-neutralizing epitopes (Lv et al. 2020). The degree of antibody-mediated cross-protection is ultimately correlated with the similarity between the prior and subsequent CoVs (Dangi et al. 2021). Pan-CoV broadly nAbs (bnAbs) are further complicated by continuous viral evolution and development of immune escape mutations over time (Martin et al. 2021). Developing bnAbs for pan-CoV sterilizing immunity therefore is a daunting task, yet bnAbs against CoVs are still a major research focus (Brinkkemper et al. 2024; Halfmann et al. 2024). Instead, future pan-CoV vaccine efforts should target more conserved regions shared by diverse CoVs that are less likely to develop escape mutations. Although these proposed pan-CoV vaccines may not provide protection against CoV infections, they will potentially reduce CoV-related disease severity and better recapitulate natural CoV-mediated heterotypic immunity. The following work aims to understand the viral targets and potential mechanisms associated with heterotypic immune protection against CoV-related disease. We hypothesize that heterotypic immunity among the CoV family members influences disease outcomes, and this protection is not mediated by neutralizing antibodies, but by alternative adaptive immune factors that target more conserved CoV regions.

The following aims were proposed to test our hypothesis:

Aim 1: Evaluate the role of neutralizing antibodies in the protection against SARS-CoV-2 reinfection

Both antibody titers and neutralization responses against SARS-CoV-2 will be compared between individuals before and after SARS-CoV-2 reinfection. These longitudinal antibody responses of individuals with SARS-CoV-2 reinfections will be compared to a matched cohort of individuals with previous SARS-CoV-2 infections with no clinical indications of reinfection. Additional demographic factors will be assessed for their association with SARS-CoV-2 reinfection. By conducting this study, we will assess whether neutralizing antibodies protect against homotypic CoV infections, which will provide insight to the potential role of nAbs in heterotypic immunity.

Aim 2: Explore differences in cross-reactive immunity towards SARS-CoV-2 in individuals with or without a recent eCoV infection.

Several antibody and T cell immunological assays will assess cross-reactivity against SARS-CoV-2 antigens in a SARS-CoV-2 infection naïve cohort. Differences in cross-reactive SARS-CoV-2 immunity will be compared between individuals with or without a suspected recent eCoV infection. This work will hope to identify viral targets and potential mechanisms of heterotypic immunity elicited by an eCoV infection against SARS-CoV-2.

Aim 3: Compare SARS-CoV-2 infection and vaccination mediated protection against subsequent symptomatic eCoV infection and identify correlates of immune protection.

The incidence of symptomatic eCoV infections will be compared between individuals with a previous SARS-CoV-2 infection, previous COVID-19 vaccination, or neither type of SARS-CoV-2 exposure. Both antibody and T cell immunity against a

range of eCoV antigens will be measured and compared between groups based on type of SARS-CoV-2 exposure. This study will compare SARS-CoV-2 spike and non-spike mediated immunity in the heterotypic immune response against eCoVs.

CHAPTER TWO: MATERIALS AND METHODS

Participants and Data Collection

Chapter Three: SARS-CoV-2 reinfection associates with unstable housing and occurs in the presence of antibodies

Demographic and clinical information was extracted from the Boston Medical Center (BMC) electronic medical records (EMR) for all patients that had a repeat SARS-CoV-2 reverse transcription (RT) – polymerase chain reaction (PCR) result at least 90 days after primary infection within March 12, 2020, to January 21, 2021 (Table 2.1). Patients who had at least 1 repeat positive test 90 days after primary infection were deemed to have reinfection. Those with only negative tests after 90 days were classified as convalescents. Demographic and clinical information was also collected for convalescents and individuals with a SARS-CoV-2 reinfection based on a 60-day reinfection window (Table 2.2). COVID-19 severity and treatments provided to the patients were only assessed at the time of first infection (Table 2.3). A SARS-CoV-2 RT-PCR positive result was required either 15 days prior to or during hospitalization for consideration as a "COVID-19 like illness". Data on housing were based on listed diagnoses or problems in the EMR, and in each case the period of unstable housing was confirmed by reviewing provider notes. Patient plasma and residual nasal swab samples were obtained from the BMC COVID-19 biorepository or in some cases during a subsequent non-COVID-19 related visit. Demographic and clinical information on the patients with available plasma were obtained from the EMR (Table 2.4).

Table 2.1. Demographics of the reinfection and convalescents individuals at the time of first infection based on a 90-day interval reinfection definition [a]

	Reinfection (n = 75)	Convalescents (n = 1,594)	Univariate p-value
Age, median (Interquartile Range (IQR))	51 (37 – 61)	48 (34 – 60)	0.81 [b]
Male	39 (52)	713 (44.7)	0.24
Race / Ethnicity			0.28 [c]
Black	21 (28)	587 (36.8)	
White	10 (13.3)	258 (16.2)	
Hispanic/Latino	39 (52)	624 (39.1)	
Other or missing	5 (6.7)	124 (7.8)	
Body mass index (IQR)	28.0 (25.0 – 33.0)	29.1 (25.2 – 34.1)	0.24 [b]
Homeless	28 (37.3)	245 (15.4)	<0.001
Pregnant	4 (5.3)	40 (2.5)	0.13
Diabetes mellitus (DM)	13 (17.3)	376 (23.6)	0.21
Heart disease [d]	9 (12)	125 (7.8)	0.19
Lung disease [e]	9 (12)	269 (16.9)	0.34
Chronic kidney disease	6 (8)	100 (6.3)	0.47
End stage renal disease	4 (5.3)	39 (2.4)	0.13
Human immunodeficiency virus (HIV)	4 (5.3)	30 (1.9)	0.06
Cancer	4 (5.3)	63 (3.9)	0.54
Smoking			0.09 [c]
Never smoker	40 (53.3)	1018 (63.9)	
Current smoker	14 (18.7)	247 (15.5)	
Former smoker	21 (28)	299 (18.8)	
Missing	0 (0)	30 (1.9)	
On immunosuppressive medication [f]	3 (4.0)	36 (2.2)	0.42
Number of co-morbidities [g]			0.93 [c]
0	41 (54.7)	904 (56.7)	
1	23 (30.7)	457 (28.7)	
≥2	11 (14.7)	233 (14.6)	

[a] Data are expressed as number (%) and p-value was calculated using Fisher's exact test unless otherwise indicated, [b] Mann Whitney U test, [c] Chi Square Test, [d] Heart disease includes coronary artery disease and/or congestive heart failure, [e] Lung disease includes chronic obstructive pulmonary disease and/or asthma, [f] Immunosuppressive medication included chronic steroid use (greater than 10mg daily Prednisone or equivalent), chemotherapeutic, or immunomodulatory agents (Bortezomib, Infliximab, Adalimumab, Cellcept, Tacrolimus, Mercaptopurine, Cyclosporine, Methotrexate, Atezolizumab), [g] Number of comorbidities accounts for diabetes mellitus, heart disease, lung disease, kidney disease, HIV, and cancer

Table 2.2. Demographics of the reinfection and convalescents individuals at the time of first infection based on a 60-day interval reinfection definition [a]

	$Reinfection_{60days}$ (n = 106)	**$Convalescent_{60days}$ (n = 1,912)**	**p-value**
Age in years, median (IQR)	48 (31 – 60)	48 (34 – 60)	0.84 [b]
Male	53 (50)	850 (44.5)	0.27
Race / Ethnicity			0.18 [c]
Black	27 (25.5)	684 (35.8)	
White	19 (17.9)	298 (15.6)	
Hispanic/Latin	52 (49.1)	787 (41.0)	
Other or missing	8 (7.5)	143 (7.5)	
Body mass index (IQR)	27.4 (24.7 – 31.9)	29.1 (25.2 – 34.0)	0.02
Homeless	32 (30.2)	261 (13.7)	<0.001
Pregnant	11 (10.4)	54 (2.8)	<0.001
Diabetes mellitus (DM)	19 (17.9)	445 (23.5)	0.24
Heart disease [d]	14 (13.2)	149 (7.8)	0.16
Lung disease [e]	10 (9.4)	316 (16.6)	0.06
Chronic kidney disease	11 (10.4)	118 (6.2)	0.10
End stage renal disease	5 (4.7)	50 (2.6)	0.21
HIV	4 (3.8)	33 (1.7)	0.13
Cancer	7 (6.6)	78 (4.1)	0.21
Smoking			0.10 [c]
Never smoker	61 (57.5)	1241 (64.9)	
Current smoker	16 (15.1)	278 (14.5)	
Former smoker	29 (27.3)	357 (18.7)	
Missing	0 (0)	36 (1.9)	
On immunosuppressive medication [f]	9 (8.5)	43 (2.2)	0.001
Number of co-morbidities [g]			0.95
0	59 (55.7)	1095 (57.3)	
1	31 (29.2)	541 (28.3)	
≥2	16 (15.1)	296 (14.4)	

Race and diagnoses are based on patient supplied information and the most current problem list in the medical record respectively, [a] Data are expressed as number (%) and p-value was calculated using Fisher's exact test unless otherwise indicated, [b] Mann Whitney U test, [c] Chi Square Test, [d] Heart disease includes coronary artery disease and/or congestive heart failure, [e] Lung disease includes chronic obstructive pulmonary disease and/or asthma, [f] Immunosuppressive medication included chronic steroid use (greater than 10mg daily Prednisone or equivalent), chemotherapeutic, or immunomodulatory agents (Bortezomib, Infliximab, Adalimumab, Cellcept, Tacrolimus, Mercaptopurine, Cyclosporine, Methotrexate, Atezolizumab), [g] Number of comorbidities accounts for diabetes mellitus, heart disease, lung disease, kidney disease, HIV, and cancer

Table 2.3. Disease severity among reinfection and convalescents individuals at the time of first infection [a]

	Reinfection (n = 75)	Convalescents (n = 1,594)	p-value
Hospitalized	20 (26.7)	373 (23.4)	0.49
Hospitalized with COVID-19 like illness	14 (18.7)	296 (18.6)	1.0
Intensive care unit (ICU) (% of hospitalized)	1 (5.0)	52 (13.9)	0.50
Mechanical ventilation (% of hospitalized)	1 (5.0)	43 (11.5)	0.72
Any COVID-19 directed medications [b]	5 (35.7)	202 (68.2)	0.01
Hydroxychloroquinine	3 (21.4)	164 (55.4)	0.01
Colchicine	0 (0)	10 (3.4)	1.0
Interleukin inhibitor [c]	2 (14.3)	72 (24.3)	0.53
Dexamethasone	0 (0)	3 (1.0)	1.0
Remdesivir	0 (0)	14 (4.7)	1.0

[a] Data are expressed as number (%) and p-value was calculated using Fisher's exact test unless otherwise indicated, [b] (%) is of hospitalized with COVID-19 like illness. The numbers include individuals enrolled in randomized double-blind placebo-controlled trials, [c] Interleukin (IL) inhibitors include tocilizumab, sarilumab, anakinra, or participation in clinical trial

Table 2.4. Demographics of the reinfection and convalescent individuals with plasma sample analysis [a]

	Reinfection Early Sample (n = 10)	Convalescent Early Sample (n = 20)	Reinfection Late Sample (n = 6)	Convalescent Late Sample (n = 12)
Age in years	56.5 (33-86)	58 (34-82)	53 (37-59)	57.5 (35-81)
Male (%)	8 (80)	13 (65)	5 (83)	5 (42)
Duration in days [b]	8.5 (2-22)	7.5 (1-31)	195.5 (154-341)	250 (156-341)
# of comorbidities	0.5 (0-2)	1 (0-3)	2 (0-3)	1.5 (0-3)
Pregnant (%)	0	0	0	0
Homeless (%)	7 (70)	8 (40)	4 (67)	3 (25)
On immunosuppressive medication	0	2	1	1

[a] Data show median and range in parenthesis unless indicated otherwise, [b] For the early sample, duration from day of symptom onset, and for the late sample day from 1st positive SARS-CoV-2 RT-PCR result.

Chapter Four: Recent Endemic Coronavirus Infection Associates With Higher SARS-CoV-2 Cross-Reactive Fc Receptor Binding Antibodies

Demographic and clinical information was extracted from the BMC EMR (Table 2.5). A representative subset of these individuals with available peripheral blood mononuclear cells (PBMC) were used in the T cell response analyses (Table 2.6). Pre-pandemic samples were collected from the HIV and aging cohort as previously described (Belkina et al. 2018). The pre-pandemic samples were collected from June 2017 to March 2020, before the first diagnosed SARS-CoV-2 infected individual at BMC. All post-pandemic samples were collected after November 2020 and prior to December 2021 before the widespread Omicron SARS-CoV-2 surge (Clarke et al. 2022). The post-pandemic blood samples were obtained from BMC patients during a non-COVID-19 related medical visit. Prior to sample collection, all individuals' status regarding COVID-19 vaccination and prior SARS-CoV-2 infection was confirmed during the consent process. Previous SARS-CoV-2 infections were based on available prior SARS-CoV-2 RT-PCR test results. Vaccinated individuals had received at least two doses of the Pfizer BioNTech BNT162b2 or Moderna mRNA-1273 COVID-19 vaccine or one dose of the Janssen / Johnson & Johnson Ad26.COV2.S COVID-19 vaccine. No individual had received a COVID-19 vaccine booster because this practice was instituted after the end of our sample collection period. Included individuals were greater than 18 years of age. Documented eCoV infections were based on prior documented positive test results for HCoV-229E, HCoV-HKU1, HCoV-NL63, or HCoV-OC43 in the comprehensive respiratory panel (CRP) -PCR test. CRP-PCR tests were used to evaluate patients that

present with acute respiratory symptoms, but we did not confirm the medical reasons for the testing in all cases. The CRP-PCR was done at the discretion of the treating physician.

Table 2.5. Demographics of the individuals with collected blood specimens for analyses of SARS-CoV-2 and eCoV mediated antibody responses

	Prior SARS-CoV-2 infection / No COVID-19 vaccine (n = 20)	Prior COVID-19 vaccine / No SARS-CoV-2 infection (n = 29)	No prior SARS-CoV-2 infection or COVID-19 vaccine (n = 72)	p-value [a]
Age (years), median (IQR)	53 (48 – 59)	62 (54 – 70)	56 (46 – 64)	0.0215 [b]
Male	8 (40)	19 (66)	36 (50)	0.1834
Race/Ethnicity [c]				0.3087
Black	14 (70)	13 (45)	35 (49)	
White	3 (15)	13 (45)	26 (36)	
Hispanic/Latino	3 (15)	3 (10)	8 (11)	
Other/missing	0 (0)	0 (0)	3 (4)	
Diabetes mellitus	5 (25)	10 (34)	21 (29)	0.7640
Heart disease [d]	3 (15)	12 (41)	15 (21)	0.0520
Lung disease [e]	5 (25)	8 (28)	26 (36)	0.5322
CKD [f]	1 (5)	3 (10)	6 (8)	0.7997
HIV [g]	7 (35)	5 (17)	26 (36)	0.1686
Cancer	1 (5)	2 (7)	1 (1)	0.3367
Number of co-morbidities [h]				0.6309
0	3 (15)	5 (17)	12 (17)	
1	12 (60)	12 (41)	33 (46)	
≥2	5 (25)	12 (41)	17 (38)	
Pre-Pandemic [i]	-	-	14 (26)	-

Data shows number and percent unless otherwise indicated, [a] Chi-square test unless otherwise indicated, [b] Kruskal-Wallis test and Dunn's multiple comparison test, [c] As specified in the EMR; an individual may be in more than 1 category, [d] Heart disease includes coronary artery disease and congestive heart failure, [e] Lung disease includes chronic obstructive pulmonary disease and asthma, [f] Chronic kidney disease, [g] Human immunodeficiency virus, [h] Number of comorbidities accounts for diabetes mellitus, heart disease, lung disease, chronic kidney disease, HIV, and cancer, [i] Samples collected before March 2020

Table 2.6. Demographics of the individuals with available PBMCs for analyses of eCoV mediated cellular responses against SARS-CoV-2 antigens

	Prior SARS-CoV-2 infection / No COVID-19 vaccine (n = 20)	Presumed or documented recent eCoV infection (n = 32)	No presumed recent eCoV infection (n = 42)	p-value [a]
Age (years), median (IQR)	53 (48 – 59)	65 (57 – 71)	57 (44 – 62)	0.0057 [b]
Male	8 (40)	17 (53)	22 (52)	0.6004
Race/Ethnicity [c]				0.3752
Black	14 (70)	12 (38)	21 (50)	
White	3 (15)	14 (44)	15 (36)	
Hispanic/Latino	3 (15)	5 (16)	5 (12)	
Other/missing	0 (0)	1 (3)	1 (2)	
Diabetes mellitus	5 (25)	12 (38)	15 (36)	0.6216
Heart disease [d]	3 (15)	13 (41)	9 (21)	0.0751
Lung disease [e]	5 (25)	13 (41)	8 (19)	0.1156
CKD [f]	1 (5)	2 (6)	5 (12)	0.5633
HIV [g]	7 (35)	8 (25)	14 (33)	0.6716
Cancer	1 (5)	1 (3)	1 (2)	0.8601
Number of co-morbidities [h]				0.3873
0	3 (15)	5 (16)	8 (19)	
1	12 (60)	11 (34)	19 (45)	
≥2	5 (25)	16 (50)	15 (36)	
Pre-Pandemic [i]	-	4 (13)	14 (33)	0.0553 [j]
COVID-19 Vaccine	-	13 (41)	10 (24)	0.1368 [j]

Data shows number and percent unless otherwise indicated, [a] Chi-square test unless otherwise indicated, [b] Kruskal-Wallis test and Dunn's multiple comparison test, [c] As specified in the EMR; an individual may be in more than 1 category, [d] Heart disease includes coronary artery disease and congestive heart failure, [e] Lung disease includes chronic obstructive pulmonary disease and asthma, [f] Chronic kidney disease, [g] Human immunodeficiency virus, [h] Number of comorbidities accounts for diabetes mellitus, heart disease, lung disease, chronic kidney disease, HIV, and cancer, [i] Samples collected before March 2020, [j] Fisher's Exact Test

Chapter Five: Heterotypic immunity from prior SARS-CoV-2 infection but not COVID-19 vaccination associates with lower endemic coronavirus incidence

The retrospective analysis of the incidence of eCoV and non-CoV infections included all available CRP-PCR results in the BMC EMR performed from November 30, 2020, to October 8, 2021. In general, CRP-PCRs are used to evaluate patients that present with acute respiratory symptoms, but we did not confirm the medical reasons for the testing in all cases. The CRP-PCR was done at the discretion of the treating physician. All individuals less than 18 years of age were excluded from analyses. The start date was chosen because the first eCoV infection at BMC for the 2020 fall/winter season was documented on November 30, 2020. The end date of October 8, 2021, was chosen because this was before the first documented SARS-CoV-2 Omicron infection at BMC. The less symptomatic and widely spread Omicron infections made it difficult to reliably differentiate individuals into the three pre-specified groups (Clarke et al. 2022). Individuals with a positive test for HCoV-229E, HCoV-HKU1, HCoV-NL63, or HCoV-OC43 were classified as having an eCoV infection. Each individual only contributed a single data point, although they may have had multiple CRP-PCR results during the study period. The date of the positive eCoV infection was used in the analysis for an individual even if they had other CRP-PCR results during the study period. Incidence of non-CoVs served as a control to assess for differences in exposure and susceptibility to respiratory pathogens among the three groups. For those individuals with positive non-CoV test results, the date of the first positive non-CoV result was used in the analysis, although the individual may have had other pathogens, besides eCoVs, in subsequent CRP-PCRs.

Among individuals with multiple CRP-PCRs that did not detect eCoVs or non-CoVs, the date of the last test was used in the analyses.

Individuals were initially classified into three groups: 1) those with a prior documented SARS-CoV-2 infection; 2) those with no prior documented SARS-CoV-2 infection and with at least one prior COVID-19 vaccine dose; and 3) those with no prior SARS-CoV-2 infection or COVID-19 vaccination. Vaccinated individuals were deemed fully vaccinated if they had received at least two doses of the Pfizer BioNTech BNT162b2 or Moderna mRNA-1273 COVID-19 vaccine or one dose of the Janssen / Johnson & Johnson Ad26.COV2.S COVID-19 vaccine a minimum of 14 days prior to the CRP-PCR test. No individual had received a COVID-19 vaccine booster because this practice was instituted after the end of the study period. SARS-CoV-2 detected on the CRP-PCR tests was not classified as either eCoV or non-CoV. If an individual had SARS-CoV-2 on a CRP-PCR test and a subsequent CRP-PCR test, that person was placed in the prior SARS-CoV-2 infection group. On the other hand, an individual with SARS-CoV-2 detected on last CRP-PCR test within the study time-period was categorized based on prior history. Demographic and clinical information were obtained for these individuals from the EMR (Table 2.7).

Blood samples were obtained from BMC patients during a non-COVID-19 related medical visit. Prior to sample collection, all individuals' status regarding COVID-19 vaccination and prior SARS-CoV-2 infection was confirmed during the consent process. Included individuals were greater than 18 years of age, and medical visits were for non-COVID-19 diagnosis. Exclusion criteria included a SARS-CoV-2 positive nasal swab

within past 14 days or use of an immunosuppressive drug. Demographic and clinical information were obtained for these individuals from the EMR (Table 2.8).

Table 2.7. Demographics of the groups in the retrospective cohort analyses of SARS-CoV-2 mediated protection against symptomatic eCoV incidence

	Prior SARS-CoV-2 infection (n = 501)	**Prior COVID-19 vaccine / No SARS-CoV-2 infection (n = 1,565)**	**No prior SARS-CoV-2 exposure (n = 2,869)**	**p-value [a]**
Age (years), median (IQR)	53 (36 – 67)	59 (43 – 71)	47 (32 – 62)	<0.0001 [b]
Male	266 (53)	737 (47)	1366 (48)	0.0524
Race/Ethnicity [c]				<0.0001
Black	264 (53)	776 (50)	1,610 (56)	
White	101 (20)	464 (30)	629 (22)	
Hispanic/Latino	124 (25)	261 (17)	501 (17)	
Other/missing	20 (4)	82 (5)	156 (5)	
Diabetes mellitus	143 (29)	442 (28)	551 (19)	<0.0001
Heart disease [d]	67 (13)	217 (14)	209 (7)	<0.0001
Lung disease [e]	154 (31)	510 (33)	717 (25)	<0.0001
CKD [f]	40 (8)	116 (7)	126 (4)	<0.0001
HIV [g]	22 (4)	56 (4)	74 (3)	0.0369
Cancer	41 (8)	171 (11)	176 (6)	<0.0001
Smoking				<0.0001
Never	234 (47)	648 (41)	1,332 (46)	
Current	107 (21)	349 (22)	677 (24)	
Former	122 (24)	425 (27)	490 (17)	
Missing	38 (8)	143 (9)	370 (13)	
Number of co-morbidities [h]				<0.0001
0	206 (41)	625 (40)	1,541 (54)	
1	179 (36)	530 (34)	921 (32)	
≥2	116 (23)	410 (26)	407 (14)	
Level of care [i]				0.6465
Ambulatory	10 (2)	26 (2)	42 (1)	
Hospital	491 (98)	1,539 (98)	2,827 (99)	

Data shows number and percent unless otherwise indicated, [a] Chi-square test unless otherwise indicated, [b] Kruskal-Wallis test and Dunn's multiple comparison test, [c] As specified in the EMR; an individual may be in more than one category, [d] Heart disease includes coronary artery disease and congestive heart failure, [e] Lung disease includes

chronic obstructive pulmonary disease and asthma, [f] Chronic kidney disease , [g] Human immunodeficiency virus, [h] Number of comorbidities accounts for diabetes mellitus, heart, disease, lung disease, chronic kidney disease, HIV, and cancer, [i] Level of medical care at the time of the CRP-PCR test. Hospital includes an emergency department visit.

Table 2.8. Demographics of the individuals with collected blood specimens for analyses of SARS-CoV-2 mediated immune responses against eCoV antigens

	Prior SARS-CoV-2 infection / No COVID-19 vaccine (n = 20)	**Prior COVID-19 vaccine / No SARS-CoV-2 infection (n = 25)**	**No prior SARS-CoV-2 exposure (n = 28)**	**p-value [a]**
Age (years), median (IQR)	53 (48 – 59)	62 (59 – 70)	57 (45 – 65)	0.0232 [b]
Male	8 (40)	17 (68)	12 (43)	0.1003
Race/Ethnicity [c]				0.4878
Black	14 (70)	12 (48)	16 (57)	
White	3 (15)	10 (40)	8 (29)	
Hispanic/Latino	3 (15)	3 (12)	4 (14)	
Other/missing	0 (0)	0 (0)	0 (0)	
Diabetes mellitus	5 (25)	9 (36)	10 (36)	0.6788
Heart disease [d]	3 (15)	10 (40)	11 (39)	0.1359
Lung disease [e]	5 (25)	7 (28)	10 (36)	0.6982
CKD [f]	1 (5)	2 (8)	1 (4)	0.7740
HIV [g]	7 (35)	4 (16)	4 (14)	0.1697
Cancer	1 (5)	2 (8)	0 (0)	0.3328
Smoking				0.0685
Never	11 (55)	11 (44)	5 (18)	
Current	4 (20)	5 (20)	12 (43)	
Former	5 (25)	9 (36)	11 (39)	
Number of co-morbidities [h]				0.6494
0	3 (15)	5 (20)	6 (21)	
1	12 (60)	10 (40)	11 (39)	
≥2	5 (25)	10 (40)	11 (39)	
Days since last SARS-CoV-2 spike protein exposure, median (IQR)	245 (68-308)	70 (19-98)	-	0.0017 [i]

Data shows number and percent unless otherwise indicated, [a] Chi-square test unless otherwise indicated, [b] Kruskal-Wallis test and Dunn's multiple comparison test, [c] As specified in the EMR; an individual may be in more than 1 category, [d] Heart disease

includes coronary artery disease and congestive heart failure, [e] Lung disease includes chronic obstructive pulmonary disease and asthma, [f] Chronic kidney disease, [g] Human immunodeficiency virus, [h] Number of comorbidities accounts for diabetes mellitus, heart disease, lung disease, chronic kidney disease, HIV, and cancer, [i] Mann-Whitney U Test

Cells and Viral Stocks

Patient Primary Cells and Plasma

Patient blood was collected, and plasma was removed after centrifugation (1500xg for 10 minutes). PBMCs were isolated using Ficoll-paque density centrifugation methods. Briefly, after removal of plasma, the remaining blood product was suspended in 1x phosphate buffered saline (PBS) to a volume of 25mL and carefully layered on top of 20mL of Histopaque-1077 (Sigma-Aldrich, 10771). The layered solution was centrifuged for 30 minutes at 400xg without any brake on the centrifuge. The white layer of PBMCs was carefully removed and washed three times in 1x PBS. Both plasma and PBMCs were aliquoted and stored at -80°C and -196°C respectively.

Cell Lines

Human epithelial kidney (HEK)-293T cells were acquired from the National Institutes of Health (NIH) HIV Reagent Program. HEK-293T cells were cultured in complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen), 2mM L-glutamine (Invitrogen), 100U/ml penicillin (Invitrogen), 100µg/ml streptomycin (Invitrogen), and 25mM HEPES buffer (Invitrogen).

Vero E6 cells were acquired from the American Type Culture Collection (ATCC) (ATCC, CRL-11268). Vero E6 cells were cultured in DMEM containing 10% FBS.

Pseudotyped Viral Stocks

Coronavirus spike protein-expressing pseudoviruses were prepared as previously described (Whitt 2010). Briefly, HCoV-229E-spike protein (SinoBiological, VG40605-CF), HCoV-OC43-spike protein (SinoBiological, VG40607-CF), or SARS-CoV-2-spike protein (BEI Resources, NR52310) expression plasmids were transfected into HEK-293T cells. After 24 hours, the transfected cells were infected with vesicular stomatitis virus (VSV)-G pseudotyped virus (G*ΔG-VSV) containing the firefly luciferase expression cassette. The pseudovirus-containing cell supernatant was collected after an additional 24 hours, filtered, concentrated, and stored at -80°C. Virus supernatants from HEK-293T cells infected with VSV-G pseudotyped virus (G*ΔG-VSV) only and not transfected with a CoV spike protein-encoding plasmid were used as a control to assess for the amount of infection in the absence of a CoV spike protein.

Immunological Assays

SARS-CoV-2 Antibody Quantification (Simoa Assay)

Anti-SARS-CoV-2 IgG antibody titers were assessed using a previously described ultra-sensitive Single Molecule Array (Simoa) multiplexed assay (Norman et al. 2020). Briefly, plasma samples were centrifuged at 4°C for 10 minutes at 2000xg. The supernatant was then diluted 1000-fold dilution in Homebrew Sample Diluent (Quanterix) before running the assay. Anti-SARS-CoV-2 RBD and nucleocapsid protein IgG titers were calculated and normalized based on the average number of enzymes per bead (AEB).

SARS-CoV-2 and ECoV Antibody Quantification (BU ELISA)

SARS-CoV-2 and eCoV spike and nucleocapsid protein-specific IgG titers were detected by enzyme-linked immunosorbent assay (ELISA) as previously described (Yuen et al. 2021). Briefly, a 96-well plate was coated overnight at 4°C with 2μg/ml of viral antigen(s): SARS-CoV-2 RBD protein (SinoBiological, 40592-V08H), SARS-CoV-2 S2 protein (SinoBiological, 40590-V08H1), SARS-CoV-2 nucleocapsid protein (SinoBiological, 40588-V08B), HCoV-OC43 spike protein (SinoBiological, 40607-V08B), HCoV-OC43 nucleocapsid protein (SinoBiological, 40643-V07E), HCoV-229E spike protein (SinoBiological, 40605-V08B), HCoV-229E nucleocapsid protein (SinoBiological, 40640-V07E), HCoV-HKU1 nucleocapsid protein (SinoBiological, 40642-V07E), or HCoV-NL63 nucleocapsid protein (SinoBiological, 40641-V07E). The next day, wells were washed thrice with PBS and blocked with casein blocking buffer (Thermo Fisher Scientific, 37528) for 90 minutes. The wells were then washed thrice with PBS and then incubated with 3-fold dilutions of patient plasma for 1 hour. Wells were subsequently washed thrice with PBS containing 0.05% Tween 20 (PBST), and then anti-human horseradish peroxidase (HRP)-conjugated secondary antibodies for IgG detection (diluted 1:50000, Sigma-Aldrich, A0170) was added to each well for 30 minutes. The wells were washed with PBST four times, and then 3,3',5,5' Tetramethylbenzidine (TMB)-ELISA substrate solution (Thermo Fisher Scientific, 34029) was added and incubated in the dark for 15 to 20 minutes. The reaction was stopped by the addition of 2M sulfuric acid. Absorbance was measured on a SpectraMax190 Microplate Reader (Molecular Devices) at 450nm and the optical density

(OD) from the no antigen negative control wells was subtracted from all readings. A positive control standard (CR3022 IgG, Abcam, 273073) was serially diluted and measured against SARS-CoV-2 RBD to create a standard curve on each plate. The CR3022 standard curve was used to calculate titers (relative units) for each sample by interpolating a four-parameter logistic (4PL) curve.

SARS-CoV-2 and ECoV Antibody Fc Fucosylation Quantification (FEASI)

Levels of SARS-CoV-2 and eCoV spike protein-specific antibodies with the ability to bind the Fc receptor, FcγRIIIa, were detected by the fucose-sensitive ELISA-based method of antigen-specific IgG Fc fucosylation (FEASI) (Šuštić et al. 2022). The ELISA protocol described above (Yuen et al. 2021) was adapted to include measurements of antibody binding to FcγRIIIa. Briefly, a 96-well plate was coated overnight at 4°C with 2µg/ml of viral antigen(s): SARS-CoV-2 RBD protein (SinoBiological, 40592-V05H), SARS-CoV-2 S2 protein (SinoBiological, 40590-V02H), HCoV-OC43 spike protein (SinoBiological, 40607-V08B), or HCoV-229E spike protein (SinoBiological, 40605-V08B). The next day, wells were washed thrice with PBS and blocked with casein blocking buffer for 90 minutes. The wells were then washed thrice with PBS, and then incubated with a 1:5 to 1:50 dilution of patient plasma for 1 hour. Wells were subsequently washed thrice with PBST, and then wells were incubated with 1ug/mL of either His-tagged (Invitrogen, RP-87975) or biotinylated (gift from Dr. Gestur Vidarsson, PhD) FcγRIIIa for 1 hour. The wells were subsequently washed three times with PBST and then incubated with either 1µg/mL of anti-His HRP-conjugated antibody (Invitrogen, MA1-21315-HRP) or 0.125µg/mL of HRP- conjugated streptavidin (Thermo

Scientific, N100) for 30 minutes. The wells were washed with PBST four times, and then TMB-ELISA substrate solution was added and incubated in the dark for 10 to 15 minutes. The reaction was stopped by the addition of 2M sulfuric acid. Absorbance was measured on a SpectraMax190 Microplate Reader (Molecular Devices) at 450nm and the OD from the no antigen negative control wells was subtracted from all readings. As described previously for the BU ELISA protocol, the CR3022 standard curve against SARS-CoV-2 RBD was used to calculate titers (relative units) for each sample. The ratio of antigen-specific FcγRIIIa binding antibodies to antigen-specific IgG titers could then be calculated.

Pseudovirus Neutralization Assay

Pseudovirus neutralization assays were performed as previously described (Nie et al. 2020). Briefly, plasma was heat inactivated at 56°C for 1 hour. Plasma, starting at 1:40 dilution, was serially diluted using a 2-fold serially dilution series. Five serially diluted plasma samples were incubated with pseudovirus stocks at 37°C for 1 hour. After 1 hour, approximately $1.25x10^4$ Vero E6 cells were added to each well and incubated at 37°C for 48 hours. After 48 hours, luciferase expression was measured using the Promega Bright-Glo Luciferase Assay System (Thermo Scientific) on a SpectraMax190 Microplate Reader (Molecular Devices). Percent neutralization was calculated in comparison to luciferase expression in infected wells without patient plasma. Area under the curve (AUC) values were calculated from the curve generated from the neutralizations across the serially diluted plasma (X. Yu et al. 2012). All neutralizations were tested in triplicate a minimum of two independent times. Neutralization against

VSV-G pseudotyped virus (G*ΔG-VSV) was used as a control to assess activity against VSV-G protein in the absence of a CoV spike protein.

Peptide Pools for T Cell Stimulation

All peptides for a given antigen were reconstituted in dimethyl sulfoxide (DMSO), pooled at equal concentrations, and diluted in PBS. Aliquots of peptide pools were stored at -80°C. The following peptides pools consisted of overlapping peptides ranging from 13 to 18 amino acids with 10 to 12 amino acids of overlap covering the entire protein region of interest: SARS-CoV-2 spike protein (n=181, BEI Resources, NR-52402); SARS-CoV-2 nucleocapsid (n=59, BEI Resources, NR-52404); SARS-CoV-2 nsp12 protein (n=231, JPT, PM-WCPV-NSP12-2); SARS-CoV-2 nsp13 protein (n=148, JPT, PM-WCPV-NSP13-2); HCoV-OC43 spike protein (n=226, BEI Resources, NR-53728); HCoV-OC43 nucleocapsid (n=110, JPT, PM-OC43-NCAP-1); and human cytomegalovirus (CMV) pp65 (n=138, NIH HIV Reagent Program, ARP-11549). The peptide pool containing CMV, Epstein-Barr virus, and influenza (CEF) peptides consisted of 32 peptides of 8-11 amino acids covering immunodominant $CD8^+$ T cell epitopes (NIH HIV Reagent Program, ARP-9808).

The HCoV-OC43 nsp12/nsp13 peptide pool consisted of 29 peptides of 14 to 16 amino acids in length (Table 2.9). The defined SARS-CoV-2 epitopes were mapped to the HCoV-OC43 protein sequence (strain ATCC VR-759, NC_006213). Additional amino acids, corresponding to the HCoV-OC43 sequence, were added to the ends of each epitope so each peptide was 14 to 16 amino acids in length. The HCoV-OC43 nsp12/nsp13 peptides were synthesized at greater than 95% purity (GenScript).

Table 2.9. HCoV-OC43 nsp12/nsp12 peptides.

Protein (Amino Acid Position)	HCoV-OC43 Peptide	Corresponding SARS-CoV-2 Peptide	% Identity	% Similarity	Suspected HLA Activity
nsp12 (4393-4407)	SGLSTDVQ LRAFDIY	TGTSTDVV YRAFDIY	73.33	80.00	HLA-B*15:01
nsp12 (4404-4419)	FDIYNASV AGIGLHLK	FDIYNDKV AGFAKFLK	62.50	68.75	HLA-A*11:01, HLA-A*24:02, HLA-A*32:01
nsp12 (4674-4688)	HCANFNILF SMVLPN	HCANFNVLF STVFPP	73.33	80.00	HLA-DQB1*02:02, HLA-DQB1*06:02, HLA-DRB1*07:01
nsp12 (4683-4698)	SMVLPNTC FGPLVRQI	STVFPPTS FGPLVRKI	68.75	68.75	HLA-A*24:02, HLA-B*57:01
nsp12 (4889-4903)	QDEIYAYT KRNVLPT	QDALFAYT KRNVIPT	73.33	93.33	HLA-B*51:01, HLA-DRB1*07:01, HLA-DRB1*15:01, HLA-DPA1*02:02/DPB 1*05:01
nsp12 (4899-4913)	NVLPTLTQ MNLKYAI	NVIPTITQ MNLKYAI	86.67	100.00	HLA-A*01:01, HLA-A*02:06, HLA-B*07:02, HLA-C*07:01
nsp12 (4909-4923)	LKYAISAK NRARTVA	LKYAISAK NRARTVA	100.00	100.00	HLA-A*68:01, HLA-C*06:02
nsp12 (4944-4958)	IAATRGVP VVIGTTK	IAATRGAT VVIGTSK	80.00	93.33	HLA-A*11:01, HLA-B*51:01, HLA-DQB1*03:03, HLA-DRB1*07:01
nsp12 (4984-4998)	YPKCDRAM PNLLRIV	YPKCDRAM PNMLRIM	86.67	86.67	HLA class II
nsp12 (4989-5003)	RAMPNLLR IVSSLVL	RAMPNMLR IMASLVL	80.00	80.00	HLA-B*07:02, HLA-B*08:01, HLA-C*07:02, HLA-DQB1*06:02, HLA-DRB1*15:01
nsp12 (4997-5011)	IVSSLVLAR KHETCC	IMASLVLAR KHTTCC	80.00	80.00	HLA-A*33:01, HLA-A*68:01
nsp12 (5014-5028)	SDRFYRLA NECAQVL	SHRFYRLA NECAQVL	93.33	93.33	HLA-DRB1*01:01, HLA-DRB1*08:01, HLA-A*02:01,

					HLA-A*02:03, HLA-A*02:06
nsp12 (5055-5069)	ANSVFNICQAVSANV	ANSVFNICQAVTANV	93.33	100.00	HLA-A*02:03, HLA-A*02:06, HLA-A*68:02
nsp12 (5064-5078)	AVSANVCALMSCNGN	AVTANVNALLSTDGN	66.67	80.00	HLA-DRB3*02:02, HLA-DRB1*13:02
nsp12 (5103-5118)	DSTFVTEYYEFLNKHF	DTDFVNEFYAYLRKHF	56.25	75.00	HLA-A*01:01, HLA-A*02:01, HLA-A*24:02, HLA-A*33:01, HLA-B*08:01
nsp12 (5181-5195)	HTMLVKMDGDDVYLP	HTMLVKQGDDYVYLP	73.33	73.33	HLA-A*02:01, HLA-B*15:01
nsp12 (5190-5204)	DDVYLPYPNPSRILG	DYVYLPYPDPSRILG	86.67	93.33	HLA-A*24:02, HLA-B*07:02, HLA-B*51:01
nsp12 (5219-5234)	LLIERFVSLAIDAYPL	LMIERFVSLAIDAYPL	93.75	93.75	HLA-DQA1*02:01, HLA-DQA1*05:01, HLA-DQA1*01:01, HLA-DQA1*01:03, HLA-DQB1*03:01, HLA-DQB1*06:01, HLA-DQB1*02:02, HLA-DQB1*03:02, HLA-DQB1*04:02, HLA-DQB1*05:01, HLA-DQB1*06:04, HLA-DRB1*01:01, HLA-DRB1*01:03, HLA-DRB1*04:04, HLA-DRB1*07:01, HLA-DRB1*08:03, HLA-DRB1*10:01,

					HLA-DRB1*12:02, HLA-DRB1*13:02, HLA-DRB1*15:01, HLA-A*02:01, HLA-B*35:01
nsp12 (5229-5243)	IDAYPLVY HENEEYQ	IDAYPLTK HPNQEYA	66.67	73.33	HLA class II
nsp12 (5237-5250)	HENEEYQK VFRVYL	HPNQEYAD VFHLYL	57.14	78.57	HLA-A*29:02, HLA-B*44:02, HLA-B*44:03, HLA-C*07:02
nsp12 (5244-5258)	KVFRVYLA YIKKLYN	DVFHLYLQ YIRKLHD	53.33	80.00	HLA-DRB1*04:04, HLA-DRB1*13:02, HLA-C*07:02
nsp13 (5425-5439)	ERLKLFAA ETQKATE	ERLKLFAA ETLKATE	93.33	93.33	HLA-A*03:01
nsp13 (5459-5473)	ELILSWEIG KVKPPL	ELHLSWEVG KPRPPL	73.33	86.67	-
nsp13 (5469-5483)	VKPPLNKN YVFTGYH	PRPPLNRN YVFTGYR	73.33	93.33	-
nsp13 (5504-5518)	NGVYYRAT TTYKLSV	DAVVYRGT TTYKLNV	66.67	86.67	HLA-A*11:01, HLA-A*24:02
nsp13 (5513-5527)	TYKLSVGD VFVLTSH	TYKLNVGD YFVLTSH	86.67	86.67	HLA-A*02:01
nsp13 (5682-5697)	VINARIRAK HYVYIGD	VVNARLRAK HYVYIGD	87.50	100.00	HLA-A*11:01
nsp13 (5693-5707)	VYIGDPAQ LPAPRVL	VYIGDPAQ LPAPRTL	93.33	93.33	HLA-A*24:02, HLA-C*07:01
nsp13 (5853-5867)	NVNRFNVA ITRAKKG	NVNRFNVA ITRAKVG	93.33	93.33	HLA-DRB1*08:03, HLA-DRB1*15:01, HLA-DQA1*05:01, HLA-DQB1*03:01, HLA-DQA1*01:02, HLA-DQB1*06:02

Activation-Induced Marker (AIM) Assay

The AIM assay was performed as described previously (Dan et al. 2016; Reiss et al. 2017). Briefly, PBMCs were thawed, washed in Roswell Park Memorial Institute 1640 Medium (RPMI-1640), re-suspended in RPMI-1640 media with 5% human serum, 1% L-glutamine, 2% penicillin/streptomycin, and 1% HEPES buffer. Cells were rested overnight. The cells were plated at concentration of $1x10^6$ cells/well in a 96-well round bottom plate. For each stimulation, peptides were added to the well at a final concentration of 1µg/mL, containing less than 0.1% DMSO. Peptide pools containing CEF or CMV pp65 were used as positive controls and media with 0.1% DMSO was used as a negative control. After 24 hours, the cell supernatant was removed, and the cells were collected for additional analysis. Stimulation experiments were performed twice at independent times.

Cell Staining and Flow Cytometry

Cells from the stimulation experiment were washed twice in PBS and then stained for live/dead cell marker (1:200 dilution, Thermo Fisher, L23105) on ice for 20 minutes. Cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FBS and 2 mM Ethylenediaminetetraacetic acid (EDTA)) and then fixed on ice for 15 minutes using cold BD Cytofix Fixation Buffer (1:10 dilution, BD Biosciences, 554655). After fixation, the cells were washed in FACS buffer and subsequently incubated with human Fc receptor block (1:100 dilution, BioLegend, 422302) for 15 minutes on ice. Cells were then stained with the following antibodies on ice for 30 minutes: Alexa Fluor 647 anti-human CD3 (1:100 dilution, BioLegend, clone HIT3a, 300321), Alexa Fluor

488 anti-human CD4 (1:200 dilution, BioLegend, clone SK3, 344618), allophycocyanin (APC)/Fire 750 anti-human CD8a (1:50 dilution, BioLegend, clone HIT8a, 300931), phycoerythrin (PE)/Cyanine7 anti-human CD69 (1:50 dilution, BioLegend, clone FN50, 310911), Brilliant Violet 421 anti-human CD134 (OX40) (1:25 dilution, BioLegend, clone Ber-ACT35 (ACT-35), 350013), PE anti-human CD137 (4-1BB) (1:100 dilution, BioLegend, clone 4B4-1, 309803). After staining, the cells were washed twice in FACS buffer and samples were analyzed on either a BD LSR II Flow Cytometer (BD Biosciences) or Cytek Aurora 5L (Cytek Biosciences).

Calculation of Activated T Cells

The resulting flow cytometry data was analyzed using FlowJo software. The following gating strategy was used (Figure 2.1A): Lymphocytes were gated to only include single cells. Dead cells (Live/Dead marker $^{+}$) were excluded. Live (Live/Dead marker $^{-}$) T cells ($CD3^{+}$) were then gated on either $CD4^{+}$ or $CD8^{+}$. Activated $CD4^{+}$ were defined by the double-positive $CD134^{+}$ $CD137^{+}$ population, while activated $CD8^{+}$ were defined by the double-positive $CD69^{+}$ $CD137^{+}$ population. The percent of activated T cells for a given antigen stimulation condition was then either background subtracted against the negative control (DMSO only) condition, or a stimulation index (SI) was calculated by dividing the percent of T cell activation from antigen stimulation by the percent of activated T cells in the negative control condition (Fig. 2.1B).

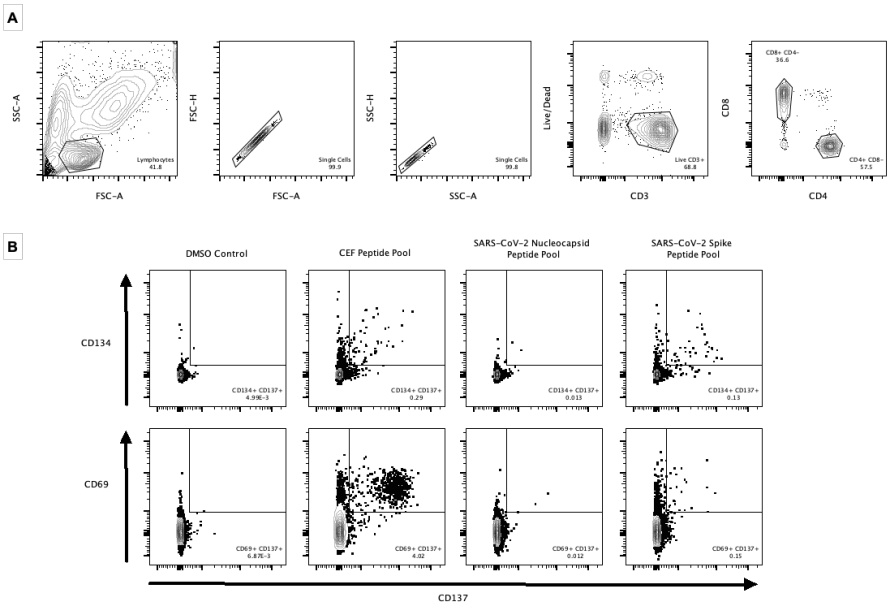


Figure 2.1. Activation induced marker (AIM) assay gating strategy and analysis. (**A**) Representative charts depicting the flow cytometry gating strategy for defining $CD4^+$ and $CD8^+$ T cell populations. (**B**) Examples of the flow cytometry gating strategy for measuring antigen specific $CD4^+$ ($CD134^+$ $CD137^+$) and $CD8^+$ ($CD69^+$ $CD137^+$) T cells after stimulation with DMSO control or experimental conditions. Of note, this individual had received a coronavirus disease 2019 (COVID-19) vaccination but had no history of a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.

Virus Sequence Analysis

SARS-CoV-2 genomic fragments were amplified from discarded nasal swab sample derived total RNA using a modified ARTIC-primer based protocol (ARTIC-V3) and sequenced on an Illumina platform. Nucleotide substitutions, insertions, and deletions were identified with LoFreq (Wilm et al. 2012) following alignment to the SARS-CoV-2 Wuhan Hu-1 reference sequence (NC_045512.2) with Bowtie2 (Langmead and Salzberg 2012). We used a quality threshold of greater than or equal to 10 reads for

determining a change from reference. Lineages of sequenced viruses were determined using the Pangolin algorithm (O'Toole et al. 2021).

Statistical Analysis

General

Univariate comparisons were done using Chi-square or Fisher's exact test for categorical variables, and the Kruskal-Wallis or Mann-Whitney U test for continuous variables, as appropriate. Kaplan Meier (KM) curve analysis and Cox proportional hazard models were used for time to event analyses. Available demographic factors, co-morbidities, and hospitalization characteristics were used as covariates in the multivariate analyses (Tables 2.1-2.5). Age was dichotomized into above and below 50 years of age. Inclusion of age as a continuous variable did not yield any significant findings. All covariates in univariate models with p values less or equal to 0.15 level were initially included in multivariable models. Covariates with p values greater than 0.10 in multivariable models were then removed. All model assumptions were found to be reasonable. Statistical analyses were performed using Stata v17, SAS v9.4, and GraphPad Prism 9.0.2. A two-sided p-value less than 0.05 was considered statistically significant.

Chapter Three: SARS-CoV-2 reinfection associates with unstable housing and occurs in the presence of antibodies

Multivariate linear regression analysis was conducted independently for RBD IgG, nucleocapsid IgG, and neutralization AUC with the predictors: 1) days from symptom onset for the early sample or days from 1st positive PCR result for the late sample; 2) gender; 3) age; and 4) reinfection or convalescent group categorical variable.

Chapter Four: Recent Endemic Coronavirus Infection Associates With Higher SARS-CoV-2 Cross-Reactive Fc Receptor Binding Antibodies

Individuals with a previous documented SARS-CoV-2 infection or previous COVID-19 vaccination were grouped together as those previous SARS-CoV-2 spike exposure for all spike-directed immune response assessments. Individuals with a previous COVID-19 vaccination were grouped based on their classified eCoV infection status for the non-spike directed immune response examinations. Composite alpha or beta eCoV nucleocapsid antibody scores were created by Log2 transforming the average of HCoV-229E and HCoV-NL63 or HCoV-HKU1 and HCoV-OC43 IgG titers, respectively. Individuals with either a documented eCoV infection in the past 550 days or those within the top 25% with the highest alpha or beta eCoV nucleocapsid antibody scores were classified as presumed or documented eCoV infection. The remaining SARS-CoV-2 infection naïve individuals were considered without a recent eCoV infection. Comparison of responses in individuals with a previous SARS-CoV-2 infection or SARS-CoV-2 spike exposure to the SARS-CoV-2 infection naïve groups was conducted using Kruskal-Wallis tests and corrected with Dunn's multiple comparisons test. Mann-Whitney U tests were used for direct comparisons between individuals with or without a presumed recent eCoV infection. Correlations were assessed using Pearson and Spearman tests as appropriate. Categorical differences were examined using Fischer's exact test or Chi-square test when comparing more than 2 groups. In the multivariable logistic regression model, the presence or absence of a SARS-CoV-2 antibody binding FcγRIIIa response was the dependent variable. Alpha and beta eCoV nucleocapsid IgG levels, prior SARS-

CoV-2 spike experience (categorical variable), and available demographic factors and co-morbidities were independent predictors in the multivariate analysis. All covariates in a univariate model with p values less or equal to 0.15 level were initially included in the multivariable model. Covariates with p values greater than 0.10 in the multivariable model were then removed.

Chapter Five: Heterotypic immunity from prior SARS-CoV-2 infection but not COVID-19 vaccination associates with lower endemic coronavirus incidence

Cox proportional hazards models were developed examining time to either eCoV or non-CoV infection using SARS-CoV-2 infection and COVID-19 vaccination as time-varying covariates. In the KM and Cox proportional hazards models, 11/30/2020 was used as the index or "start" date if SARS-CoV-2 infection or COVID-19 vaccination occurred before 11/30/2020. The start date was the day of exposure if SARS-CoV-2 infection or first COVID-19 vaccination was after 11/30/2020. The event day was the positive CRP-PCR test of interest or the date with the last negative CRP-PCR test. There was no loss to follow up.

Spike protein and nucleocapsid indices were generated by summing log normalized (Log_2 (result + 1)) spike protein and nucleocapsid SIMOA IgG, AIM-$CD4^+$, and AIM-$CD8^+$ measurements, respectively. The accuracy of clinical classifications based on SARS-CoV-2 adaptive immune responses, including sensitivity, specificity, positive predictive value and negative predictive value were examined along with asymptotic 95% confidence intervals. In the case of perfect prediction, exact binomial confidence intervals are presented.

In the multivariable linear regression models, antibody or T cell responses were the dependent variables. The independent predictors varied in the models but included the group categorization or a combined classification based on SARS-CoV-2 spike protein exposure (SARS-CoV-2 infection and COVID-19 vaccination group) or no known SARS-CoV-2 infection (COVID-19 vaccination and no SARS-CoV-2 antigen experience group). A multiplicative interaction term between SARS-CoV-2 infection and COVID-19 vaccination was examined in Cox proportional hazards models but then removed due to lack of statistical significance.

Ethics Statement

The BMC Institutional Review Board approved these studies.

CHAPTER THREE: SARS-COV-2 REINFECTION ASSOCIATES WITH UNSTABLE HOUSING AND OCCURS IN THE PRESENCE OF ANTIBODIES

Disclaimer: Portions of this dissertation were included in or adapted from:

Severe Acute Respiratory Syndrome Coronavirus 2 Reinfection Associates With Unstable Housing and Occurs in the Presence of Antibodies. Bean DJ, Monroe J, Turcinovic J, Moreau Y, Connor JH, Sagar M. Clin Infect Dis. 2022 Aug 24;75(1):e208-e215. Doi: 10.1093/cid/ciab940. (Bean et al. 2022)

Background

The majority of individuals develop a robust immune response after SARS-CoV-2 infection (Sette and Crotty 2021). Despite this, there have been multiple reports of reinfection and prolonged shedding (Harrington et al. 2021; Selhorst et al. 2021; Tillett et al. 2021; To et al. 2021; Van Elslande et al. 2021). While these investigations have suggested that persistent shedding is often observed among those immunosuppressed (Avanzato et al. 2020; Baang et al. 2021; B. Choi et al. 2020; Tarhini et al. 2021) or pregnant (Burgoyne et al. 2020; Molina et al. 2020), the demographic and immune characteristics that associate with reinfection are not known.

It can be difficult to distinguish between reinfection and prolonged shedding without serial longitudinal sampling and virus sequence analysis (Choudhary et al. 2022). As of July 2021, the Centers for Disease Control and Prevention (CDC) stipulates that asymptomatic patients with documented COVID-19 should not be re-tested prior to 90

days after the primary infection (CDC 2024). Repeat positive SARS-CoV-2 RT-PCR tests within 90 days of the original result may represent prolonged shedding rather than reinfection. Some cohort studies have also used serology to characterize patients with reinfection (Lumley et al. 2021; Leidi et al. 2022; Abu-Raddad et al. 2021). Retrospective and prospective cohort investigations suggest that reinfection occurs in less than 1% of health care workers (Lumley et al. 2021). In the general population, prior infection may provide greater than 80% protection against reinfection, although it may be lower among individuals older than 65 years of age (Leidi et al. 2022; Abu-Raddad et al. 2021; Sheehan, Reddy, and Rothberg 2021; Hansen et al. 2021; Vitale et al. 2021). Besides age, other patient characteristics and immune factors that may associate with reinfection have not been identified.

In this retrospective cohort study, we examined the association of diverse demographic factors and antibody responses with reinfection. We found that unstable housing, but not other baseline characteristics or antibody levels, was associated with reinfection. Our study identifies modifiable factors that may be important in preventing SARS-CoV-2 reinfection.

Results

Subjects and demographics

There were 67,688 unique patients with an available SARS-CoV-2 RT-PCR test result in the BMC EMR from March 12, 2020, to January 21, 2021. Of these, 9,910 (14.6%) unique patients had at least 1 positive SARS-CoV-2 RT-PCR positive test. Of the patients with a positive test, 1,669 (16.8%) had another SARS-CoV-2 RT-PCR result

available at least 90 days after the initial positive result (Table 2.1). Of these patients, 75 (4.5%) had 2 positive test results at least 90 days apart. Forty-nine of these 75 individuals had at least 1 or more negative RT-PCR tests in the period between the first and the repeat positive RT-PCR result a minimum of 90 days later. Twenty-five individuals did not have another result between their first and last positive test, and one person had only intervening positive results. These 75 individuals were deemed to have reinfection. The remaining 1,594 (95.5%) of the 1,669 with only negative test results at least 90 days after a positive test were classified as convalescents.

Factors associated with reinfection

The number of unique SARS-CoV-2 tests were higher among those with reinfection (median 5, range 2 – 21) as compared to the convalescents (median 3, range 2–25, $p<0.0001$) group (Fig. 3.1A). The days between the first and last positive test in the reinfection group (median 139, range 91–298) was shorter than the days between the first positive and last negative test in the convalescents group (median 172, range 90–317, $p=0.0005$) (Fig. 3.1B).

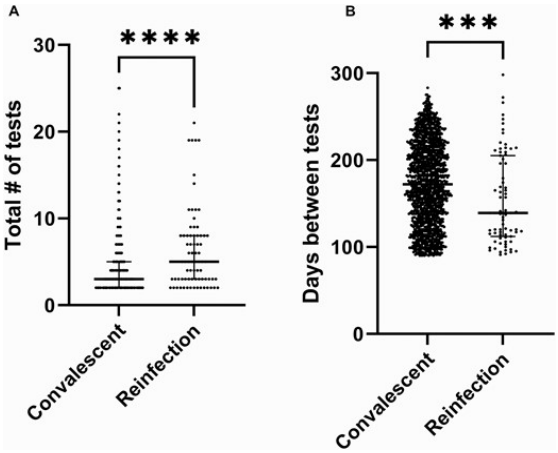


Figure 3.1. Reinfection associates with more frequent testing at shorter intervals. The total number of tests (A) and the interval in days between tests (B) among the

reinfection and convalescent group. The interval is days from the first positive result to the next positive test at least 90 days later in those with reinfection, and from the first positive to the last negative test at least 90 days later in the convalescent. The box plots show median and interquartile range. *** and **** indicate $p \leq 0.001$ and $p \leq 0.0001$ respectively by the Mann-Whitney U test.

A greater proportion of the reinfection as compared to the convalescent individuals had housing instability at the time of the first positive SARS-CoV-2 RT-PCR test (Table 2.1). Other demographics, including age, were not statistically different in the two groups. In time to event analysis, the percent of patients that had a repeat positive result at least 90 days after the first positive test was significantly higher in those with unstable housing as compared to stable housing (hazard ratio (HR) 2.71, 95% confidence interval (CI) 1.69-4.36, p<0.001, Fig. 3.2 and Table 3.1). After adjusting for demographics and other comorbidities, experiencing homelessness significantly predicted a repeat positive test 90 days later (adjusted HR (aHR) 3.26, 95% CI 1.69–6.29, p<0.001, Table 3.1). In this multivariate analysis, the number of tests did not associate with reinfection (aHR 1.04, 95% CI 0.99–1.10, p=0.12).

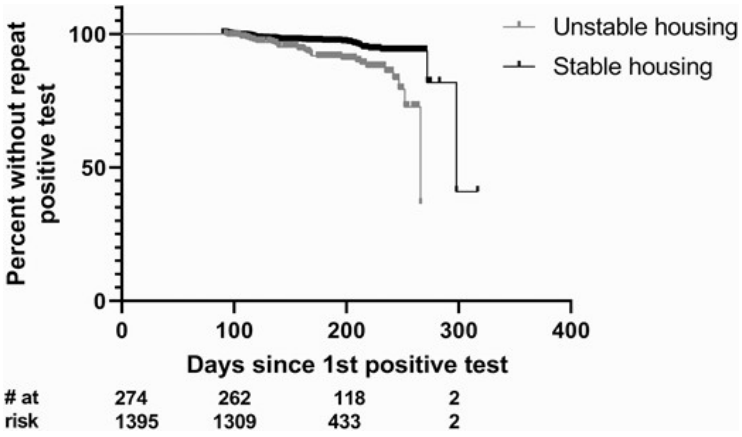


Figure 3.2. Unstable housing associates with reinfection. Kaplan-Meier survival curve for those with unstable housing (red) and stable housing (black) at the time of the first

infection. The y-axis shows the percent without a repeat positive SARS-CoV-2 RT-PCR result, and the x-axis shows days after first SARS-CoV-2 positive RT-PCR result. The tick marks denote right censoring. Number of patients at risk at different time points is displayed below the x-axis.

Table 3.1. Univariate and multivariate hazard ratios for risk factors associated with SARS-CoV-2 reinfection [a]

	Univariate HR (95% CI)	Schoefeld residuals ρ (p-value) [d]	Multivariate HR (95% CI)
Age, median (IQR)	1.00 (0.99 – 1.01)	-0.02 (0.87)	1.00 (0.98 – 1.02)
Male	1.20 (0.76 – 1.90)	-0.06 (0.60)	0.78 (0.45 – 1.34)
ETHNICITY/RACE [b]			
Black	0.63 (0.38 – 1.04)	0.03 (0.78)	0.77 (0.26 – 2.29)
White	0.68 (0.35 – 1.33)	-0.01 (0.95)	0.63 (0.19 – 2.07)
Hispanic/Latino	2.04 (1.30 – 3.22)	-0.08 (0.52)	1.87 (0.67 – 5.28)
Body mass index (IQR)	0.99 (0.96 – 1.02)	0.04 (0.74)	0.99 (0.96 – 1.03)
Homeless	2.71 (1.69 – 4.36)	0.08 (0.50)	3.26 (1.69 – 6.29)
Pregnant	2.89 (1.05 – 7.93)	-0.05 (0.69)	2.90 (0.98 – 8.60)
Diabetes mellitus	0.73 (0.40 – 1.33)	0.18 (0.14)	0.65 (0.34 – 1.27)
Heart disease	1.39 (0.69 – 2.83)	-0.18 (0.05)	1.34 (0.58 – 3.14)
Lung disease	0.64 (0.32 – 1.28)	0.22 (0.06)	0.51 (0.25 – 1.08)
CKD	1.29 (0.64 – 2.62)	0.23 (0.03)	1.54 (0.58 – 4.06)
End stage renal disease	1.20 (0.37 – 3.86)	-0.13 (0.25)	0.84 (0.20 – 3.54)
HIV	2.65 (0.97 – 7.27)	-0.03 (0.80)	2.23 (0.78 – 6.33)
Cancer	1.40 (0.51 – 3.85)	-0.00 (1.00)	1.61 (0.54 – 4.78)
Smoking [c]	1.16 (0.88 – 1.54)	0.05 (0.66)	0.95 (0.67 – 1.33)
On immunosuppressive medication	2.40 (0.77 – 7.68)	0.14 (0.23)	2.53 (0.71 – 9.01)
# of SARS-CoV-2 RT-PCR tests	1.08 (1.04 – 1.13)	0.03 (0.82)	1.04 (0.99 – 1.10)
Use of any COVID-19 medication	0.44 (0.18 – 1.10)	-0.22 (0.04)	0.30 (0.11 – 0.79)

[a] Hazard ratio is calculated based on Cox regression with Breslow method for ties, [b] Testing was a specific race/ethnicity versus not that race/ethnicity, [c] Smoking was treated as a categorical variable. Never smoker=0, former smoker=1 and current smoker = 2, [d] Proportional-hazards assumption test based on Schoenfeld residuals. A $\rho \neq 0$ with p-value < 0.05 violates the proportional-hazard assumption.

The CDC's criteria for reinfection includes a repeat positive SARS-CoV-2 RT-PCR test separated by a minimum of 90 days. Presumably shorter intervals may incorporate more individuals that have prolonged shedding rather than reinfection. Decreasing the interval to 60 days between a positive SARS-CoV-2 RT-PCR results followed by a repeat positive ($reinfection_{60days}$) or a negative ($convalescents_{60days}$) test increased the number of individuals in the two groups (Table 2.2). With this shorter time span between the repeat tests, unstable housing remained significantly higher among those with repeat positive tests. Pregnancy and being on an immunosuppressive medication were also significantly higher in the $reinfection_{60days}$ as compared to the $convalescents_{60days}$ group in univariate analysis.

Hospitalization and disease severity

Individuals with mild COVID-19 after SARS-CoV-2 infection may develop lower antibody levels and thus, individuals with mild disease after initial infection may be at greater risk for reinfection (Long, Tang, et al. 2020). The proportion of patients with hospitalization, with or without a "COVID-19 like illness", intensive care unit (ICU) stay, and need for mechanical ventilation was not different in the reinfection and the convalescent group (Table 2.3). During the first COVID-19 surge, hydroxychloroquine, colchicine, and immune modulators, such as IL inhibitors were frequently used off-label or as part of clinical trials at BMC (Sinha et al. 2020). The use of these medications, especially hydroxychloroquine, was higher in the hospitalized convalescents as compared to reinfection individuals (Table 2.3). Unstable housing remained predictive of having a repeat positive test 90 days later after adding use of any COVID-19 medications in the

previous multivariate model (aHR 3.12, 95% CI 1.62–6.00, p=0.001). Use of a COVID-19 medication during the primary infection hospitalization was also associated a lower risk of reinfection (aHR 0.30, 95% CI 0.11–0.79, p=0.02), although in univariate analysis the data violated the proportional hazard assumption (Table 3.1).

Thirty-one of the 75 (41.3%) individuals in the reinfection group were hospitalized around the time of the repeat positive SARS-CoV-2 RT-PCR test. Three of these 31 (9.7%) individuals were deemed to have a "COVID-19 like illness" during their second hospitalization. Two of these three patients were treated with dexamethasone, and one was treated with remdesivir. None of them required ICU admission or mechanical ventilation. Two other patients required mechanical ventilation at the time of their second hospitalization, but both were deemed not to have an illness consistent with COVID-19.

Antibody responses

A weakened humoral immune response to a primary SARS-CoV-2 infection could increase the probability of reinfection. Antibody levels within weeks after the first infection were compared between 10 and 20 reinfection and convalescent individuals respectively. The samples were from individuals with similar age, gender distribution, day from symptom onset, and number of comorbidities (Table 2.4). There were no significant differences between SARS-CoV-2 RBD and nucleocapsid IgG levels and ability to neutralize VSV-SARS-CoV-2-S pseudovirus between the convalescent and reinfection groups (Fig. 3.3A-C).

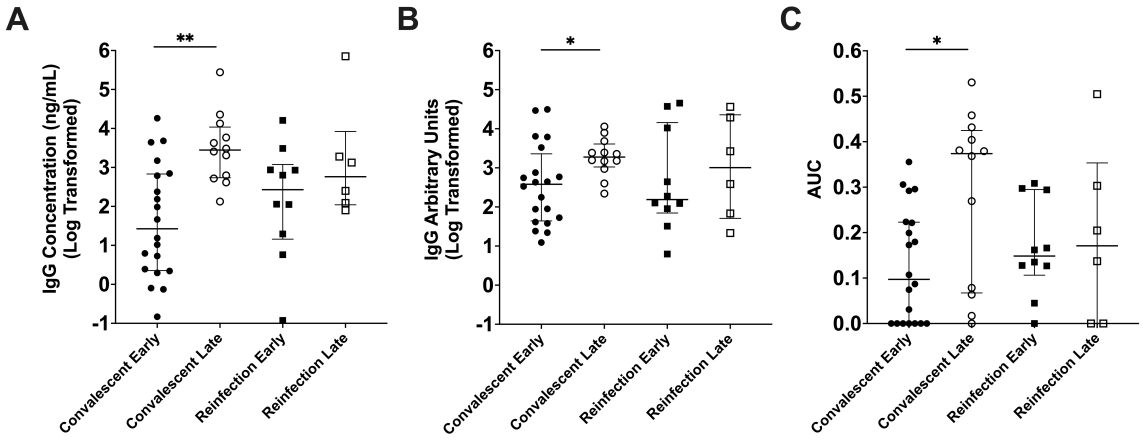


Figure 3.3. Antibody responses are not different among those with reinfection and the convalescents. Receptor binding domain (RBD) (A), nucleocapsid (B) IgG levels, and pseudovirus neutralization area under the curve (C) among those with reinfection (squares) and the convalescent group (circles). The x-axis denotes the early (collected within weeks of primary infection, filled symbols) and late (obtained at least 90 days after 1st positive RT-PCR test, unfilled symbols) samples. * and ** denote $p \leq 0.05$ and $p \leq 0.01$ respectively by the Mann-Whitney U test.

Humoral responses at least 90 days after primary infection were also compared among 6 and 12 reinfection and convalescent individuals respectively. For all the reinfection individuals, this late sample was collected after the second positive SARS-CoV-2 RT-PCR result. The individuals in the groups were well matched (Table 2.4). Although median number of days from first positive SARS-CoV-2 test to the late sample was around 55 days earlier in the reinfection group, this was not statistically different ($p=0.37$). In the convalescent but not reinfection group, antibody levels and neutralization AUC were higher in the late as compared to the early plasma sample. (Fig. 3.3A-C). In multivariate linear regression analysis, interval from primary infection to date of late sample collection, but not reinfection as compared to convalescent group, associated with

higher RBD IgG levels, nucleocapsid IgG magnitude, and pseudovirus neutralization AUC (Table 3.2).

Table 3.2. Predictors of humoral responses in multi-variable linear regression analyses [a]

	RBD IgG β (95% CI, p-value)	**Nucleocapsid IgG β (95% CI, p-value)**	**Neutralization AUC β (95% CI, p-value)**
(Intercept)	1.18 (-0.52 to 2.88, 0.17)	2.47 (1.05 to 3.90, 0.001)	-0.02 (-0.24 to 0.21, 0.88)
Reinfection	0.63 (-0.10 to 1.36, 0.09)	0.19 (-0.42 to 0.80, 0.53)	0.01 (-0.08 to 0.11, 0.83)
Interval (days) [b]	0.007 (0.004 to 0.01, <0.0001)	0.003 (0.0005 to 0.005, 0.02)	0.0004 ($1.9X10^{-5}$ to 0.0008, 0.04)
Age (years)	0.02 (-0.01 to 0.04, 0.14)	0.01 (-0.01 to 0.03, 0.46)	0.003 (-0.003 to 0.006, 0.08)
Male	-1.09 (-1.84 to -0.34, 0.005)	-0.65 (-1.28 to -0.022, 0.04)	-0.009 (-0.109 to 0.09, 0.86)

[a] Abbreviations include: RBD: receptor binding domain; CI: confidence interval,
[b] Interval is days from symptom onset for the early sample and days from 1st positive RT-PCR test for the late sample.

Sequence analysis

Longitudinal early and late nasal swabs and after the repeat positive test only samples were available from 3 and 2 reinfection individuals respectively. Only a late, without a matching early, and 3 early, without the matching late, nasal swab samples yielded quality sequences that covered the majority of the SARS-CoV-2 genome. Pangolin lineage assignment placed the 1 late and the 3 early samples into the B.1.2 and B.1 lineage respectively. The B.1.2 lineage had very low incidence in the United States when the reinfection patient was first diagnosed on March 25, 2020 and high incidence at

the time of the 2nd positive RT-PCR test on December 22, 2020 (Hodcroft et al. 2021). The B.1 lineage, however, was highly prevalent when the 3 early samples were collected in April 2020. The 1 late sample had 10 of the 11 expected single nucleotide changes consistent with B.1.2, and it did not have extended mutations that would be associated with a long-term infection and prolonged shedding (Choudhary et al. 2022).

Discussion

In this study, we identified factors associated with presumed SARS-CoV-2 reinfection. We compared demographics, disease characteristics, and humoral responses among individuals presumed to have reinfection. Reinfection was associated with unstable housing, but no other baseline demographic factor or comorbidity. Furthermore, antibody responses were not significantly different in a subset of individuals with reinfection. These observations suggest that socio-environmental factors rather than preexisting comorbidities or immunologic deficits are associated with reinfection.

We defined reinfection based on the CDC criteria of 2 separate SARS-CoV-2 RT-PCR tests separated by at least 90 days. Virus sequence analysis confirmed reinfection in only one individual, primarily because of sample limitations. Quantitative PCR cycle threshold values were also not available for the majority of reinfection samples to potentially differentiate reinfection from prolonged shedding. Interestingly, by relaxing the criteria for reinfection as 2 separate positive tests separated by 60 as opposed to 90 days demonstrated that pregnancy and use of an immunosuppressive medication, along with unstable housing, were also associated with the reinfection group in univariate analysis. Pregnancy and use of immunosuppressive medications have been associated

with prolonged virus shedding (Avanzato et al. 2020; Baang et al. 2021; B. Choi et al. 2020; Pene et al. 2003; Tarhini et al. 2021; Burgoyne et al. 2020; Molina et al. 2020). This suggests that the 90- as opposed to 60-day criteria may exclude individuals that are more likely to have prolonged virus shedding rather than reinfection.

In this BMC cohort, individuals experiencing unstable housing had more reinfection. BMC is New England's largest safety net hospital that often serves people experiencing homelessness (Barocas et al. 2021). This finding may not be generalizable; Indeed association between unstable housing and SARS-CoV-2 incidence was not been observed in another cohort (Keller et al. 2022). Major inaccuracies in EMR documentation may also skew the findings. Studies found up to 36 to 66% SARS-CoV-2 positivity rate among residents of large adult homeless shelters (Baggett et al. 2020; Mosites et al. 2020; Loubiere et al. 2021). Unstable housing likely makes it difficult to maintain the physical distance known to reduce SARS-CoV-2 transmission, and it may present challenges for other measures such as mask usage and hand hygiene (Barocas, Jacobson, and Hamer 2021). Thus, housing insecurity may be a surrogate marker for exposure to infectious SARS-CoV-2, although this was not directly measured in the study.

The reinfection as compared to convalescents group were tested more frequently and at shorter intervals. The exact reason for every repeat test was not ascertained. SARS-CoV-2 testing is often done when patients present for any hospital-based medical care. It is possible that individuals in the reinfection as compared to convalescent groups presented more frequently for medical care or were referred for SARS-CoV-2 testing

more often. Importantly, homelessness remained a significant predictor for reinfection after accounting for differential length of follow up and number of SARS-CoV-2 RT-PCR tests. Thus, the association of unstable housing with reinfection does not merely reflect changes in testing frequency or interval. It is possible that the convalescents have a high incidence of asymptomatic reinfections that were not documented.

COVID-19 severity did not differ after primary infection among those with reinfection and the convalescent group. Less COVID-19 medication use during hospitalization was associated with higher incidence of reinfection, although this association was based on a small sample size. A small minority had "COVID like illness" that required hospitalization after reinfection. This suggests that the immune response after primary infection may not prevent infection, but it does ameliorate against severe disease, which is similar to previous reports (Sheehan, Reddy, and Rothberg 2021) and observations from SARS-CoV-2 breakthrough infection after vaccination (Goldberg et al. 2021).

We also found that individuals with presumed reinfection did not have significantly lower antibody responses. Within the early weeks after SARS-CoV-2 infection, antibody levels and neutralization capacity were similar suggesting that individuals in the reinfection group did not have any obvious pre-existing deficit that prevented this early antibody response. The convalescent, but not reinfection, group demonstrated a significant increase in antibody levels and neutralization in the sample collected at least 90 days after the first positive test. This is especially surprising because the late plasma sample from the reinfection group was collected after the second positive

RT-PCR test. Presumably, reinfection should have boosted a pre-existing immune response as has been observed with vaccination among previously infected individuals (Z. Wang et al. 2021). The lack of both higher antibody levels and greater neutralization responses in the late as compared to the early plasma possibly may suggest that those with reinfection have a lower peak and/or faster decay in their antibodies over time. The data, however, were based on evaluating a small number of individuals at 2 time-points only. There could be selection bias in the samples available in the BMC biorepository. More intensive longitudinal examination of the immune responses from a larger number of individuals will be required to understand how changes in immunity along with greater exposure to the virus impacts reinfection.

The end date for the data acquisition in this study occurred prior to both the vaccine rollout in the city of Boston and emergence of the highly infectious delta (B.1.617.2) variant. Demographic and immunologic factors associated with repeat infections may be different with these changes. Vaccination reduces the chance of reinfection, but our data suggests that high levels of exposure can overcome relevant immune responses (Cavanaugh et al. 2021). Policies aimed at reducing homelessness and increasing vaccination may be helpful in reducing subsequent infections.

CHAPTER FOUR: RECENT ENDEMIC CORONAVIRUS INFECTION ASSOCIATES WITH HIGHER SARS-COV-2 CROSS-REACTIVE FC RECEPTOR BINDING ANTIBODIES

Disclaimer: Portions of this dissertation were included in or adapted from:

Recent Endemic Coronavirus Infection Associates With Higher SARS-CoV-2 Cross-Reactive Fc Receptor Binding Antibodies. Bean DJ, Liang YM, Sagar M. bioRxiv [Preprint]. 2024 Oct 23:2024.10.23.619886. Doi: 10.1101/2024.10.23.619886. (Bean, Liang, and Sagar 2024)

Background

Prior to the emergence of SARS-CoV-2 and COVID-19, studies suggested that humans are infected with an eCoV, such as HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43, about every 18 months to 2 years (Hamre and Beem 1972; Edridge et al. 2020). It has been hypothesized that cross-reactive immune responses generated from one CoV may protect against infection or ameliorate disease severity from a heterologous CoV (Dangi et al. 2021). In a large retrospective cohort study, we observed that individuals with a documented recent eCoV infection had less severe COVID-19 although there was no difference in SARS-CoV-2 incidence (Sagar et al. 2021). The immune mechanism behind this observed protection has not been definitively elucidated. Some, but not all, studies suggest that the pre-existing humoral and cellular immunity may decrease SARS-CoV-2 incidence and COVID-19 severity (Swadling et al. 2022;

Loyal et al. 2021; Ortega et al. 2021). Few studies, however, have directly looked at how the cross-reactive immune response changes after a recent eCoV infection.

Recent eCoV infections are difficult to diagnose because disease is mild, and the etiology for a "common cold" is usually not investigated if one presents for medical care (Walsh, Shin, and Falsey 2013). Many studies define recent eCoV infections either by detecting viral RNA through PCR-based methods or changes in eCoV directed antibody levels (Edridge et al. 2020; Gaunt et al. 2010). CRP-PCR (BioFire) tests detect active eCoV infections (Leber et al. 2018), but they are not widely used and most individuals do not present for medical care when they have the "common cold" due to an eCoV (Gaunt et al. 2010). Detecting changes in antibody levels require longitudinal sampling, and these types of studies are hindered by the number of participants and longevity of the study. Inability to identify individuals with recent eCoV infections with confidence makes it difficult to assess the effect on SARS-CoV-2 immunity.

In this study, we used a combination of PCR documented eCoV infections with eCoV nucleocapsid antibody titer data to classify individuals with presumed recent eCoV infections. The cross-reactive immune responses to various SARS-CoV-2 antigens were compared between those individuals with or without a presumed recent eCoV infection. We found that eCoV spike specific antibodies are boosted after a recent eCoV infection and are likely mediating the cross-reactivity against SARS-CoV-2 spike. These cross-reactive antibodies were not associated with higher SARS-CoV-2 neutralization, but they were capable of binding to the Fc receptor, FcγRIIIa, and potentially mediating enhanced Fc effector functions. Our results suggest a recent infection with an eCoV boosts the Fc

effector function potential of CoV-specific antibodies and offers an additional possible mechanism for the protection against severe COVID-19.

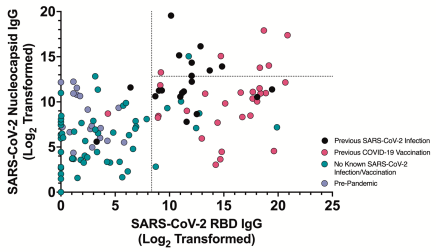
Results

Plasma IgG levels indicate recent CoV infections

We examined immune responses in blood samples from individuals that either had a confirmed previous SARS-CoV-2 infection (n=20), prior COVID-19 vaccination (n=29), or no known history of SARS-CoV-2 infection or COVID-19 vaccination (n=72) (Table 2.5). Of those with no documented SARS-CoV-2 infection or COVID-19 vaccination, 18 were sampled prior to the start of the COVID-19 pandemic in the United States. Thus, these individuals could not have had undocumented or asymptomatic SARS-CoV-2 infection. The remaining people (n=54), however, possibly could have had prior undiagnosed or asymptomatic SARS-CoV-2 infection because samples were collected after March 2020. We used previous methodology described by our group and others to identify individuals that may have had undocumented asymptomatic SARS-CoV-2 infection (Bean et al. 2024; Burbelo et al. 2020). Briefly, we measured plasma IgG levels against SARS-CoV-2 RBD and SARS-CoV-2 nucleocapsid by ELISA. These ELISA results on samples collected prior to the pandemic, those with documented SARS-CoV-2 infection, and those with known COVID-19 vaccination were used to set cutoffs for the SARS-CoV-2 RBD and nucleocapsid IgG levels that would differentiate individuals with possible asymptomatic undocumented SARS-CoV-2 infections (Fig. 4.1A). As expected, most of the individuals with either a previous SARS-CoV-2 infection (18/20, 90%) or COVID-19 vaccination (28/29, 97%) had elevated anti-SARS-CoV-2

RBD IgG titers. Furthermore, all the pre-pandemic samples (18/18, 100%) were below the set cutoff for SARS-CoV-2 antigen exposure. In summary, the overall classification accuracy based on our assigned cutoffs was 87% (95% CI 76–94%), which is within the range of previous methodologies (73 to 91%) (Fig. 4.1B) (Bean et al. 2024). Seven individuals classified in the no known SARS-CoV-2 infection or COVID-19 vaccination group had IgG levels against SARS-CoV-2 RBD above the established cutoff, indicative of a potential previous SARS-CoV-2 infection. Furthermore, anti-SARS-CoV-2 nucleocapsid IgG levels above the established cutoff indicated that 6 individuals with a prior COVID-19 vaccination may have had a previous SARS-CoV-2 infection. These 13 individuals were excluded from the subsequent groups classified according to possible recent eCoV infection.

A



B

			Clinical Classification				
			Prior SARS-CoV-2 infection (n = 20)	COVID-19 vaccination, but no SARS-CoV-2 infection (n = 29)	Pre-pandemic (n = 18)	Positive predictive value	Negative predicative value
Cutoff Classification	Prior infection	RBD IgG ≥ 7.95	18	6	0	75% (53%, 90%)	95% (84%, 99%)
	Vaccination, no infection	RBD IgG ≥ 7.95 & NC IgG ≤ 12.2	0	22	0	100% (85%, 100%)	84% (71%, 94%)
	No SARS-CoV-2 exposure	RBD IgG < 7.95	2	1	18	86% (64%, 97%)	100% (92%, 100%)
	Sensitivity		90% (68%, 99%)	76% (56%, 90%)	100% (81%, 100%)	Overall Accuracy 87% (76%, 94%)	
	Specificity		87% (74%, 95%)	100% (91%, 100%)	94% (83%, 99%)		

Figure 4.1. Classification of presumed SARS-CoV-2 infections based on antibody levels. (A) IgG antibody levels against SARS-CoV-2 RBD and nucleocapsid protein were measured in individuals with a previous SARS-CoV-2 infection (black), previous COVID-19 vaccination with no documented history of SARS-CoV-2 infections (pink), no known SARS-CoV-2 infection or COVID-19 vaccination (green), or pre-pandemic samples collected before March 2020 (purple). The dotted lines specify cutoffs that were established to classify individuals with presumed undiagnosed SARS-CoV-2 infection. (B) The accuracy and other test characteristics of the classification based on the established cutoffs for classifying individuals into the specified groups. The values in parentheses display the 95% CI.

Similarly, plasma IgG antibody levels against the nucleocapsid antigen have been previously used to identify recent eCoV infections (Blanchard et al. 2011). We measured plasma IgG levels against all four human eCoVs nucleocapsid protein to identify presumed recent eCoV infections within our COVID-19 vaccinated and no known SARS-CoV-2 exposure groups. Nucleocapsid antibody titers against the two alpha (HCoV-229E and HCoV-NL63, Pearson ρ=0.920, $p<0.0001$, Fig. 4.2A) and the two beta (HCoV-OC43 and HCoV-HKU1, Pearson ρ=0.478, $p<0.0001$, Fig. 4.2B) eCoVs showed significant correlations. On the other hand, there was no correlation between alpha and beta nucleocapsid (Fig. 4.2C). Thus, we generated a composite alpha and beta eCoV anti-nucleocapsid IgG metric.

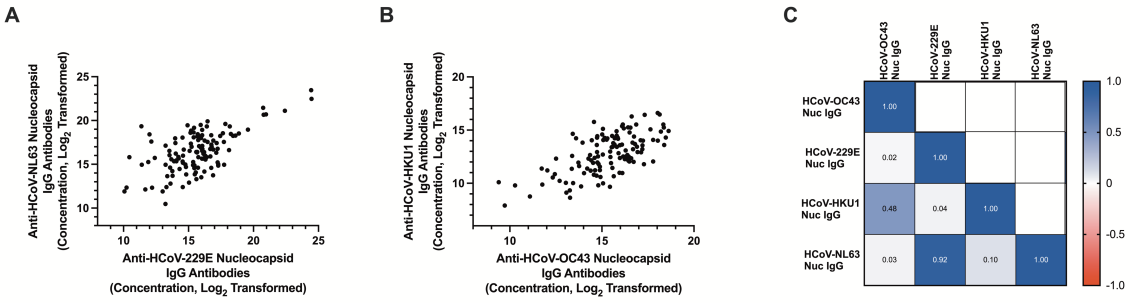


Figure 4.2. Correlations between IgG antibody responses to alpha and beta eCoV nucleocapsid proteins. Anti-nucleocapsid antibody responses were measured in all individuals in the study. (A) Correlation of IgG antibody responses against HCoV-229E and HCoV-NL63 nucleocapsids. (B) Correlation of IgG antibody responses against HCoV-HKU1 and HCoV-OC43 nucleocapsids. (C) The matrix shows Pearson correlation r values between IgG antibody responses against HCoV-OC43, HCoV-229E, HCoV-HKU1, and HCoV-NL63 nucleocapsid proteins. Blue represents stronger positive correlations, while red signifies stronger negative correlations.

Longitudinal sampling in prior studies suggests a reinfection with an eCoV occurs on average every 1 to 3 years (Hamre and Beem 1972; Edridge et al. 2020). Furthermore, duration between peak antibody responses after a presumed eCoV infection suggest that a subsequent infection with a heterologous eCoV occurs around every 1.5 years (Edridge et al. 2020). Based off this timeframe and the BMC EMR, we identified 22 individuals within the no known SARS-CoV-2 infection that had documented eCoV RNA (11 HCoV-OC43, 6 HCoV-NL63, 4 HCoV-229E, 1 HCoV-HKU1) on a prior CRP-PCR test less than 550 days prior to sample collection. Thus, these individuals were definitively classified as having a recent eCoV infection. In the remaining with no documented or presumed SARS-CoV-2 infection (n=72), the top 25% of individuals (n=18) with the highest nucleocapsid antibody levels (either anti-alpha or beta eCoV) were also classified as having a recent eCoV infection. The rest (n=54) were classified as not having a recent eCoV infection. Other than a clinically small but statistically significant age difference, the groups classified as either with or without a recent eCoV infection had no meaningful demographic differences (Table 4.1).

Table 4.1. Comparison of demographics of the individuals with or without a presumed recent eCoV infection.

	Presumed or documented recent eCoV infection (n = 40)	No presumed recent eCoV infection (n = 54)	p-value [a]
Age (years), median (IQR)	63 (50 – 70)	56 (45 – 63)	0.0470 [b]
Male	19 (48)	28 (52)	0.6765
Race/Ethnicity [c]			0.8105
Black	18 (45)	29 (54)	
White	16 (40)	18 (33)	
Hispanic/Latino	5 (13)	5 (9)	
Other/missing	1 (3)	2 (4)	
Diabetes mellitus	14 (35)	18 (33)	0.8661
Heart disease [d]	15 (38)	11 (20)	0.0664
Lung disease [e]	17 (43)	15 (28)	0.1364
CKD [f]	3 (8)	6 (11)	0.7283 [g]
HIV [h]	8 (20)	18 (33)	0.1530
Cancer	1 (3)	2 (4)	>0.9999 [g]
Number of co-morbidities [i]			0.4396
0	7 (18)	8 (15)	
1	14 (35)	26 (48)	
≥2	19 (48)	20 (37)	
Pre-Pandemic [j]	4 (10)	14 (26)	0.0656 [g]
COVID-19 Vaccine	13 (33)	10 (19)	0.1190

Data shows number and percent unless otherwise indicated, [a] Chi-square test unless otherwise indicated, [b] Mann-Whitney test, [c] As specified in the EMR; an individual may be in more than 1 category, [d] Heart disease includes coronary artery disease and congestive heart failure, [e] Lung disease includes chronic obstructive pulmonary disease and asthma, [f] Chronic kidney disease, [g] Fisher's Exact Test, [h] Human immunodeficiency virus, [i] Number of comorbidities accounts for diabetes mellitus, heart disease, lung disease, chronic kidney disease, HIV, and cancer, [j] Samples collected before March 2020

Recent eCoV infection associates with increased SARS-CoV-2 S2 specific FcγRIIIa binding antibody responses

We examined cross-reactive humoral immunity after a recent eCoV infection because antibody responses are the best correlate of protection against SARS-CoV-2

infection and severe COVID-19 (Garcia-Beltran, Lam, et al. 2021; Khoury et al. 2021). Individuals with a previous COVID-19 vaccination were excluded from the groups classified as with (n=13) or without (n=10) a presumed recent eCoV infection when comparing heterotypic SARS-CoV-2 spike immune responses (Table 4.1). Instead, they were included in a composite group along with those with a documented previous SARS-CoV-2 infection, classified as previous SARS-CoV-2 spike exposure. There was no significant difference in anti-SARS-CoV-2 RBD IgG (Fig. 4.3A) and anti-SARS-CoV-2 S2 (Fig. 4.3B) IgG antibodies in those with and without a recent eCoV infection. As expected, anti-SARS-CoV-2 spike antibodies were significantly higher in the individuals with prior SARS-CoV-2 spike exposure (Fig. 4.3A and B). Neutralization responses against VSV pseudotyped with SARS-CoV-2 spike (Wuhan variant) were higher in those with previous SARS-CoV-2 spike exposure, but there was no difference in neutralization between individuals with or without a presumed recent eCoV infection (Fig. 4.3C).

Another potential functional role of these anti-SARS-CoV-2 antibodies is through FcR binding, which contributes to the clearance of virally infected cells through mechanisms such as ADCC or ADCP (Y. Yu et al. 2021). We used a previously validated ELISA method (Šuštić et al. 2022) to measure SARS-CoV-2 specific FcγRIIIa (CD16a) binding antibodies, which is a predictor for NK cell mediated antibody effector function. As expected, those individuals with prior SARS-CoV-2 spike exposure had higher levels of anti-SARS-CoV-2 RBD and S2 antibodies binding to FcγRIIIa (Fig. 4.3D-G). Individuals with or without a presumed recent eCoV infection had similar anti-SARS-CoV-2 RBD FcγRIIIa binding antibodies and FcγRIIIa binding antibodies to IgG ratio

(Fig. 4.3D and E). However, anti-SARS-CoV-2 S2 FcγRIIIa binding antibodies were 7.8-fold higher (p=0.0234) and the FcγRIIIa binding antibodies to IgG ratio was 85-fold higher (p=0.0501) in the individuals classified as having a recent eCoV infection (Fig. 4.3F and G).

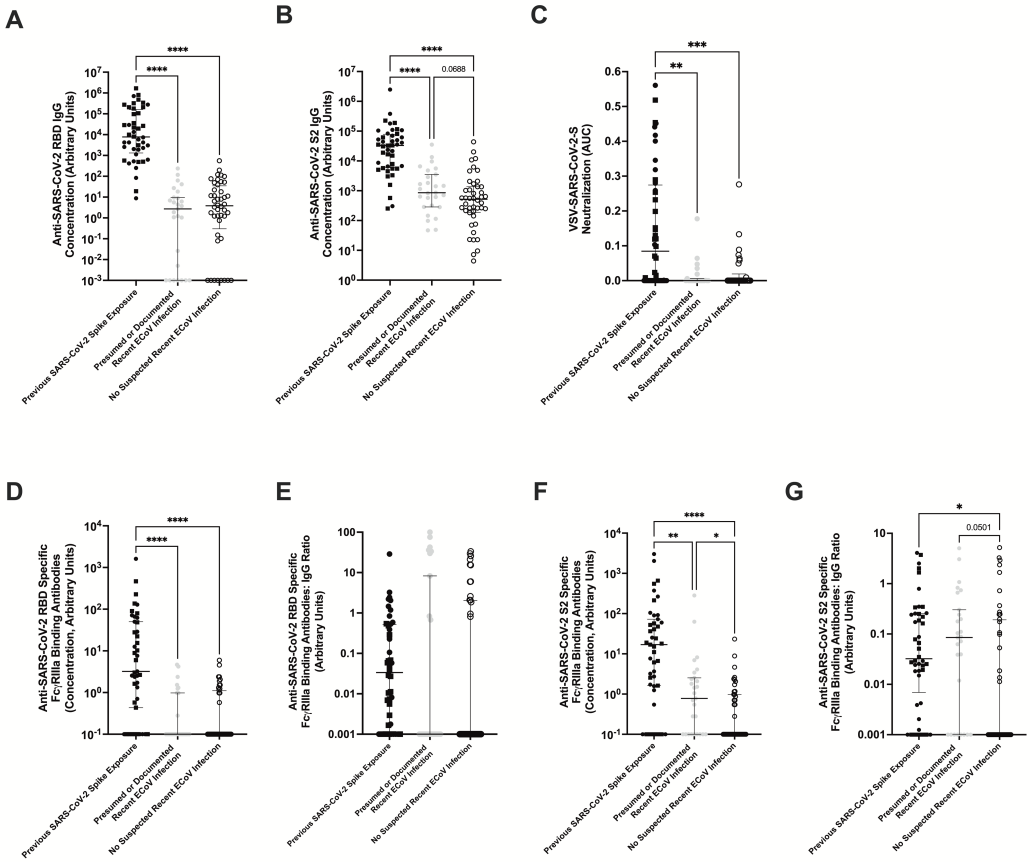


Figure 4.3. Humoral immune responses to SARS-CoV-2 spike antigens. Plasma antibody responses in those with documented SARS-CoV-2 spike exposure (previous SARS-CoV-2 infection (black circle) or prior COVID-19 vaccination (black square)), presumed or documented recent eCoV infection (gray circle), or without a presumed recent eCoV infection (white circle). (A and B) IgG antibody levels against SARS-CoV-2 RBD (A) or SARS-CoV-2 spike S2 subunit (B). (C) Neutralization responses against a VSV-ΔG pseudovirus expressing the SARS-CoV-2-Wuhan spike protein. (D-G) Titer of SARS-CoV-2 RBD (D) or SARS-CoV-2 S2 (F) specific antibodies binding to the Fc receptor, FcγRIIIa, or the ratio of those FcR binding antibodies to the SARS-CoV-2 RBD (E) or SARS-CoV-2 S2 (G) IgG levels in panel (A and B). The dark horizontal lines in

each scatter dot plot denote the median and interquartile range. Statistical analyses were performed using either Kruskal-Wallis test with Dunn multiple comparison test or Mann-Whitney U test. P-values less than 0.1 are displayed. *, **, ***, and **** represent p-values <0.05, <0.01, <0.001, and <0.0001, respectively.

In the FcγRIIIa binding assay, individuals could be separated into responders and non-responders based on FcγRIIIa binding activity above or below a designated cutoff (Fig. 4.4A). We used a multiple logistic regression model to identify characteristics associated with SARS-CoV-2 S2-specific FcγRIIIa antibody binding. As expected, previous SARS-CoV-2 spike exposure was associated with 4.055-fold higher odds (95% CI: 1.581 to 11.55, p=0.0053) of having a SARS-CoV-2 S2 FcγRIIIa binding antibody response. There were 1.379-fold higher odds (95% CI: 1.060 to 1.859, p=0.0249) of having a SARS-CoV-2 S2 FcγRIIIa binding antibody response for every 2-fold increase in anti-alpha eCoV nucleocapsid antibody levels (Fig. 4.4B). Levels of anti-beta eCoV nucleocapsid antibodies, age, gender, and other demographic characteristics were not associated with detectable SARS-CoV-2 S2 FcγRIIIa binding antibody.

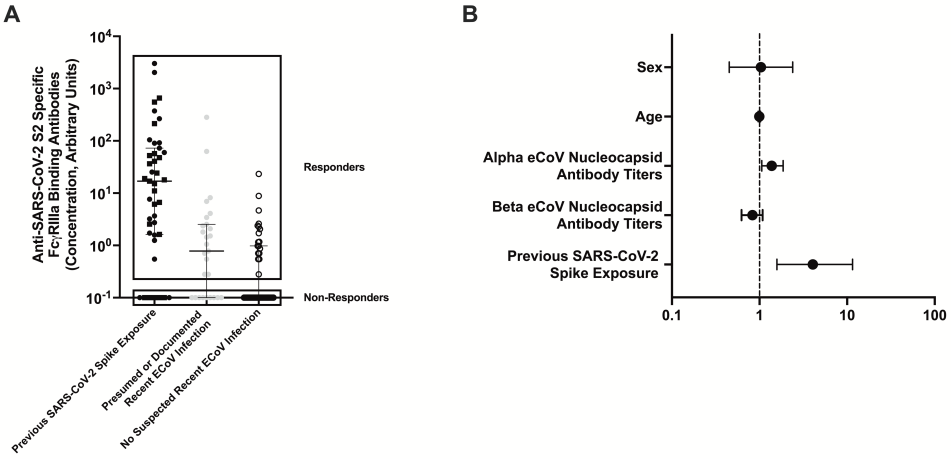


Figure 4.4. Higher alpha eCoV nucleocapsid antibody responses predicts positive responses in the SARS-CoV-2 S2 specific antibody FcR binding assay. (A)

Individuals were separated into two groups based on positive (responders) or absent (non-responders) FcγRIIIa SARS-CoV-2 S2 specific antibody binding. This responder and non-responder classification was used in a multivariate logistic regression model. This data is the same as Fig. 2F. (B) The odds ratio of variables used in the multivariate logistic regression model to predict positive SARS-CoV-2 S2 antibody FcR binding responses. The error bars represent the 95% CI.

SARS-CoV-2 S2 FcγRIIIa binding antibody responses are correlated with eCoV spike specific FcγRIIIa binding antibody responses

Next, we assessed if eCoV spike protein directed FcγRIIIa binding antibodies possibly account for the anti-SARS-CoV-2 S2 FcγRIIIa antibodies (Fig. 4.3 and 4.4). We used the antigen specific IgG and FcγRIIIa binding antibody ELISA to measure antibody levels against HCoV-229E spike and HCoV-OC43 spike proteins as representative antibody responses to alpha and beta eCoV infections respectively (Fig. 4.5). As expected, IgG levels were higher against HCoV-229E spike ($p=0.0241$, Fig. 4.5A) and HCoV-OC43 spike ($p=0.2086$, Fig. 4.5B) in the individuals with a presumed recent eCoV infection, although only the comparison with HCoV-229E spike antibody levels reached statistical significance. Furthermore, HCoV-229E ($p=0.0688$, Fig. 4.5C) and HCoV-OC43 ($p=0.0282$, Fig. 4.5D) spike specific FcγRIIIa binding antibody levels were also higher in the individuals with a presumed recent eCoV infection, and these comparisons showed a statistical trend and significance. There were no statistical differences in the HCoV-229E ($p=0.4689$, Fig. 4.5F) and HCoV-OC43 spike specific

(p=0.2044, Fig. 4.5E) FcγRIIIa binding antibodies to IgG ratio between those with or without a presumed recent eCoV infection.

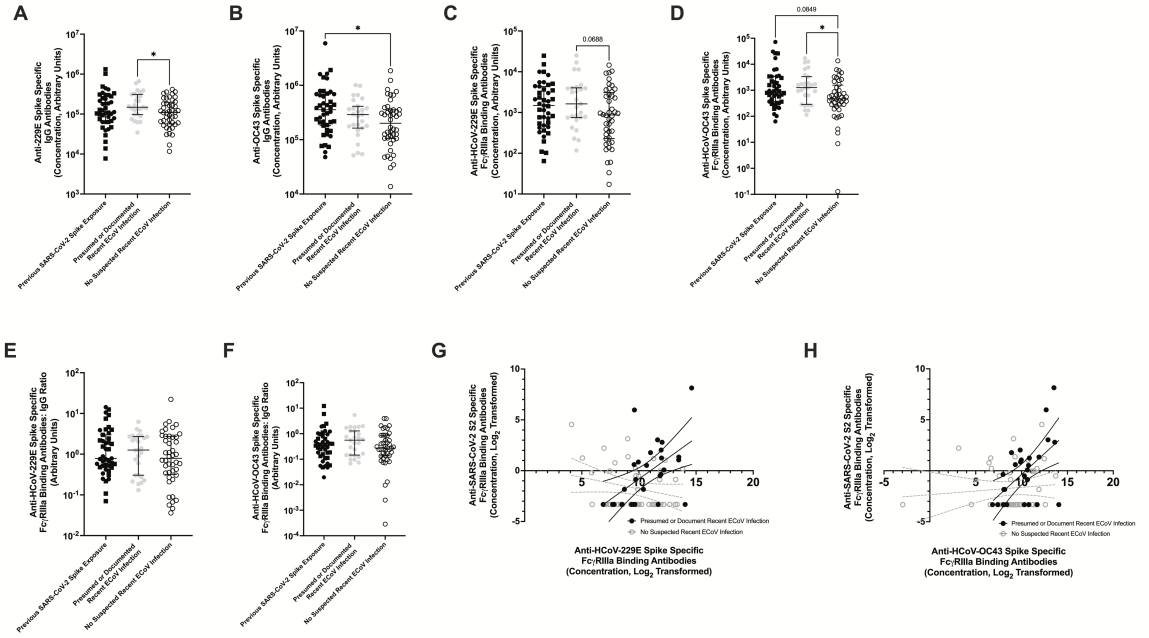


Figure 4.5. ECoV specific antibody FcR binding responses correlate with SARS-CoV-2 S2 antibody responses. Plasma antibody responses were measured in individuals with either a previous SARS-CoV-2 spike exposure (previous SARS-CoV-2 infection (black circle) and previous COVID-19 vaccination (black square)), presumed or documented recent eCoV infection (gray circle), or without a presumed recent eCoV infection (white circle). (A and B) IgG antibody responses against HCoV-229E (A) or HCoV-OC43 (B) spikes. (C and D) Level of HCoV-229E (C) or HCoV-OC43 (D) spike-specific antibodies binding to the Fc receptor, FcγRIIIa. (E and F) Ratio of Fc receptor antibody binding (C and D) to antigen specific IgG (A and B) in HCoV-229E (E) or HCoV-OC43 (F) spike-specific antibody responses. (G and H) Correlations between HCoV-229E (E) or HCoV-OC43 (F) spike-specific antibody FcR binding to SARS-CoV-2 S2 specific antibody FcR binding responses. Black dots and lines represent individuals with presumed or documented recent eCoV infection, while gray represents those without a presumed recent eCoV infection. The lines show a simple linear regression with the 95% CI. The dark horizontal lines in each scatter dot plot denote the median and interquartile range. Statistical analyses were performed using either Kruskal-Wallis test with Dunn multiple comparison test or Mann-Whitney U test. P-values less than 0.1 are displayed. * represents p-values <0.05.

We compared the SARS-CoV-2 S2 and eCoV spike FcγRIIIa binding antibody responses to better understand the extent of cross-reactivity of the antibodies. Moderate positive correlations were observed between the SARS-CoV-2 S2 and HCoV-229E (Spearman r=0.5153 and p=0.010, Fig. 4.5G) and HCoV-OC43 (Spearman r=0.4793 and p=0.010 Fig. 4.5H) spike FcγRIIIa binding antibody responses in those individuals with presumed or documented recent eCoV infections. There were no significant correlations in SARS-CoV-2 and eCoV FcR binding antibody responses in individuals without a presumed recent eCoV infection. These observations suggest eCoV antibodies spike capable of binding to FcγRIIIa are elevated after a recent eCoV infection, and these antibodies correlate with increased cross-reactivity against SARS-CoV-2 spike S2 region.

Recent eCoV infection does not improve T cell responses against SARS-CoV-2 antigens

Pre-existing T cells against SARS-CoV-2 antigens were found in pre-pandemic samples, suggesting a previous eCoV infection elicits cross-reactive T cells (Grifoni et al. 2020; Mateus et al. 2020). We tested T cell responses against SARS-CoV-2 spike, nucleocapsid, and nsp12/nsp13 antigens in the subset of individuals with available PBMC and either a confirmed previous SARS-CoV-2 infection (n=20), presumed or documented recent eCoV infection (n=32), or no presumed recent eCoV infection (n=42). The subset of individuals with available PBMCs were representative of the full cohort (Table 2.6). As before, individuals with a previous COVID-19 vaccination were included in the prior SARS-CoV-2 spike exposure group when testing responses against SARS-CoV-2 spike. As expected, individuals with a previous SARS-CoV-2 antigen experience had significantly higher activated ($CD134^+$ $CD137^+$) $CD4^+$ and ($CD69^+$ $CD137^+$) $CD8^+$ T cell

responses to SARS-CoV-2 spike (Fig. 4.6A and B) and nucleocapsid (Fig. 4.6C and 5D). Nsp12/nsp13 $CD4^+$ (p=0.1120, Fig. 4.6E) and $CD8^+$ T cell responses (p=0.0327, Fig. 4.6F) were also higher in those with prior SARS-CoV-2 infection as compared to those with no previous SARS-CoV-2 antigen experience, although there were no consistent differences when compared with the two groups classified based on recent eCoV infection individually. Importantly, there were no significant differences in activated $CD4^+$ or $CD8^+$ T cell responses to SARS-CoV-2 spike, nucleocapsid, or nsp12/nsp13 peptides between those individuals with or without a presumed recent eCoV infection (Fig. 4.6A-F). This suggests that cross-reactive T cell levels in the peripheral blood are not significantly boosted after a recent eCoV infection.

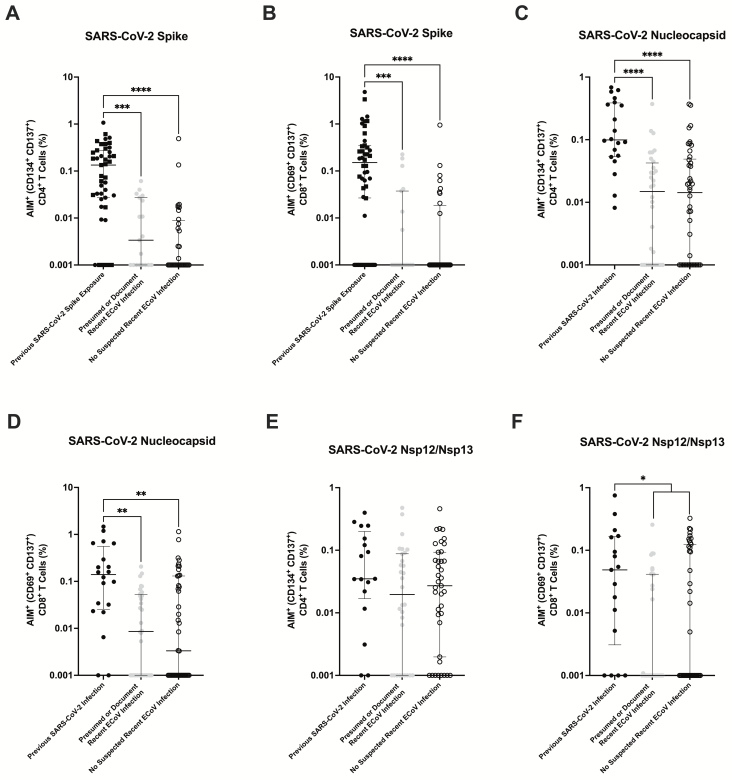


Figure 4.6. T cell responses against SARS-CoV-2 antigens are similar between individuals with or without presumed recent eCoV infections. T cell responses were

measured in individuals with either a previous SARS-CoV-2 spike exposure (previous SARS-CoV-2 infection (black circle) and previous COVID-19 vaccination (black square)), presumed or documented recent eCoV infection (gray circle), or without a presumed recent eCoV infection (white circle). Cells were stimulated with SARS-CoV-2 peptide pools and percent of activated ($CD134^+$ $CD137^+$) $CD4^+$ T cells and ($CD69^+$ $CD137^+$) $CD8^+$ T cells were measured. (A-B) $CD4^+$ (A) and $CD8^+$ (B) T cell activation was measured after stimulation with SARS-CoV-2 spike peptides. (C-D) $CD4^+$ (C) and $CD8^+$ (D) T cell activation was measured after stimulation with SARS-CoV-2 nucleocapsid peptides. (E-F) $CD4^+$ (E) and $CD8^+$ (F) T cell activation was measured after stimulation with SARS-CoV-2 nsp12/nsp13 peptides. Data were background subtracted against the negative control (DMSO only). The dark horizontal lines in each scatter dot plot denote the median and interquartile range. Note, the y axis varies among the different panels. Statistical analyses were performed using either Kruskal-Wallis test with Dunn multiple comparison test or Mann-Whitney U test. P-values less than 0.1 are displayed. *, **, ***, and **** represent p-values <0.05, <0.01, <0.001, and <0.0001, respectively.

Discussion

Multiple different highly pathogenic CoV have emerged over the past 25 years (Peiris et al. 2003; de Groot et al. 2013; P. Zhou et al. 2020), and thus developing a pan-CoV vaccine remains a major priority. The efficacy of this pan-CoV vaccine against future emerging CoVs may be judged by how well it performs against the known currently circulating HCoVs. Such broad protection against the diverse CoVs requires knowledge of both the conserved immunodominant regions and the components of the immune system mediating this protection. Understanding the role of heterotypic immunity in the protection against SARS-CoV-2 and severe COVID-19 is a key step towards developing a pan-CoV vaccine. Most studies suggest that heterotypic immunity from a recent eCoV infection protects against severe COVID-19, while some studies indicate there is no cross-reactive protection (Sagar et al. 2021; Abela et al. 2024; Aguilar-Bretones et al. 2021; Anderson et al. 2021). These varying conclusions may

differ based on the cohorts studied and criteria used to define protection against SARS-CoV-2 or severe COVID-19. Besides resolving this controversy, it is important to understand how a recent eCoV infection impacts immune responses directed against SARS-CoV-2 because it will highlight immune mechanisms that mediate cross-reactive protection. Here in this study, we identify a potential role of antibody Fc effector functions in the eCoV-derived heterotypic immune response against SARS-CoV-2.

Studies have measured heterotypic immunity against SARS-CoV-2 by correlating eCoV and SARS-CoV-2 immune responses (Yuen et al. 2021). In general, investigations have not identified individuals with a recent eCoV infection and assessed subsequent SARS-CoV-2 immune responses. We used both clinically documented cases and antibody levels against eCoV nucleocapsids to classify presumed recent eCoV infections. These are the most reliable and accepted ways to classify recent eCoV infection. This classification, however, is complicated by the prevalence and frequency of eCoV infections. By the age of 5, most children have had at least 1 eCoV infection with reinfections occurring frequently (Gaunt et al. 2010; Dijkman et al. 2008). The protective immunity towards these eCoVs lasts for a short duration, and reinfections can even happen within the same year despite detectable antibody levels (Edridge et al. 2020). Thus, it is difficult to accurately define a recent eCoV infection without complete PCR-based results or longitudinal sampling of the entire cohort. Our results and comparisons between groups largely depend on this definition of a recent eCoV infection. SARS-CoV-2 directed FcR antibodies still trended higher if the prior eCoV infection group was defined by documented infection on CRP-PCR test within the past 550 days and up to

35% of those with the highest alpha and beta nucleocapsid IgG levels (Table 4.2). This sensitivity analysis provides further confidence for our conclusions. Regardless, additional studies with well-defined eCoV infections will further validate our findings.

Table 4.2. Statistical comparison of SARS-CoV-2 S2-specific antibody FcγRIIIa binding responses between individuals with or without a presumed recent eCoV infection

Percent of individuals with highest anti-alpha and beta eCoV nucleocapsids IgG levels included in the presumed recent eCoV group	Presumed or documented recent eCoV infection (median, IQR)	No presumed recent eCoV infection (median, IQR)	p-value[a]
25%	1.662 (0.587-3.165)	0.100 (0.100-0.977)	0.0234
30%	1.436 (0.280-2.369)	0.100 (0.100-0.977)	0.0629
35%	1.071 (0.145-2.298)	0.100 (0.100-1.021)	0.1037

[a] Mann-Whitney test between groups with or without a presumed recent eCoV infection

In our study, SARS-CoV-2 S2, but not RBD, directed antibodies were elevated after a recent eCoV infection (Fig. 4.3). These results align with previous studies because many individuals have pre-existing anti-SARS-CoV-2 antibodies, with a preference towards more conserved regions, like S2, compared to more variable regions, like RBD (Grobben et al. 2021). In general, the higher degree of similarity between different CoVs predicts higher levels of antibody-mediated protection against heterologous infections (Dangi et al. 2021). Sterilizing protection against a heterologous CoV requires neutralizing antibodies against conserved spike regions. In accordance with our study and previous work, eCoV directed antibodies poorly cross-react with SARS-CoV-2 RBD and

thus have limited neutralizing ability (Aguilar-Bretones et al. 2021). Antibodies targeted against SARS-CoV-2 S2 can neutralize the virus, but they are rare and less potent than anti-SARS-CoV-2 RBD neutralizing antibodies (Geanes et al. 2022). This agrees with clinical observations that a recent eCoV infection does not reduce the number of SARS-CoV-2 infections, but instead associates with protection against COVID-19 severity (Sagar et al. 2021; Abela et al. 2024). The protection against severe COVID-19, but not SARS-CoV-2 infection, indicates components of the immune system other than neutralizing antibodies may be mediating this protection.

The Fc effector function of cross-reactive SARS-CoV-2 directed antibodies could potentially contribute to the protection against severe COVID-19 (Adeniji et al. 2021). SARS-CoV-2 S2 FcγRIIIa binding antibodies were higher in those classified as having a recent eCoV infection, increased levels were associated with higher alpha eCoV nucleocapsid levels, and there was a direct association with eCoV spike IgG levels. In aggregate, this implies that recent eCoV infection enhances cross-reactive SARS-CoV-2 S2 FcγRIIIa binding antibodies. We used an ELISA which measures antigen specific antibodies capable of binding to FcγRIIIa, which primarily depends of afucosylation of the antibody Fc portion (Šuštić et al. 2022). We focused our study on FcγRIIIa, which is expressed on NK cells and macrophages, and its interaction is crucial for ADCC and ADCP activity (Maucourant et al. 2020). Other Fc receptors, like FcγRI, have a higher affinity toward IgG antibodies, but they are not expressed on NK cells and do not play as critical of a role in Fc mediated effector functions (Kiyoshi et al. 2015). We found that both SARS-CoV-2 S2 and eCoV spike antibodies capable of binding FcγRIIIa were

increased in those individuals with a recent eCoV infection. Both higher afucosylated antibody levels and greater ADCC activity are correlated with COVID-19 severity (Chakraborty et al. 2022; Larsen et al. 2021). Yet, stronger ADCC activity is also associated with protection against fatal COVID-19 cases (Y. Yu et al. 2021). The Fc portion of antibodies can also enhance viral infections and contribute to disease pathogenesis. Antibody dependent enhancement (ADE) may increase SARS-CoV-2 infection in monocytes, but its role in COVID-19 severity is not clear (Junqueira et al. 2022). We only measured levels and not the function of FcγRIIIa binding antibodies, and thus we can only speculate about increases in ADCC, ADCP, and ADE. Furthermore, we restricted our analysis to peripheral blood immune responses, and cross-reactive tissue-based SARS-CoV-2 immunity may be different after recent eCoV infection

Elevated levels of T cells early in a SARS-CoV-2 infection are associated with protection against severe COVID-19 (Bergamaschi et al. 2021; M. Yu et al. 2023). These initial protective T cells against SARS-CoV-2 are speculated to be cross-reactive memory T cells from a previous eCoV infection. Although other studies have noted a boost in cross-reactive T cells after a SARS-CoV-2 infection, few studies have looked at cross-reactive T cell levels after an eCoV infection (Bean et al. 2024; Low et al. 2021). Cross-reactive T cells against SARS-CoV-2 antigens were detected in nearly all SARS-CoV-2 naïve individuals, but they were at similar levels among those with or without a presumed recent eCoV infection. Our data suggests that an eCoV infection does not preferentially expand T cell responses against SARS-CoV-2 spike, nucleocapsid, or nsp12/nsp13. Alternatively, eCoV-specific T cells have a lower avidity toward their corresponding

SARS-CoV-2 peptides, so the cross-reactive T cells may not respond sufficiently in our assay (Bacher et al. 2020). Furthermore, we only stimulated cells with peptides from SARS-CoV-2 spike, nucleocapsid, nsp12, and nsp13. We chose these proteins because they are either immunodominant or associated with immune protection against CoV related diseases, but other antigens may contribute to the heterotypic immune response (Swadling et al. 2022; Bean et al. 2024; Tarke, Sidney, Kidd, et al. 2021). Humans have a limited T cell repertoire against SARS-CoV-2 epitopes, and thus it is possible that the appropriate peptides were not included in our peptide pools (Tarke, Sidney, Kidd, et al. 2021). Cross-reactive T cells may still contribute to reducing COVID-19 severity, but in our cohort, they were not associated with a recent eCoV infection.

Our observations suggest that antibody Fc effector function may play a critical role in the reduction of COVID-19 severity. Antibody Fc effector functions are important in the battle against other viruses and disease, including HIV and cancer (Thomas et al. 2021; Junttila et al. 2010). Most clinical versions of monoclonal antibodies are now designed to have afucosylated Fc regions, so they are more capable of mediating ADCC and ADCP activities. As work continues toward developing a pan-CoV vaccine or therapy, it will be important to understand how to elicit optimal FcR functionality.

CHAPTER FIVE: HETEROTYPIC IMMUNITY FROM PRIOR SARS-COV-2 INFECTION BUT NOT COVID-19 VACCINATION ASSOCIATES WITH LOWER ENDEMIC CORONAVIRUS INCIDENCE

Disclaimer: Portions of this dissertation were included in or adapted from:

Heterotypic immunity from prior SARS-CoV-2 infection but not COVID-19 vaccination associates with lower endemic coronavirus incidence. Bean DJ, Monroe J, Liang YM, Borberg E, Senussi Y, Swank Z, Chalise S, Walt D, Weinberg J, Sagar M. Sci Transl Med. 2024 Jun 12;16(751):eado7588. Doi: 10.1126/scitranslmed.ado7588. (Bean et al. 2024)

Background

Numerous SARS-CoV-2 vaccines, primarily targeting the spike protein, are highly effective in reducing the incidence of hospitalization and progression to severe disease after infection (Andrews, Tessier, et al. 2022). The SARS-CoV-2 vaccines or prior SARS-CoV-2 infection, however, are less likely to prevent breakthrough or re-infection especially with the most recent lineages, such as the Omicron variants and subvariants (Bean et al. 2022; Andrews, Stowe, et al. 2022). Numerous studies have demonstrated that antibodies generated either from previous vaccination, prior SARS-CoV-2 infection, or both have lower neutralization potency against Omicron subvariants (Rössler et al. 2022; Miller et al. 2023). The less potent nAbs potentially account for the

limited protection against subsequent infections among those with prior SARS-CoV-2 exposure by vaccination or infection.

Besides nAbs, cellular responses are also likely important in reducing the incidence of symptomatic infection and onset of severe disease (A. T. Tan et al. 2021). nAbs generally target the SARS-CoV-2 spike protein, whereas T cells respond to various peptides present across the entire SARS-CoV-2 coding genome (Grifoni et al. 2020; Tarke, Sidney, Kidd, et al. 2021). In general, spike protein-encoding sequences are more variable than other parts of the genome, both when comparing different SARS-CoV-2 variants and when comparing to other CoVs (Jungreis, Sealfon, and Kellis 2021). In addition, non-structural proteins, such as the viral RNA dependent RNA polymerase (nsp12) and viral helicase (nsp13), show greater genetic conservation among the different CoVs (Jungreis, Sealfon, and Kellis 2021). Furthermore, in contrast to nAbs, adaptive T cell responses show slower decay over time (C. Feng et al. 2021; Bilich et al. 2021). Thus, cellular responses generated by vaccination and infection may be especially important in protecting against the development of symptomatic disease by the SARS-CoV-2 variants and the other circulating CoVs.

Previous studies have demonstrated that individuals without prior SARS-CoV-2 exposure have pre-existing antibodies and cellular responses against SARS-CoV-2 spike, nucleocapsid, nsp12, nsp13, and other proteins (K. W. Ng et al. 2020; Le Bert et al. 2020). It has been speculated that these immune reactions may arise from prior eCoV infections. Studies from our group and others suggests that prior infection with eCoVs and the subsequent immune response can attenuate COVID-19 severity after SARS-CoV-

2 infection (Sagar et al. 2021; Kundu et al. 2022). These studies imply that an eCoV infection may generate heterotypic immunity against other CoV viral family members. Deciphering the immune basis for this potential heterologous immunity is important because of the ongoing threat of yet another coronavirus as the etiologic agent for a future pandemic.

In this study, we examined the effect of heterotypic immunity generated from previous SARS-CoV-2 antigen exposure, either by infection or COVID-19 vaccination. We find that prior SARS-CoV-2 infection, but not COVID-19 vaccination alone, associates with protection against subsequent symptomatic disease from the eCoVs. Non-CoV incidence was similar among those with different prior SARS-CoV-2 antigen exposure histories, implying the heterotypic immunity is specific to CoVs. This heterologous immunity may be mediated by $CD8^+$ T cell responses targeting non-structural proteins, such as nsp12 and nsp13, and not nAbs. Our observations have important implications for future pan-CoV vaccines and other disease prevention strategies.

Results

Individuals with prior SARS-CoV-2 infection have lower incidence of subsequent symptomatic eCoVs

We examined the incidence symptomatic eCoV and non-CoV infections in a retrospective cohort (Fig. 5.1A), and we analyzed *ex vivo* immune responses in a separate prospective cohort (Fig. 5.1B). We collected all eCoV and non-CoV infection instances documented on CRP-PCR tests among individuals who presented for clinical evaluation

during the study period of November 30, 2020, to October 8, 2021, at BMC (Fig. 5.1A). The CRP-PCR test detects the four eCoVs, SARS-CoV-2, and sixteen other common non-CoV respiratory pathogens. The follow-up time was a median of 170 days with an IQR of 156 days. All individuals were categorized into three groups based on their pre-CRP-PCR test history: 1) prior documented SARS-CoV-2 infection; 2) antecedent COVID-19 vaccination but no known SARS-CoV-2 infection; 3) no previous SARS-CoV-2 antigen exposure. There were 5,713 CRP-PCR results from 4,935 (median 1 test per person, range 1–8) different individuals. The majority of individuals in all three groups had 1 CRP-PCR test with an IQR from 1 to 1.

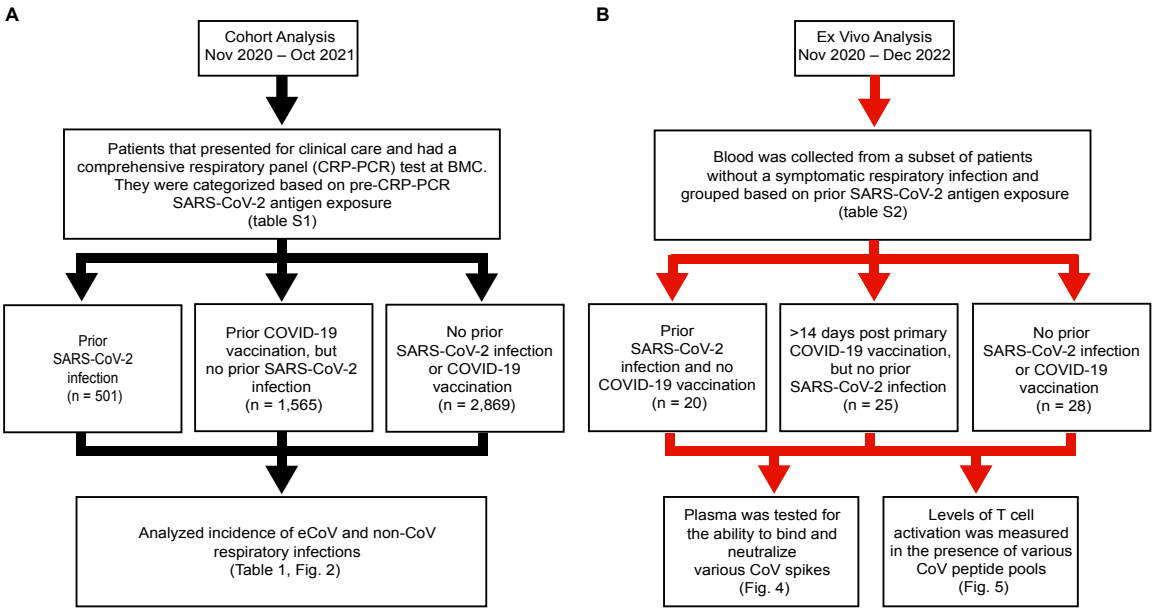


Figure 5.1. Overall study design used in the investigation. (A) Black arrows detail the grouping and analysis for the incidence of respiratory infections at Boston Medical Center (BMC). (B) Red arrows detail the recruitment and grouping of individuals for the *ex vivo* assessment of CoV specific immunity.

Early in the pandemic, testing and vaccination were limited, and SARS-CoV-2 infection was documented more often in and COVID-19 vaccines were preferentially given to older individuals with pre-existing comorbidities. Thus, as expected, the individuals with no prior SAR-CoV-2 antigen exposure were younger and healthier compared with the people in the other two groups (Table 2.7). In general, the differences in age, gender, and the proportion with pre-existing diagnoses was smaller among those with previous documented SARS-CoV-2 infection and those with prior COVID-19 vaccination but no known SARS-CoV-2 infection.

Among the 4,935 individuals, 617 had a non-SARS-CoV-2 respiratory pathogen detected on the CRP-PCR test at some time during the study period (Table 5.1). There were 103 eCoVs, and rhinovirus/enterovirus (n=409) was the most common non-CoV infection detected on the CRP-PCR tests (Table 5.1). Of the eCoV infections, 79 were HCoV-OC43, 19 were HCoV-229E, and 5 were HCoV-NL63. Individuals with prior SARS-CoV-2 infection had the lowest incidence of subsequent symptomatic eCoV as compared with the two other groups as judged by outcome alone (Table 5.1). Those with prior documented SARS-CoV-2 infection had lower incidence of alpha but not beta eCoVs. In contrast, non-CoV and enterovirus/rhinovirus detection was not different in the three groups. Time to event analysis also showed that eCoV ($p<0.0001$, log-rank test, Fig. 5.2A) but not non-CoV ($p=0.2823$, Fig. 5.2B) infections occurred less frequently among those with prior documented SARS-CoV-2 infections as compared with the individuals with COVID-19 vaccination or no known antigen exposure.

Table 5.1. Infections detected on CRP-PCR tests.

	Prior SARS-CoV-2 infection (n = 501)	Prior COVID-19 vaccine / No SARS-CoV-2 infection (n = 1,565)	No prior SARS-CoV-2 exposure (n = 2,869)	p-value[a]
Positive CRP-PCR [b]	51 (10.2)	226 (14.4)	340 (11.9)	0.0113
eCoV	5 (1.0)	45 (2.9)	53 (1.8)	0.0145
alpha-eCoV [c]	0 (0.0)	13 (0.8)	11 (0.4)	0.0316
beta-eCoV [d]	5 (1.0)	32 (2.0)	42 (1.5)	0.1777
eCoV – full vaccine [e]	3/209 (1.4) [f]	44/1463 (3.0)	N/A	0.2638 [h]
eCoV – partial vaccine [g]	0/17 (0.0)	1/102 (1.0)	N/A	0.9999 [h]
eCoV – no vaccine	2/275 (0.7)	N/A	53/2869 (1.8)	0.2301 [h]
Non-CoV	46 (9.2)	181 (11.6)	287 (10.0)	0.1688
Rhinovirus/Enterovirus	31 (6.2)	127 (8.1)	251 (8.7)	0.1518

[a] Chi-Square test unless noted otherwise, [b] For one of the twenty non-SARS-CoV-2 respiratory pathogens detected, [c] Includes HCoV-229E and HCoV-NL63, [d] Includes HCoV-OC43 and HCoV-HKU-1, [e] Received at least two doses of the Pfizer BioNTech BNT162b2 or Moderna mRNA-1273 or one dose of the Janssen / Johnson & Johnson Ad26.COV2.S COVID-19 vaccine a minimum of 14 days prior to the CRP-PCR test, [f] 2/124 (1.6%) – Moderna mRNA-1273; 1/66 (1.5%) – Pfizer BioNTech BNT162b2; and 0/19 (0%) - Janssen / Johnson & Johnson Ad26.COV2.S, [g] Received only one dose of the Pfizer BioNTech BNT162b2 (n = 43) or Moderna mRNA-1273 COVID-19 (n = 76) vaccine, [h] Fisher's exact test, N/A: not applicable

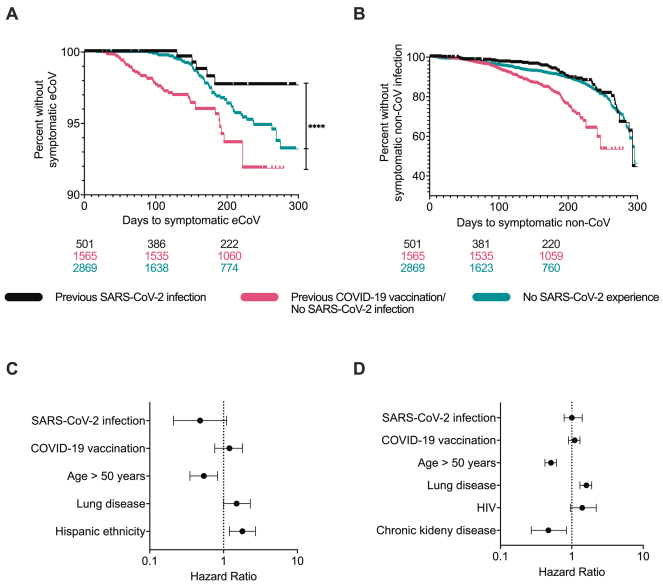


Figure 5.2. Previous SARS-CoV-2 infection is associated with lower incidence of symptomatic eCoV infections. (A and B) Shown are Kaplan Meier plots for eCoV (A)

and non-CoV (B) in those with no prior documented SARS-CoV-2 antigen exposure (teal), prior COVID-19 vaccination but no known SARS-CoV-2 infection (pink), and prior SARS-CoV-2 infection (black). Plots show unadjusted analyses with number of individuals at risk in each group at the bottom. Time to event analysis was performed using a log-rank test. (C and D) Shown are hazard ratios (HR) of eCoV (C) and non-CoV (D) incidence in time varying adjusted models. The figures show the covariates that had a p-value ≤ 0.1 in the multivariable Cox-proportional hazard model. No prior documented SARS-CoV-2 antigen exposure group is the reference category. The horizontal lines indicate the HR for each variable along with the 95% confidence interval, and the vertical dotted line indicates a HR of 1.0. **** represent p-values <0.0001.

Among those with prior COVID-19 vaccination but no documented SARS-CoV-2 infection, 1,463 of the 1,565 (93%) were deemed fully vaccinated because they had received the last dose of the recommended primary vaccine series at least fourteen days before the CRP-PCR result (Table 5.1). During the study period, individuals that had received two doses of the Pfizer BioNTech BNT162b2 or Moderna mRNA-1273 COVID-19 vaccine or one dose of the Janssen / Johnson & Johnson Ad26.COV2.S COVID-19 vaccine were considered to have completed the recommended primary vaccination series according to national guidelines. In these 1,463 individuals, there was no difference in eCoV incidence among those that got two doses of the Moderna mRNA-1273 (29/831, 3.5%), Pfizer BioNTech BNT162b2 (12/483, 2.5%) or one dose of the Janssen / Johnson & Johnson Ad26.COV2.S (3/149, 2.0%) COVID-19 vaccine (p=0.4448, Chi-square test). Among the 501 individuals with prior SARS-CoV-2 infection, 226 (45%) had at least one COVID-19 vaccine dose antecedent to the subsequent CRP-PCR test (Table 5.1). Thus, these individuals possibly had hybrid immunity associated with enhanced responses (Reynolds et al. 2021). Importantly, incidence of symptomatic eCoV infection was significantly lower in those with prior

SARS-CoV-2 infection and no vaccination (2 of 275, 0.7%) as compared with the individuals that had been deemed fully vaccinated but had no known prior SARS-CoV-2 infection (44 of 1,463, 3.0%, p=0.0245, Fisher's exact test, Table 5.1). One dose of the Janssen / Johnson & Johnson Ad26.COV2.S was often deemed to induce an inadequate immune response (Zeli Zhang et al. 2022). Even after excluding individuals with Janssen / Johnson & Johnson Ad26.COV2.S immunization, those with prior documented SARS-CoV-2 infection (5/482, 1.0%) had lower subsequent symptomatic eCoV disease as compared with those with prior mRNA based COVID vaccine (42/1416, 3.0%, p=0.0169, Fisher's exact test).

The prior results may lead to an incorrect conclusion that COVID-19 vaccination enhances disease likelihood from respiratory pathogens. The previous analyses did not account for the various differences among the three groups (Table 2.7). Thus, we conducted time-varying covariate analysis to account for the baseline demographics and because some individuals had SARS-CoV-2 infection or COVID-19 vaccination during and not prior to the study period. In these instances, the CRP-PCR test of interest also occurred after the exposure. Previous COVID-19 infection (HR)0.48, 95% CI 0.21-1.1, p=0.0810) associated with around 50% reduced risk of a future symptomatic eCoV (Fig. 5.2C), but it did not impact non-CoV incidence (Fig. 5.2D). On the other hand, COVID-19 vaccination showed no effect on the risk for eCoV or non-CoV infections. There was no statistically significant interaction between prior SARS-CoV-2 infection and antecedent COVID-19 vaccination in these models. Age greater than 50 years had significantly lower eCoV (HR 0.54, 95% CI 0.35, 0.83, p=0.0121) and non-CoV (HR

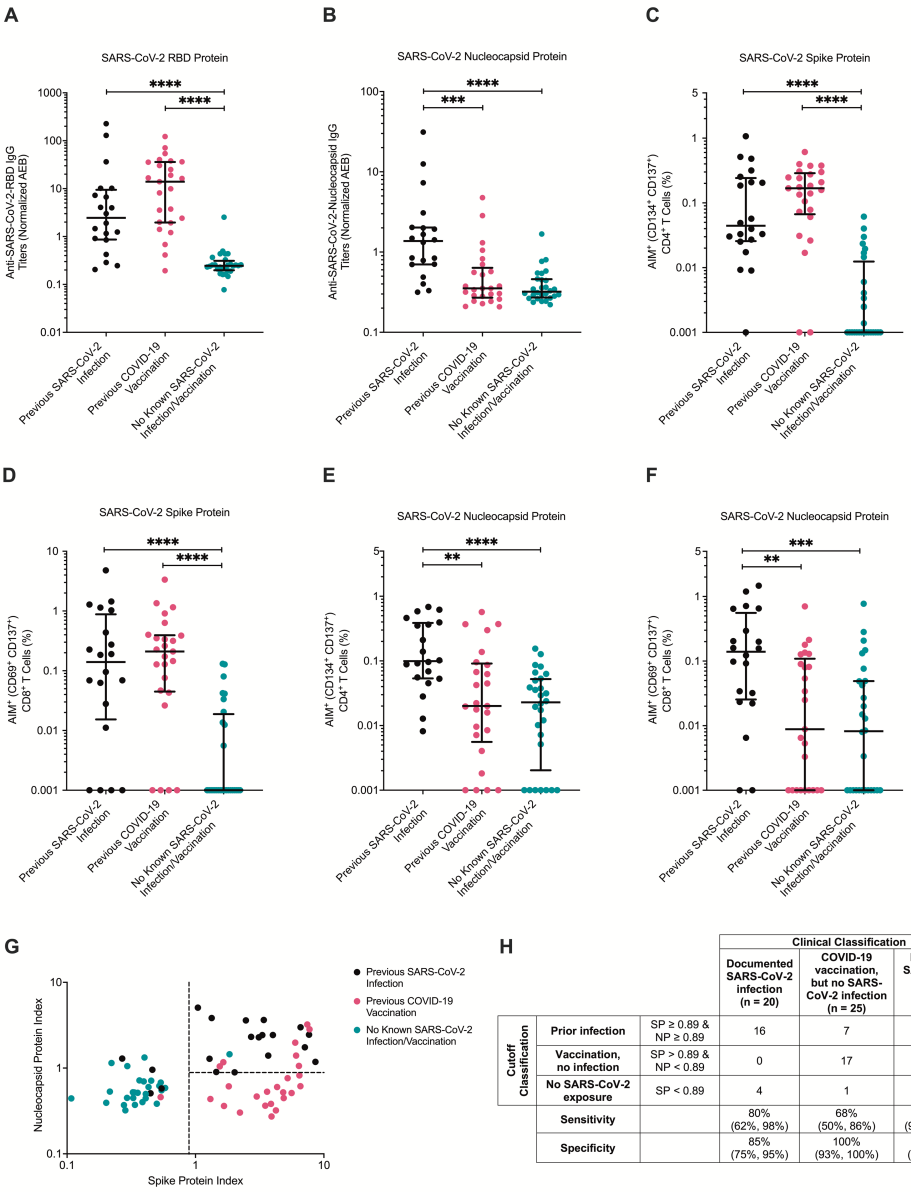
0.51, 95% CI 0.42-0.61, p<0.0001) incidence. The lower hazard ratio for both eCoV and non-CoV suggests decreased overall exposure or lower likelihood of developing symptomatic disease in this older age group. Those with preexisting lung disease, however, had higher risk for both eCoVs (HR 1.5, 95% CI 1.0-2.3, p=0.0419) and non-CoVs (HR 1.6, 95% CI 1.3-1.9, p<0.0001) infection. This may suggest that individuals with pre-existing lung disease have greater physiologic risk for developing symptomatic disease after exposure to respiratory pathogens.

Ex vivo immunological differences to SARS-CoV-2 antigens defines SARS-CoV-2 exposure groups

We collected blood samples from November 2020 to December 2022 to examine immune differences associated with the observed protection against symptomatic eCoVs. In contrast to the retrospective analysis, individuals in this independent cohort were grouped into three categories with stricter definitions (Fig. 5.1B). First, none of those with prior PCR documented SARS-CoV-2 infection had COVID-19 vaccination (n=20). Second, all the individuals in the COVID-19 vaccine group had received the second dose of either the Pfizer BioNTech BNT162b2 or Moderna mRNA-1273 COVID-19 vaccine at least 14 days prior to blood collection (n=25). Finally, individuals with no known SARS-CoV-2 antigen exposure (n=28) and COVID-19 vaccination alone were interviewed regarding prior suspected infection during the consent process before blood collection. We specifically did not choose pre-pandemic samples for the no antigen exposure group because eCoV circulation was substantially higher in the preceding years as compared with the year when COVID-19 was declared a public health emergency in

the city of Boston (Sinha et al. 2021). Thus, samples collected prior to as compared with after pandemic onset may have higher eCoV immune responses from greater community virus circulation. The three groups had relatively similar demographics other than age distribution (Table 2.8). Additionally, the duration between the last documented exposure to SARS-CoV-2 spike protein was shorter among the individuals in the COVID-19 vaccination as compared with the SARS-CoV-2 infection group.

Individuals classified as not having a previous SARS-CoV-2 infection may have had prior undiagnosed or asymptomatic COVID-19 (Kalish et al. 2021). Thus, there is a possibility of misclassification. We used comprehensive antibody- and T cell-based assessments to evaluate this possibility. First, we measured anti-SARS-CoV-2 RBD and nucleocapsid plasma IgG titers by the Simoa SARS-CoV-2 IgG antibody test (Norman et al. 2020). RBD antibody response has been demonstrated as highly indicative of SARS-CoV-2 spike protein exposure either through infection or vaccination (Indenbaum et al. 2020). As expected, individuals with prior SARS-CoV-2 spike protein exposure by infection or vaccination had higher RBD antibody titers compared with those with no known antigen exposure (Fig. 5.3A). Nucleocapsid IgG reactivity was used to further classify some COVID-19 vaccine recipients that may have had prior SARS-CoV-2 infection (Burbelo et al. 2020). Again, as expected, nucleocapsid IgG titers were higher in individuals with documented SARS-CoV-2 infection as compared with the two other groups (Fig. 5.3B).



H

			Clinical Classification				
			Documented SARS-CoV-2 infection (n = 20)	COVID-19 vaccination, but no SARS-CoV-2 infection (n = 25)	No known SARS-CoV-2 antigen exposure (n = 28)	Positive predictive value	Negative predictive value
Cutoff Classification	Prior infection	SP ≥ 0.89 & NP ≥ 0.89	16	7	1	67% (48%, 86%)	92% (80%, 98%)
	Vaccination, no infection	SP > 0.89 & NP < 0.89	0	17	0	100% (80%, 100%)	86% (77%, 95%)
	No SARS-CoV-2 exposure	SP < 0.89	4	1	27	84% (72%, 97%)	98% (93%, 100%)
	Sensitivity		80% (62%, 98%)	68% (50%, 86%)	96% (90%, 100%)	Overall Accuracy 82% (73%, 91%)	
	Specificity		85% (75%, 95%)	100% (93%, 100%)	89% (80%, 89%)		

Figure 5.3. *Ex vivo* immune responses to SARS-CoV-2 antigens differentiates individuals with different SARS-CoV-2 immune histories. (A to F) Shown are antibody (A and B) and cellular responses (C to F) against SARS-CoV-2 spike protein (A, D, E) and SARS-CoV-2 nucleocapsid (B, D, E) in those with no prior SARS-CoV-2 exposure (teal), prior COVID-19 vaccination but no SARS-CoV-2 infection (pink), and prior documented SARS-CoV-2 infection (black). (A and B) SARS-CoV-2 IgG was calculated based on the average enzyme per bead (AEB) against RBD (A) or nucleocapsid (B) measured using Simoa detection system. Statistical analyses were performed using a Kruskal-Wallis test with Dunn multiple comparison test. (C to F) Shown are results of AIM assays with SARS-CoV-2 spike protein (C and D) or SARS-

CoV-2 nucleocapsid (E and F) peptide pools. T cell activation was measured on $CD4^+$ ($CD134^+$ $CD137^+$) (C and E) and $CD8^+$ ($CD69^+$ $CD137^+$) (D and F) T cells by flow cytometry. Data were background subtracted against the negative control (DMSO only). The dark horizontal lines in each scatter dot plot denote the median and interquartile range. Statistical analyses were performed using a Kruskal-Wallis test with Dunn multiple comparison test. (G) The antibody and T cell responses against SARS-CoV-2 (A to F) were log transformed and combined to create a spike protein and nucleocapsid protein index. The dotted lines represent the cutoff values to best differentiate the groups. (H) Accuracy and 95% confidence intervals of clinical classifications based on SARS-CoV-2 spike protein (SP) and nucleocapsid protein (NP) immunity index values from panel H. **, ***, **** represent p-values <0.01, <0.001, <0.0001 respectively.

We also examined the number of activated T cells (both $CD4^+$ and $CD8^+$) after exposure to various SARS-CoV-2 peptide pools using the AIM assay (Fig. 2.1) (Dan et al. 2016; Reiss et al. 2017). As expected, higher percentages of activated $CD4^+$ ($CD134^+$ $CD137^+$, Fig. 5.3C) and $CD8^+$ ($CD69^+$ $CD137^+$, Fig. 5.3D) T cells were observed after SARS-CoV-2 spike peptide pool stimulation in individuals with either a previous SARS-CoV-2 infection or COVID-19 vaccination compared with those individuals without a known history of SARS-CoV-2 exposure. The activated SARS-CoV-2 spike protein-specific $CD4^+$ and $CD8^+$ T cell frequencies were similar between individuals with either a previous SARS-CoV-2 infection or COVID-19 vaccination. Also as expected, individuals with a previous documented SARS-CoV-2 infection had elevated SARS-CoV-2 nucleocapsid responsive $CD4^+$ (Fig. 5.3E) and $CD8^+$ (Fig. 5.3F) T cells compared with individuals with COVID-19 vaccination only or no history of SARS-CoV-2 antigen experience.

Log-transformed values from the three nucleocapsid (IgG, AIM $CD4^+$ and AIM $CD8^+$) and three spike protein (RBD, AIM $CD4^+$ and AIM $CD8^+$) measurements were

combined to generate a nucleocapsid and spike protein index. Spike protein and nucleocapsid cutoffs were chosen that best separated the three groups (Fig. 5.3G). These cutoffs yielded relatively similar test characteristics and overall accuracy (82%, 95% CI 73-91%, Fig. 5.3H) compared to another method (accuracy range 84 to 90%) that was based on examining SARS-CoV-2 cellular responses only (E. D. Yu et al. 2022). One and seven in the no antigen exposure and COVID-19 vaccine only group, respectively, clustered with the majority of individuals with prior documented SARS-CoV-2 infection based on the established cutoffs. Thus, these eight individuals may have had prior undocumented or asymptomatic SARS-CoV-2 infection. These potentially "misclassified" individuals are highlighted in the ensuing figures, and the subsequent analyses were conducted both including and excluding these people. Furthermore, four individuals with documented SARS-CoV-2 positive PCR results had either a low spike protein or nucleocapsid index. Two of these four had severe COVID-19 with admission to the ICU, whereas the other two were not hospitalized during their primary infection. Seven and nine of the remaining sixteen individuals with documented SARS-CoV-2 infection were not hospitalized and not admitted to an ICU floor during their disease course, respectively.

Humoral immune responses are unlikely to account for the decreased symptomatic eCoV incidence

We first investigated humoral immune responses because nAbs have been described as the correlate of protection for SARS-CoV-2 (C. Feng et al. 2021; Khoury et al. 2021). We measured the ability of patient plasma to neutralize pseudoviruses that

express the spike protein of one of three CoVs (SARS-CoV-2-Wuhan, HCoV-OC43, and HCoV-229E) (Fig. 5.4). Even though spike protein exposure was more recent in those with vaccination (Table 2.8), neutralization of the SARS-CoV-2 spike protein-based pseudovirus was similar in the individuals with a previous SARS-CoV-2 infection as compared with COVID-19 vaccination, even when seven vaccinated individuals with possible misclassification were excluded (Fig. 5.4A). Individuals with SARS-CoV-2 spike protein exposure either by infection or vaccination had higher SARS-CoV-2 nAbs as compared with the no SARS-CoV-2 antigen exposure group ($p=0.0439$, Fig. 5.4B), although this was not the case when the one "misclassified" person from the no antigen group was excluded ($p=0.0566$). Similar to previous reports, multiple individuals categorized into the no known SARS-CoV-2 spike protein exposure group had SARS-CoV-2 nAbs (K. W. Ng et al. 2020; Galipeau et al. 2021; Shrwani et al. 2021). These humoral responses may occur from a previous unknown SARS-CoV-2 infection or heterotypic responses from prior eCoV infections. It should be noted that SARS-CoV-2 RBD IgG binding titers weakly correlated with pseudovirus neutralization (Spearman $\rho=0.1810$, $p=0.03$). Weak association may have been observed because we only quantified SARS-CoV-2 RBD IgG binding antibodies. On the other hand, plasma contains both nAbs that target other spike protein domains, besides RBD, and non-IgG isotypes, which can also affect neutralization (Voss et al. 2021; Sterlin et al. 2021).

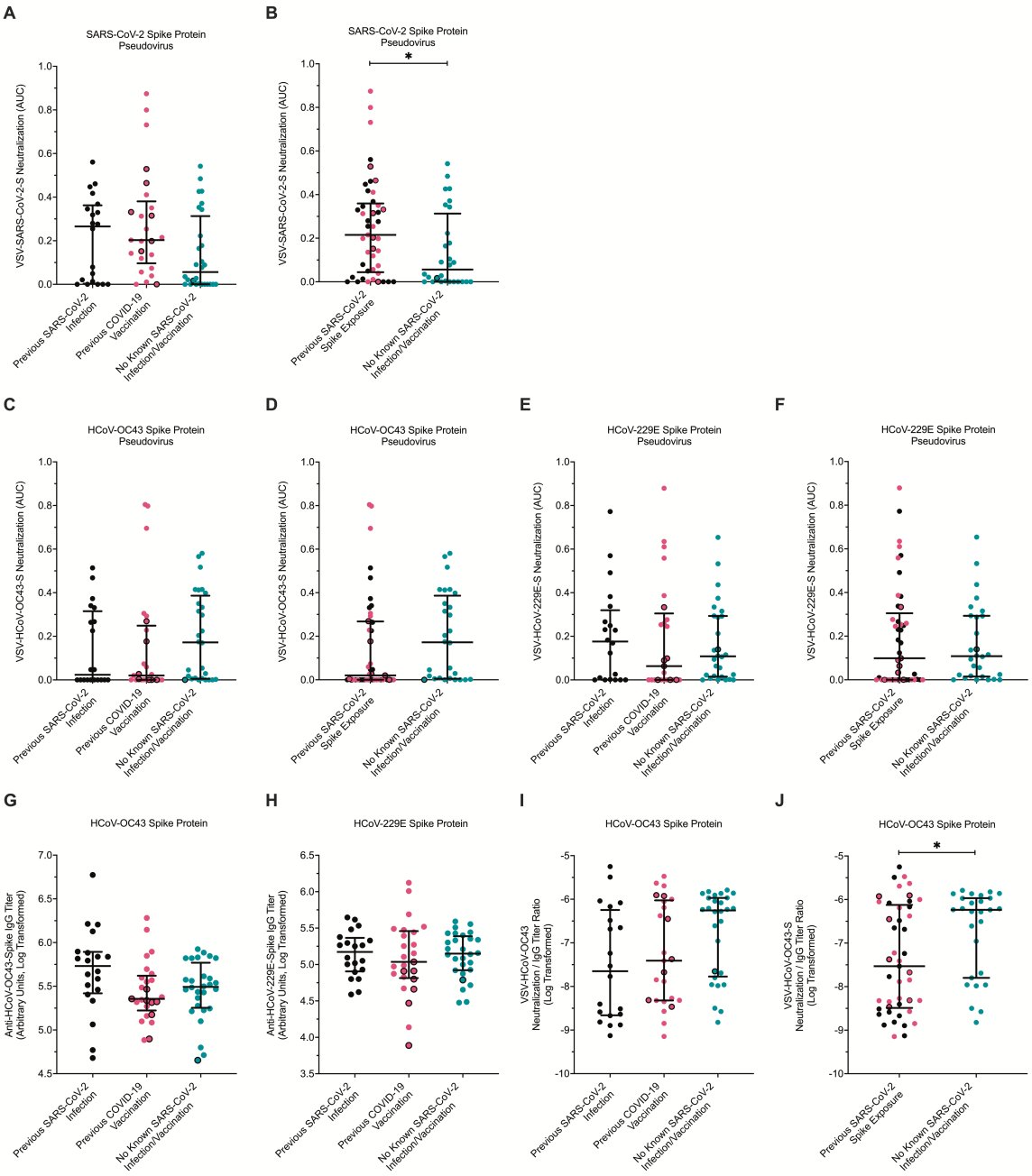


Figure 5.4. Prior SARS-CoV-2 spike protein exposure associates with more HCoV-OC43 non-neutralizing antibodies. Area under the neutralization curve (AUC) (A to F) and spike protein binding (G and H) antibody responses to various CoV spike proteins in those with no prior SARS-CoV-2 exposure (teal), prior COVID-19 vaccination but no SARS-CoV-2 infection (pink), and prior documented SARS-CoV-2 infection but no COVID-19 vaccination (black). (A and B) Neutralization responses against pseudoviruses expressing SARS-CoV-2 spike protein are shown for groups categorized

by type of exposure (infection versus vaccination) (A) or with any exposure combined (B). (C and D) Neutralization responses against pseudoviruses expressing HCoV-OC43 spike protein are shown for groups categorized by type of exposure (infection versus vaccination) (C) or with any exposure combined (D). (E and F) Neutralization responses against pseudoviruses expressing HCoV-229E spike protein are shown for groups categorized by type of exposure (infection versus vaccination) (E) or with any exposure combined (F). (G and H) HCoV-OC43 (G) and HCoV-229E (H) spike protein binding IgG antibody titers were measured by ELISA. (I and J) Ratios of HCoV-OC43 spike protein neutralization to binding antibodies are shown for groups categorized by type of exposure (infection versus vaccination) (I) or with any exposure combined (J). Black borders represent the eight individuals identified as potentially having prior asymptomatic SARS-CoV-2 infection. The dark horizontal lines denote the median and interquartile range. Statistical analyses were performed using either Kruskal-Wallis test with Dunn multiple comparison test (A, C, E, G, H, I) or Mann-Whitney U test (B, D, F, J). * represents p-values <0.05.

No differences in HCoV-OC43 neutralization were observed between the prior SARS-CoV-2 infection group compared to the COVID-19 vaccinated group with or without excluding the seven people with possible prior occult infection (Fig. 5.4C). Individuals with any SARS-CoV-2 spike protein exposure either by SARS-CoV-2 infection or COVID-19 vaccination had lower HCoV-OC43 neutralization responses as compared with the no SARS-CoV-2 antigen experience individuals, but only when excluding the one “misclassified” person from the no antigen group ($p=0.0407$ when excluding the one “misclassified” person from the no antigen group, otherwise $p=0.0645$, Fig. 5.4D). On the other hand, there were no differences in the ability to neutralize HCoV-229E spike protein-containing pseudoviruses between any of the three groups (Fig. 5.4E) or between those with as compared to without previous spike protein exposure (Fig. 5.4F).

A previous investigation demonstrated that SARS-CoV-2 infection enhances antibodies against the beta, but not alpha eCoVs (Cohen et al. 2021)**.** We observed that binding antibodies against HCoV-OC43 (Fig. 5.4G) and HCoV-229E (Fig. 5.4H) spike proteins as measured by ELISA were not different between the three groups. Though, in multi-variate linear regression analysis that accounted for age, gender, and comorbidities, prior SARS-CoV-2 infection associated with higher HCoV-OC43 binding antibody titers (Table 5.2). Individuals with no known prior SARS-CoV-2 antigen experience had the highest ratio of HCoV-OC43 neutralization relative to total binding antibodies (Fig. 5.4I). The ratio of neutralization relative to total binding antibodies was lower in those with any previous SARS-CoV-2 spike protein experience as compared with those with no spike protein antigen exposure (p=0.0473, p=0.0412 after excluding the one person with possible prior asymptomatic infection, Fig. 5.4J). These results possibly suggest that any SARS-CoV-2 spike protein exposure, either by infection or vaccination, enhances non-neutralizing binding antibodies more than nAbs against HCoV-OC43.

Table 5.2. Multi-variable linear regression analysis for predictors of HCoV-OC43 spike protein-binding antibodies.

Variable	Estimate (β)	95% confidence interval	p-value
Intercept	5.5604	5.3999 to 5.7217	<0.0001
Prior SARS-CoV-2 infection	0.2586	0.0545 to 0.4626	0.0140 [a]
HIV	-0.1613	-0.3867 to 0.0639	0.1580
Hypertension	-0.1343	-0.3197 to 0.0511	0.1530

[a] β=0.2261 and p=0.0340 after excluding eight with possible occult SARS-CoV-2 Infection

HCoV-OC43 non-structural protein directed cellular immune responses are higher among those with prior SARS-CoV-2 infection

We next examined differences in cellular immune responses among the different groups using the AIM assay. We focused our testing to HCoV-OC43 peptides because HCoV-OC43 was the primary eCoV circulating in Boston during the study period, and PBMC quantity limitations prevented testing against a large number of peptide pools. We also focused our study on spike protein, nucleocapsid, and nsp12/nsp13 T cell activity, which allowed us to compare spike protein, non-spike protein, structural protein, and non-structural protein responses. Similar HCoV-OC43-reactive $CD4^+$ and $CD8^+$ T cells were observed between the three groups when PBMCs were stimulated with HCoV-OC43 spike protein (Fig. 5.5A and B) and HCoV-OC43 nucleocapsid (Fig. 5.5C and D) peptide pools, even when the individuals with possible prior asymptomatic infection were excluded from the analysis. We next examined cellular responses to non-structural proteins nsp12 and nsp13, because pre-existing responses against these viral targets have been associated with lack of detectable SARS-CoV-2 infection after presumed exposure (Swadling et al. 2022). We generated an HCoV-OC43 nsp12/nsp13 peptide pool based on previously described SARS-CoV-2 nsp12 and nsp13 reactive T cell epitopes (Tarke, Sidney, Kidd, et al. 2021; Kundu et al. 2022; Saini et al. 2021; Kared et al. 2021; Ferretti et al. 2020; Mateus et al. 2020; Schulien et al. 2021; Nelde et al. 2021). Based on the Immune Epitope Database and Analysis Resource (IEDB) population coverage analysis tool, this combined set of epitopes with their known HLA interactions potentially covers 98.05% (HLA class I) and 99.3% (HLA class II) of the world population, and averages

7.82 (HLA class I) and 5.61 (HLA class II) epitopes per HLA combination (Bui et al. 2006). The defined SARS-CoV-2 epitopes were mapped to the HCoV-OC43 protein sequence, and HCoV-OC43 nsp12/nsp13 peptides were synthesized and pooled together (Table 2.9).

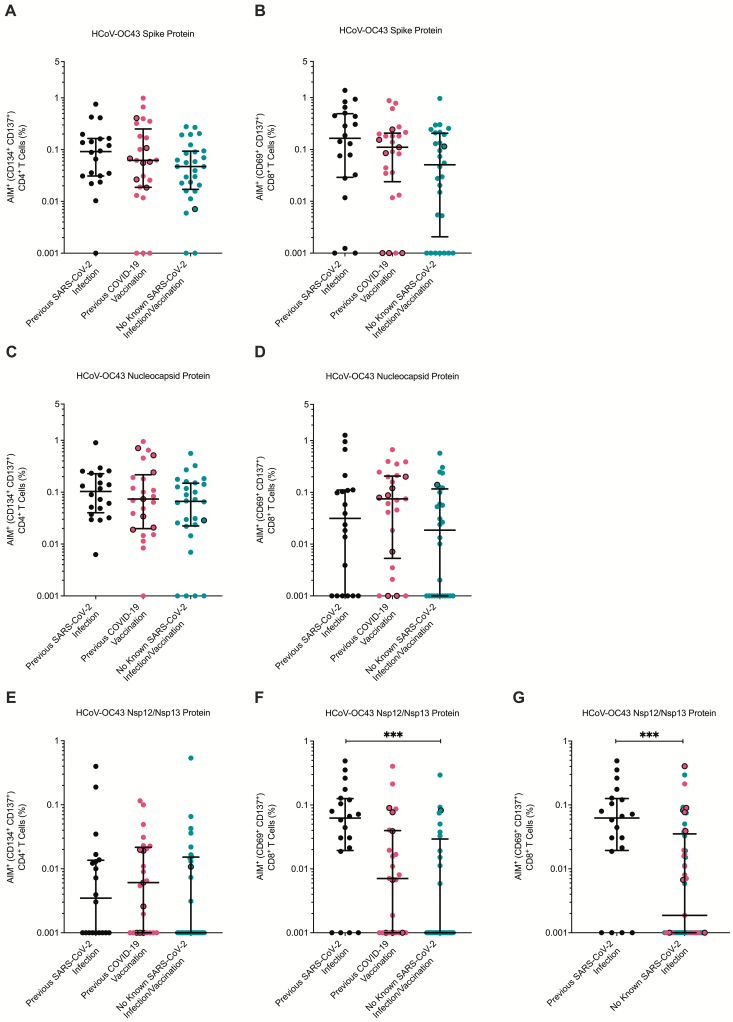


Figure 5.5. Prior SARS-CoV-2 infection associates with elevated CD8$^+$ T cell responses to HCoV-OC43 non-structural proteins. (A to G) The percent of activated CD4$^+$ and CD8$^+$ T cells was measured by flow cytometry in response to various HCoV-OC43 proteins in those with no prior SARS-CoV-2 exposure (teal), prior COVID-19 vaccination but no SARS-CoV-2 infection (pink), and prior documented SARS-CoV-2 infection (black). (A and B) Peripheral blood mononuclear cells (PBMC) were stimulated with peptides from HCoV-OC43 spike protein and CD4$^+$ (A) or CD8$^+$ (B) T cell

responses were measured. (C and D) PBMCs were stimulated with peptides from HCoV-OC43 nucleocapsid protein and $CD4^+$ (C) or $CD8^+$ (D) T cell responses were measured. (E and F) PBMCs were stimulated with peptides from HCoV-OC43 nsp12 and nsp13 proteins and $CD4^+$ (E) or $CD8^+$ (F) T cell responses were measured. (G) As in (F), but groups are categorized according to those with and without documented SARS-CoV-2 infection. Black borders represent the eight individuals identified as potentially having prior undocumented or asymptomatic SARS-CoV-2 infection. Data were background subtracted against the negative control (DMSO only). The dark horizontal lines in each scatter dot plot denote the median and interquartile range. Note, the y-axis varies among the different panels. Statistical analyses were performed using either Kruskal-Wallis test with Dunn multiple comparison test (A to F) or Mann-Whitney U test (G). *** represents p-values <0.001.

Although no difference was observed between $CD4^+$ T cells (Fig. 5.5E), HCoV-OC43 nsp12/nsp13 reactive $CD8^+$ T cells (Fig. 5.5F) were more frequent in individuals with a previous SARS-CoV-2 infection compared with individuals without a known history of SARS-CoV-2 antigen exposure (p=0.0018, p=0.0009 after excluding the one with possible "misclassification") or with a prior COVID-19 vaccination only (p=0.0693, p=0.0279 without the seven with possible prior occult infection). Individuals with prior SARS-CoV-2 infection had significantly higher $CD8^+$ T cell responses to HCoV-OC43 nsp12/nsp13 peptide pools as compared with a combined group containing those with COVID-19 vaccination and no antigen exposure (Fig. 5.5G, p=0.0009, p=0.0002 when excluding the eight with possible prior infection). Previous SARS-CoV-2 infection associated with 0.0714 higher percent of nsp12/nsp13-responsive $CD8^+$ T cells in multivariable linear regression analysis that accounted for baseline demographics and co-morbidities (Table 5.3). In this multi-variate model, age greater than 50 years was not a predictor for HCoV-OC43 nsp12/nsp13 $CD8^+$ T cell responses. This suggests that the lower incidence of both eCoVs and non-CoVs among those greater than 50 years (Fig.

5.2C and D) is unlikely due to higher pre-existing cellular responses. Importantly, no differences were observed between the groups in the $CD4^+$ or $CD8^+$ T cell responses to control peptide pools, human CMV pp65 or CEF (Fig. 5.6). Similar results were observed when stimulation index was used to calculate the relative T cell activation compared with the negative control condition (Fig. 5.7). This suggests the individuals in the groups did not have baseline differences in cellular reactivity.

Table 5.3. Multi-variable linear regression analysis for predictors of HCoV-OC43 nsp12/nsp13 $CD8^+$ T cell responses.

Variable	Estimate (β)	95% confidence interval	p-value
Intercept	-0.0544	-0.1490 to 0.0402	0.2555
Prior SARS-CoV-2 infection	0.0714	0.0223 to 0.1198	0.0040 [a]
Age > 50 years	0.00180	-0.0199 to 0.0742	0.2530

[a] β=0.0799 and p=0.0010 after excluding eight with possible occult SARS-CoV-2 infection

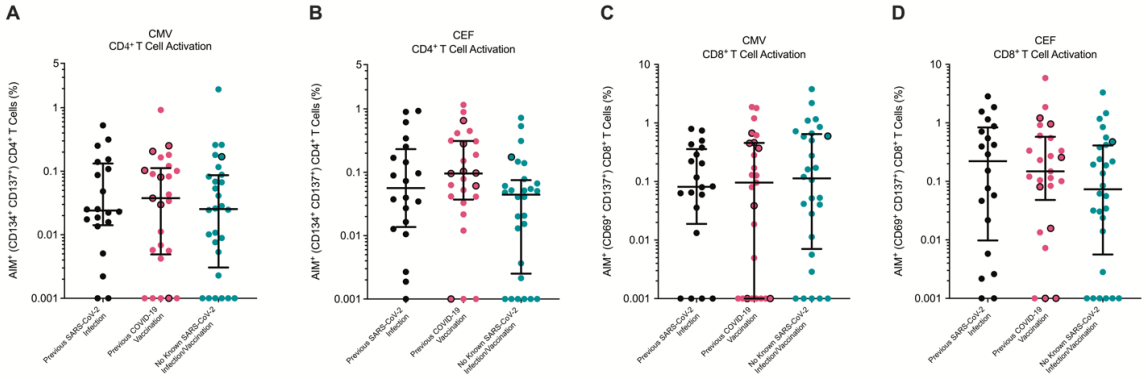


Figure 5.6. T cell responses to CMV and CEF peptide pools among those with different SARS-CoV-2 antigen exposure. (A to D) Percent of activated $CD4^+$ (A, B) and $CD8^+$ T cells (C, D) in response to CMV pp65 (A, C) or CEF(B, D) peptide pools in those with no prior SARS-CoV-2 exposure (teal), prior COVID-19 vaccination but no SARS-CoV-2 infection (pink), and prior documented SARS-CoV-2 infection (black). Black borders represent the eight individuals identified as potentially having prior undocumented or asymptomatic SARS-CoV-2 infection. Data were background subtracted against the negative control (DMSO only). The dark horizontal lines in each

scatter dot plot denote the median and interquartile range. Note, the y-axis varies among the different panels.

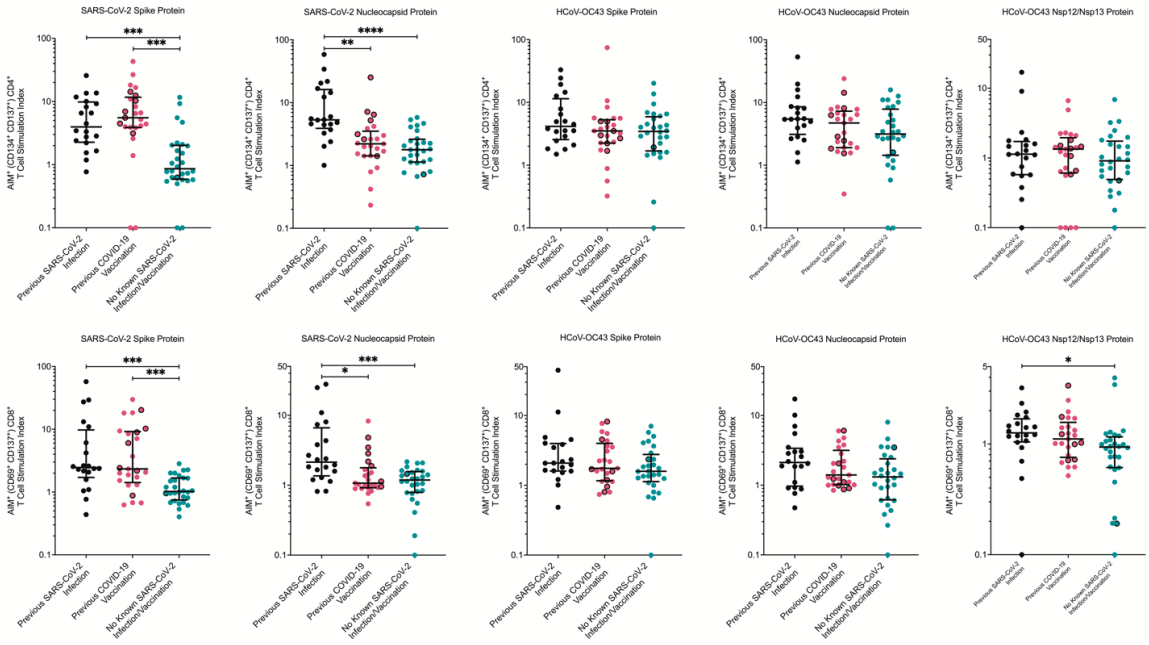


Figure 5.7. Alternative analysis of T cell responses to CoV-derived peptide pools among those with different SARS-CoV-2 antigen exposure. A calculation of relative $CD4^+$ (top row) and $CD8^+$ T (bottom row) cell responses to various CoV peptide pools in those with no prior SARS-CoV-2 exposure (teal), prior COVID-19 vaccination but no SARS-CoV-2 infection (pink), and prior documented SARS-CoV-2 infection (black). Black borders represent the eight individuals identified as potentially having prior undocumented or asymptomatic SARS-CoV-2 infection. The data is the same as in Fig. 5.3 and 5.5, but the percent of activated T cells for each experimental condition was divided by the negative control (DMSO only) condition to calculate a stimulation index. The dark horizontal lines in each scatter dot plot denote the median and interquartile range. Note, the y-axis varies among the different panels. Statistical analyses were performed using a Kruskal-Wallis and Dunn's multiple comparison test. *, **, ***, **** represent p-values <0.05, <0.01, <0.001, <0.0001, respectively.

Discussion

Some, but not all, prior studies have implied that previous eCoV immunity can provide protection against SARS-CoV-2 infection and COVID-19 morbidity (Kundu et al. 2022; Dugas et al. 2021; Abela et al. 2021). At BMC, which was the focus for the

epidemiological examination in this study, we have previously shown that recent documented eCoV infection was associated with less COVID-19 disease severity and morbidity. We previously reasoned that nAbs are unlikely to mediate this effect because there was no difference in SARS-CoV-2 incidence (Sagar et al. 2021). The biological mechanism for this observation remains uncertain, although numerous groups have suggested that prior eCoV infection can generate heterotypic humoral and cellular responses against SARS-CoV-2 (Le Bert et al. 2020; Song et al. 2021). Examining if SARS-CoV-2 infection or COVID-19 vaccination protects against a subsequent eCoV disease can provide further insights into the nature of heterotypic immune responses among CoVs. Although nearly all adults have preexisting eCoV immune responses, these "common cold" CoVs share less genetic similarity with SARS-CoV-2 as compared with the relatedness among different SARS-CoV-2 lineages. Thus, even with the prior complex immunological history, eCoVs as compared with the SARS-CoV-2 variants are a better, although not a perfect, surrogate for a possible future genetically distant novel CoV. The incidence of eCoVs after SARS-CoV-2 infection and COVID-19 vaccination provide insights into the prospects for preventing disease from another CoV outbreak. Furthermore, the immunological characterization can inform future pan-CoV vaccine design.

We found that individuals with a prior SARS-CoV-2 infection, but not COVID-19 vaccination alone, had a lower likelihood of having subsequent symptomatic eCoV disease, although statistically significant differences were only observed for the more distantly related alpha-CoVs. This suggests that SARS-CoV-2 infection, as compared

with COVID-19 vaccination, may provide greater protection against genetically disparate CoVs. Similar to our results, SARS-CoV-2 spike protein immunization alone provided minimal protection against subsequent HCoV-OC43 infection in mice (Dangi et al. 2021). In some respects, these animal results support our observations because COVID-19 vaccines predominantly only contain the SARS-CoV-2 spike antigen and no other viral protein. Along with spike protein-directed immunity, individuals with prior SARS-CoV-2 infection harbor additional immune responses against non-spike viral proteins, such as nucleocapsid, nsp12, and nsp13. The non-spike proteins are more conserved among the different CoVs, and thus the immune responses are likely cross-reactive (Westphal et al. 2023). The SARS-CoV-2 infection- and COVID-19 vaccine-generated heterotypic immunity, especially nAbs, may not be able to fully prevent a heterologous CoV infection. After initial infection, however, SARS-CoV-2 heterotypic non-spike protein-specific cellular responses could lower virus load and reduce the onset of symptomatic disease (Dagotto et al. 2022).

There are several strengths with our observational retrospective results. The data incorporates a relatively large number of individuals even though it was acquired from a single medical center. The large population size allowed us to adjust for various demographics that are likely important for symptomatic eCoVs, such as age, gender, and pre-existing morbidities (Cummings et al. 2021). Furthermore, we also analyzed non-CoV incidence as a control among the groups. Prior SARS-CoV-2 infection associated with lower subsequent eCoV incidence with all the statistical methods used to analyze the data. Importantly, in the time-varying multi-variable models that accounted for baseline

demographics and pre-existing comorbidities, non-CoV incidence was not different among those with prior SARS-CoV-2 infection or COVID-19 vaccination as compared with the no SARS-CoV-2 antigen experience group. This finding is important because it suggests that behaviors and unmeasured confounders, such as physician's reasons for testing and patient's health seeking behaviors, were likely similar in these groups. On the other hand, older age associated with lower eCoV infections. This likely reflects differences in exposure to respiratory pathogens because non-CoV incidence was also lower in the older age group. Conversely, the higher incidence of both eCoV and non-CoV incidence among those with pre-existing lung disease likely reflects physiological predisposition to developing symptomatic disease after exposure to a respiratory pathogen. The impact of SARS-CoV-2 infection as compared with COVID-19 vaccination on eCoV incidence should be examined in other cohorts also, although the widespread and often undocumented infection with the SARS-CoV-2 Omicron variant will make a prospective analysis extremely challenging now (Clarke et al. 2022).

There are also limitations with this retrospective observational data. First, the sample size was based on a specific time period, not statistical power, so lack of statistical significance may not imply lack of clinical relevance. There is a risk of misclassification because some individuals may have had asymptomatic COVID-19. In our *ex vivo* analysis, 8 of the 53 (15%) individuals classified as not having prior SARS-CoV-2 infection may have had prior occult disease (Fig. 5.3). Prior SARS-CoV-2 infection, however, would still be associated with lower eCoV incidence even with the worst-case assumption of non-differential misclassification of up to 10% of the

vaccinated and no previous antigen exposure group (Table 5.4). Analyses may also be subject to residual confounding and formal tests for statistical interaction may be underpowered. In addition, hazard ratios may be subject to selection bias. Our observational data includes symptomatic individuals that present for clinical care; it does not record possible asymptomatic infections. It remains uncertain if prior SARS-CoV-2 infection potentially also associates with lower incidence of asymptomatic eCoV infections.

Table 5.4. ECoV incidence difference assuming different misclassification rates.

	Prior SARS-CoV-2 infection	**Prior COVID-19 vaccine / No SARS-CoV-2 infection**	**No prior SARS-CoV-2 exposure**	**p-value [a]**
Observed data	5/501 (1.0)	45/1565 (2.9)	53/2869 (1.8)	0.0113
10% misclassification	14/944 (1.5)	41/1409 (2.9)	48/2582 (1.8)	0.0300
15% misclassification	20/1166 (1.7)	38/1330 (2.9)	45/2439 (1.8)	0.0690
20% misclassification	25/1418 (1.8)	36/1252 (2.9)	42/2295 (1.8)	0.0705

[a] Chi-Square test

We conducted *ex vivo* analyses to identify immune mechanisms for the observed protection against symptomatic eCoV infections. These *ex vivo* studies were restricted to individuals with more strict definitions for the different groups. Individuals in the three pre-specified groups were sampled during the same time-period because this assured that there was similar community eCoV exposure across the entire population. This, along with the emergence of both the highly infectious Omicron variant and widespread home

testing, limited sample sizes for these *ex vivo* analyses because we aimed to avoid individuals that may have had an undocumented SARS-CoV-2 infection. Importantly, we conducted numerous tests to identify misclassified individuals because it can be difficult to decipher previous asymptomatic and undocumented SARS-CoV-2 infections using single tests alone. We used six different independent measurements to identify misclassified individuals. In general, our methodology revealed similar results as other T cell-based methods to differentiate routes of SARS-CoV-2 spike protein exposure (E. D. Yu et al. 2022). It should be noted, however, that no assay or methodology can identify prior undocumented SARS-CoV-2 infection with complete certainty (Loesche et al. 2022).

Numerous previous studies have reported on the importance of nAbs in preventing CoV infections and ameliorating disease severity (S. Feng et al. 2021; Khoury et al. 2021). Indeed, nAbs are deemed as a correlate of protection against SARS-CoV-2, and as a corollary against CoVs in general (A. T. Huang et al. 2020). Animal models also suggest that cross-reactive nAbs can protect against heterologous CoV challenge if the two CoVs have high degrees of genetic conservation (Dangi et al. 2021). In our analysis, we found that nAbs against SARS-CoV-2, HCoV-OC43, and HCoV-229E were not different among those with prior SARS-CoV-2 infection or COVID-19 vaccination alone. Previous reports have suggested SARS-CoV-2 spike protein exposure boosts antibody titers against the beta-CoVs, such as HCoV-OC43, and not necessarily against the alpha-CoVs, such as HCoV-229E (Anderson et al. 2021). We also found that binding antibodies against HCoV-OC43 spike protein were increased after SARS-CoV-2 infection, though

we did not observe a boost in HCoV-OC43 antibodies in our COVID-19 vaccinated group. In contrast, we observed that HCoV-OC43 neutralization was generally lower in those with prior documented SARS-CoV-2 infection or COVID-19 vaccination as compared with those without any known SARS-CoV-2 antigen exposure. The lower HCoV-OC43 neutralizing to total antibody binding ratio in those with prior SARS-CoV-2 spike protein exposure suggests that SARS-CoV-2 spike protein preferentially induces antibodies that are not critical for HCoV-OC43 neutralization (Crowley et al. 2022). It should be noted that we did not assess mucosal antibodies or Fc-mediated antibody functions against HCoV-OC43 or HCoV-229E among the groups, and differences in these parameters may be important for protection against symptomatic eCoVs (Tang et al. 2022). Collectively, our studies suggest that pre-existing or de-novo generated plasma nAbs titers do not associate with the lower incidence of symptomatic eCoVs.

In contrast to nAbs, our *ex vivo* investigations imply that cellular responses are important in lowering the incidence of symptomatic eCoVs. Spike protein-based T cell immunity was similar between those individuals with either a previous SARS-CoV-2 infection and a previous COVID-19 vaccination, which agrees with previous work in the field (Hurme et al. 2022). In general, the T cell responses against HCoV-OC43 structural proteins were relatively common in all individuals, highlighting the ubiquity of prior eCoV infections (Grifoni et al. 2020). The primary difference between the individuals with a previous SARS-CoV-2 infection and those with a COVID-19 vaccination were in the elevated $CD8^+$ T cell responses to the non-structural antigens, nsp12 and nsp13. Memory T cell responses to these non-structural antigens have been implicated in the

protection against SARS-CoV-2 infections (Kundu et al. 2022; Swadling et al. 2022). Animal models have also implied that cellular responses against non-structural proteins are important for protection against infection and disease severity (Dagotto et al. 2022; Tai et al. 2023). Importantly, the non-structural regions of the different CoVs are generally more conserved, and thus cross-reactive responses are more likely preserved (Westphal et al. 2023; Nesterenko et al. 2021). The heterotypic boost of these cross-reactive T cells likely contributes to the killing of CoV infected cells and the clearance of the infection before the onset of symptomatic disease. In our *ex vivo* analysis, greater than 50 years of age did not associate with higher HCoV-OC43 nsp12 and nsp13 directed $CD8^+$ T cell responses (Table 5.3). This further argues that the protection against symptomatic eCoV observed in those greater than 50 years of age in the retrospective analysis potentially reflects lower exposure to respiratory pathogens rather than enhanced immunity from a greater number of prior HCoV-OC43 infections with increasing age (Chow, Uyeki, and Chu 2023).

There are limitations to our conclusions from the *ex vivo* analyses. Most importantly, our results demonstrate associations and do not prove causation. We chose to examine spike protein, nucleocapsid, and nsp12/nsp13 cellular responses only. Cellular responses against other structural and non-structural proteins are likely also important. Cell quantities limited the breadth of the cellular responses that we could examine, and we focused on those that have been deemed most important for disease prevention (Swadling et al. 2022; Hajnik et al. 2022). Additionally, we only tested cellular responses against HCoV-OC43. Although there is a relatively high degree of similarity between

alpha- and beta-CoV non-spike proteins, cellular responses against an alpha-CoV, such as HCoV-229E, should be tested to make our results more generalizable. We also did not incorporate COVID-19 disease parameters for the individuals with prior SARS-CoV-2 infection in our analyses. The magnitude of the immune responses differ based on COVID-19 disease severity, and associations between eCoV incidence and mild versus severe COVID-19 disease may further highlight the potentially protective immune responses (Dan et al. 2021). Documenting lower eCoV incidence among those with as compared to without pre-existing cellular responses against non-structural CoV proteins generated by vaccination or infection will be required to conclude biological mechanisms. These types of human studies will be exceedingly difficult because of the relatively low incidence of symptomatic, clinically recognized eCoV infections. Animal studies may also be challenging because there are not great non-human models to examine symptomatic eCoV disease after SARS-CoV-2 infection. Indeed, interferon-γ receptor-deleted transgenic mice expressing specific HLAs were recently used to document that cellular responses from prior HCoV-OC43 infection could mitigate SARS-CoV-2-induced disease (Dos Santos Alves et al. 2024). In these highly immunocompromised mice, however, HCoV-OC43 exposure generated immune responses but minimal to no disease. Future studies that examine cellular reactivity against SARS-CoV-2 nsp12/nsp13 among those with no previous SARS-CoV-2 infection and prior known eCoV infection history will also provide valuable insights into the heterotypic immunity observed in our study. Furthermore, examining nAbs, as has been done previously (Garcia-Beltran, Lam, et al. 2021; C.-W. Tan et al. 2021), and cellular

responses against truly novel CoVs that currently only exist in non-human species, such as bats, could also further highlight the extent of heterotypic immunity from SARS-CoV-2 infection and COVID-19 vaccination.

Prior SARS-CoV-2 infection associates with better protection against the heterologous eCoVs compared with spike protein-based COVID-19 vaccination alone. Even though acute COVID-19 severity has been greatly reduced with the widespread institution of COVID-19 vaccination and immunity from prior SARS-CoV-2 infections, our results should not be used to promote SARS-CoV-2 infections as a means to limit future heterologous CoV disease (Tenforde et al. 2021). SARS-CoV-2 infections continue to pose a substantial risk for developing long-term symptoms, termed Long COVID or PASC (Groff et al. 2021). Most efforts aimed at developing universal CoV vaccines are focused on either eliciting broadly nAbs and inducing T cell responses that target conserved domains in structural proteins shared among diverse CoVs (S. Su, Li, and Jiang 2022). Although these efforts should continue, we propose that the addition of non-structural antigens to future universal CoV vaccines may induce a more diverse T cell repertoire and recapitulate the heterotypic immune benefit of a natural infection.

CHAPTER VI: CONCLUDING REMARKS

Summary of Major Findings

The work presented in this dissertation discussed differences in immune responses after a CoV infection and associated these changes in immunity with protection against subsequent homologous and heterologous CoV infections and their corresponding diseases. In Chapter 3, I compared the longitudinal antibody responses of individuals with or without a clinically confirmed SARS-CoV-2 reinfection. Those individuals without a SARS-CoV-2 reinfection had significant increases in SARS-CoV-2-specific antibody IgG titers and neutralizing ability at later time points, but this change in the humoral immune response was not observed in individuals with a SARS-CoV-2 reinfection. Ultimately, there were no differences in SARS-CoV-2-specific antibody IgG levels and neutralization titers between the groups of individuals with or without a SARS-CoV-2 reinfection. Instead, I found higher rates of SARS-CoV-2 reinfection were associated with unstable housing of the individuals and that SARS-CoV-2 reinfection viruses had accumulated viral mutations over time. SARS-CoV-2 reinfection can still occur due to increased viral exposure and neutralization escape by viral variants despite the presence of an adequate SARS-CoV-2-specifc antibody response.

In Chapter 4, I measured a variety of antibody and T cell responses against SARS-CoV-2 spike antigens in SARS-CoV-2 naïve individuals. The SARS-CoV-2 naïve individuals were divided into those with or without a suspected recent eCoV infection, based on clinical PCR test results and eCoV nucleocapsid antibody levels. There were no differences in neutralizing antibody and T cell responses against SARS-CoV-2 between

those individuals with or without a recent eCoV infection. Elevated levels of SARS-CoV-2 spike S2 antibodies in individuals with a suspected recent eCoV infection had more effector function potential as more bound to the Fc receptor, FcγRIIIa (Illustration 6.1). These SARS-CoV-2 spike S2-directed antibody FcR binding responses correlated with eCoV spike-directed antibody FcR binding responses. The increase in cross-reactive antibody Fc effector function after an eCoV infection may contribute to the heterotypic protection against severe COVID-19.

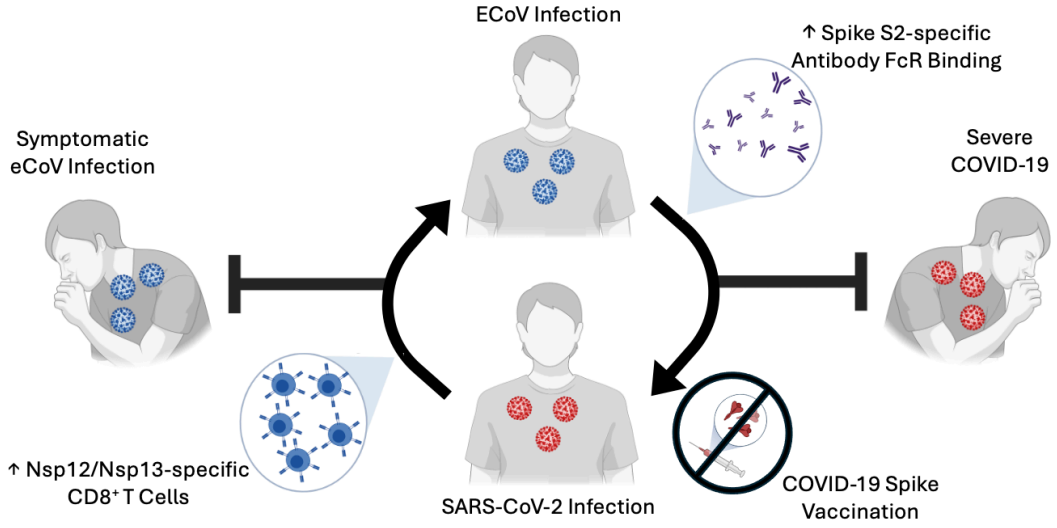


Illustration 6.1. Graphical summary of elevated heterotypic immune responses after CoV infections. After a recent eCoV infection, cross-reactive spike S2-directed antibodies with increased binding to FcγRIIIa are elevated, which could mediate increased Fc effector functions and prevent against severe COVID-19. After a SARS-CoV-2 infection, but not COVID-19 vaccination, cross-reactive nsp12/nsp13-directed $CD8^+$ T cells are increased, which could help protect against subsequent symptomatic eCoV infections.

In Chapter 5, I compared how SARS-CoV-2 immunity protects against subsequent symptomatic eCoV infections. I first compared the incidence rate of

symptomatic eCoV infections in those individuals with different types of SARS-CoV-2 exposure. Lower rates of symptomatic eCoV infections were identified in those individuals with a prior SARS-CoV-2 infection, but not those with a previous COVID-19 vaccination. I then measured a variety of antibody and T cell responses against eCoV antigens in individuals with different types of SARS-CoV-2 exposure. Individuals with a prior SARS-CoV-2 spike exposure had a lower percent of HCoV-OC43 neutralizing antibodies, but otherwise individuals with either a previous SARS-CoV-2 infection or COVID-19 vaccination had similar antibody responses to eCoV antigens. Individuals with either a previous SARS-CoV-2 infection or COVID-19 vaccination had similar T cell responses to eCoV structural proteins, spike and nucleocapsid. The key difference between the groups was that individuals with a previous SARS-CoV-2 infection had higher amounts of eCoV nsp12/nsp13-reactive $CD8^+$ T cells (Illustration 6.1). These elevated levels of nsp12/nsp13-reactive $CD8^+$ T cells after a SARS-CoV-2 infection may provide heterotypic protection against subsequent symptomatic CoV infections.

Discussion

Heterotypic immunity protects against CoV-related disease but not against CoV infections, which we observed in the context of both SARS-CoV-2 (Sagar et al. 2021) and eCoV infections (Bean et al. 2024). Our results suggest that nAbs are not the primary mediator of heterotypic immunity. Based on the data presented in this dissertation, antibodies elicited from a CoV infection and/or vaccination may provide some cross-reactive protection, but not neutralizing sterility against subsequent heterologous CoV infections. Cross-reactive nAbs are not elevated after either an eCoV or SARS-CoV-2

infection. Conversely, HCoV-OC43 nAbs are decreased after SARS-CoV-2 spike exposure, though there are no changes HCoV-229E nAbs. Although cross-reactive antibodies may be boosted upon CoV exposure, individuals predominantly still make new nAbs toward the newly infecting virus (Teng et al. 2024). Cross-reactive nAbs are difficult to elicit largely due to CoV neutralizing epitopes are located in less conserved areas of spike protein (Barnes et al. 2020). Neutralization responses are also quickly evaded by even more similarly related CoVs, as evidenced by our observation that SARS-CoV-2 reinfection occurs despite measurable antibody levels and neutralization responses. Since our SARS-CoV-2 reinfection study, the rise of SARS-CoV-2 VOCs and their associated escape mutations has further validated our conclusions that CoV-directed nAbs do not provide long-lasting protection from subsequent CoV infections (K. M. Hastie et al. 2021). Despite the short-term protection of CoV-directed nAbs, they do contribute by reducing the severity of subsequent CoV infections (Khoury et al. 2021). Eliciting nAbs and providing sterilizing protection against viral infections is the gold standard for vaccine efficacy, but as previously mentioned, this goal remains difficult against CoVs for long periods of time. Instead, pan-CoV vaccine efforts should focus on the more attainable goal of reducing severe CoV-related disease.

The reduction of viral loads is crucial to reduce the severity of COVID-19 and likely other CoV-related diseases (Fajnzylber et al. 2020). The transition from the innate immune response to the adaptive immune response is critical as a delay in adaptive immunity against SARS-CoV-2 correlates with COVID-19 severity (Bergamaschi et al. 2021; Lucas et al. 2021; M. Yu et al. 2023). Instead of the old model of COVID-19

severity (Illustration 1.8), I propose that heterotypic immunity can potentially overcome delays in the innate and *de novo* adaptive immune responses (Illustration 6.2).

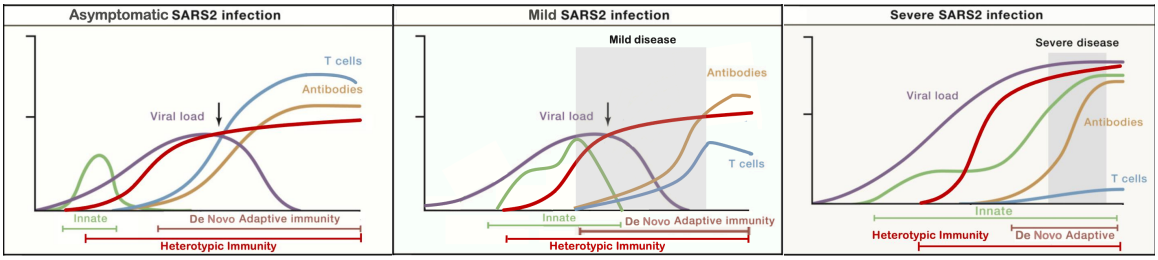


Illustration 6.2. Updated model of SARS-CoV-2 immunity and COVID-19 severity. Proposed model depicting the relationship between the timing of the heterotypic immune response to SARS-CoV-2 and COVID-19 severity. Early induction of the heterotypic immune response can limit viral loads and potentially overcome deficiencies in the innate and *de novo* adaptive immune responses. Uncontrolled viral loads and delays in the immune response will lead to increased disease severity. Image modeled after figure from (Sette and Crotty 2021).

Heterotypic immunity may not be as potent and specific as the eventual *de novo* immune response, but the cross-reactive immune response may reduce or at least maintain the viral load by limiting viral replication and clearing infected cells through T cell and antibody effector functions. The low virus levels may allow the body's adaptive immune system to generate new antibodies and T cells to further limit virus spread and disease severity. This initial heterotypic immune response may be able to overcome delays in either the innate or *de novo* adaptive immune responses. In the absence or delay of the cross-reactive immune response, high virus levels may induce an exuberant and inappropriate "cytokine storm" that prevents the timely generation of new adaptive immune responses, which eventually leads to increased disease severity. Understanding

the immune mechanisms mediating this initial cross-reactive protection is critical for the development of future pan-CoV vaccines.

My work in this dissertation suggests that heterotypic immune protection is not defined by a single immune mechanism, but through a combination of immune responses against conserved regions of CoVs. Cross-reactive nAbs likely contribute to this protection against CoV-related disease, but other immune mechanisms play more important roles. As suggested by the work in this dissertation and previously by others, a recent CoV infection produces more afucosylated antibodies with higher affinity to Fc receptors and more potential to mediate Fc effector functions (Larsen et al. 2021). These afucosylated non-neutralizing antibodies may mediate the killing of CoV-infected cells through mechanisms like ADCC. Elimination of these CoV-infected cells will lower viral loads and prevents an exaggerated and harmful immune response. In other fields, such as cancer research, most therapeutic monoclonal antibodies are manufactured to be afucosylated to enhance antibody Fc effector functions (Pereira et al. 2018). Whether more afucosylated antibodies is protective or detrimental to CoV-related disease severity remains a question, as antibody Fc effector functions can also lead to increased inflammation and viral infection enhancement (Y. Yu et al. 2021; Adeniji et al. 2021; Chakraborty et al. 2022).

Cross-reactive memory T cells also contribute to the clearance of virally infected cells, as well as coordinate the adaptive immune response. Although cross-reactive T cells are mostly reactive towards structural proteins, this work, along with previous reports, suggests T cell responses against the initially translated, non-structural proteins

may be critical in preventing CoV related disease symptoms (Swadling et al. 2022; Bean et al. 2024). Current HCoV vaccine efforts target the immunodominant structural protein, spike, but the addition of non-structural proteins to future vaccines would improve the breadth of T cell responses against heterologous CoVs. Animal studies have shown that vaccination against the RdRp can reduce the viral load in the mucosal tissue (Dagotto et al. 2022). Incorporation of non-structural proteins, such as the RdRp, to future vaccines could provide additional T cell help to reduce initial viral loads and disease severity. Antibodies with Fc effector functions and early T cell responses working together may be the recipe for a protective heterotypic immune response, but more work needs to be done to see if and how these immune responses work in concert to prevent severe CoV-related disease.

Significance

My work in this dissertation greatly expands on the concept of CoV-directed heterotypic immunity and highlights understudied immune mechanisms underlying this heterotypic immune protection. Most prior work has focused on eCoV-induced immunity in its protection against SARS-CoV-2, but we expand this concept and explore SARS-CoV-2-mediated protection against eCoVs. Prior studies have primarily focused on SARS-CoV-2 immune protection from heterologous CoVs using *in vitro* studies or animal models, but this work is one of the first examples to show the clinical relevance of SARS-CoV-2-mediated heterotypic immunity in humans. To our knowledge, we are the first to show differences in the protection against symptomatic eCoV infections between SARS-CoV-2 infection and COVID-19 vaccine elicited immunity.

Lastly, the HCoV research field is primarily focused on spike (mainly RBD) immunity, whether its nAbs or cytotoxic T cells. This spike-reactive immunity is commonly the primary indicator of an efficacious immune response to SARS-CoV-2 and other HCoVs. My work highlights the potential importance of more conserved immune targets, such as non-structural proteins, and also suggests a potential role of non-neutralizing antibody targets. Functional capacity of spike S2 directed non-neutralizing antibodies and T cell responses against non-structural proteins are rarely measured in most studies. My work will encourage future studies to explore additional immune targets and mechanisms as correlates of protection against CoV-related disease. Additionally, strategies for developing pan-CoV vaccines should also be reexamined based on my observations. The incorporation of non-structural proteins in future pan-CoV vaccines could improve the protection against severe disease from future HCoV infections.

Study Limitations

A major limitation of our work is the cohorts used throughout the studies. Although there is diversity amongst our cohorts and we account for demographic factors between the groups in each of our studies, the cohorts consist of individuals from the same geographical area. Our work should be repeated in additional cohorts from other geographical areas, ideally across the world, which would generalize the findings. Furthermore, it would document if the observed important immune responses are universally conserved. Additionally, the sample sizes of each of our cohorts is relatively small, especially in the analyses of the immunological assays. These small sample sizes are largely limited by the availability of patient samples, particularly PBMCs. PBMCs are

not always collected for each patient and the number of available cells may not be sufficient to run a range of different conditions in a variety of assays. The inclusion of more individuals would provide greater power to our analyses. Our cohorts are also complicated by our definitions of previous CoV exposures. Our studies relied on differentiating individuals based on their prior history to CoV infections, but there is currently no assay available to definitively identify recent CoV infections. Many eCoV and SARS-CoV-2 infections are asymptomatic and the resulting CoV-directed immune responses vary depending on the severity and time since the infection, which makes detection of recent CoV infections exceedingly difficult. Individuals may have been misclassified in our cohorts, especially with our classification of recent eCoV infections, which could affect our results and interpretations.

The primary limitation of our studies is that we only identify associations and correlates of immune protection against CoV-related disease. These correlates of immune protection only offer potential immune mechanisms of protection, but they do not imply causation. The important immune components to heterotypic immunity we identify in these studies may not provide sufficient protection against CoV-related disease. Other immune factors, which we did not assess or sufficiently measure, may be more important in the protection against CoV related disease. Directly testing our proposed mechanisms of immune protection, *in vivo*, will provide clarity on their efficacy and clinical significance.

Future Directions

The associations between antibody Fc effector functions or non-structural protein directed T cell responses with heterotypic protection against CoV-related disease can first be tested *in vivo* using animal models. Animal models offer a convenient opportunity to directly test mechanisms of immune protection against COVID-19 and other CoV-related diseases (H. Chu, Chan, and Yuen 2022). Follow-up studies to my work would likely use a mouse model because of their adaptability and relative low-cost. Transgenic mice, such as the commonly used K18-hACE2 mice, are often used for CoV infection studies, but they poorly represent the range of disease severity observed with HCoV infections in humans (McCray et al. 2007). The primary endpoint of my future *in vivo* studies relies on differences in disease severity, which makes it difficult to use these transgenic mice. Additionally, cross-reactive CoV protection studies will be difficult to conduct in transgenic mice unless all known HCoV receptors are appropriately expressed. Humanized mouse models, especially with human lung xenografts, offer a more realistic model to study HCoV infections with the potential to better represent HCoV-related disease (Kenney et al. 2022). CoV-related disease will vary amongst these different mouse models, so clearly defining these disease manifestations will be critical for determining the efficacy of various treatments. Careful consideration of the specific mouse model will need to be addressed before future *in vivo* studies are conducted.

Initial tests for the efficacy of antibody Fc effector function in reducing CoV-related disease will first test infusion of monoclonal antibodies in the humanized mice (R. E. Chen et al. 2021). Ideally the mouse model should have functional human NK cells

and macrophages to engage in ADCC and ADCP activity (Kenney et al. 2022). These monoclonal antibodies will require varying levels of fucosylation (Šuštić et al. 2022). By varying the level of antibody fucosylation, we can directly compare the role of fucosylation on monoclonal antibody efficacy and induction of antibody-dependent Fc effector functions. These monoclonal clonal antibodies can be designed to target both neutralizing and non-neutralizing epitopes in variable and conserved regions of spike. After monoclonal antibody infusion, the mice can be challenged with different heterologous CoVs with varying levels of genetic similarity (Dangi et al. 2021). Alternatively, the mice can be either infected with or vaccinated against one CoV and then subsequently challenged with a heterologous CoV (Illustration 6.3). The mechanisms that control antibody fucosylation in response to viral antigens is not completely understood. Enveloped viruses induce more afucosylated antibodies compared to antibodies produced against purified proteins or non-enveloped viruses (Larsen et al. 2021). The method of immunization can potentially change the level of antibody afucosylation. These varying levels of antibody afucosylation can be used to stratify mice and compare levels of protection against severe CoV-related disease.

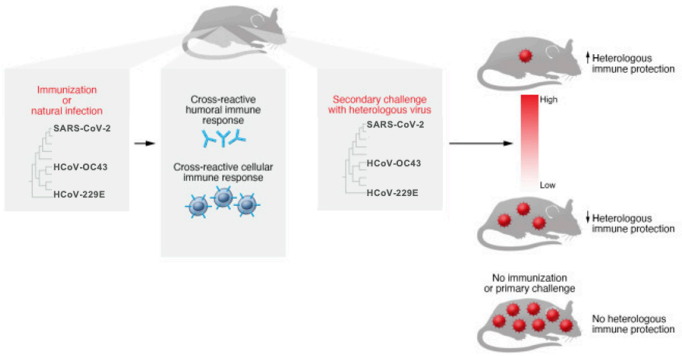


Illustration 6.3. Design of future mouse experiments to study CoV heterotypic immunity. Transgenic and/or humanized mice will be either be infected or vaccinated

against one HCoV and then cross-reactive immune responses can be measured. The mice will then be challenged against a heterologous CoV. If there is a high degree of heterotypic immune protection from the initial infection or vaccination, the subsequent CoV infection will be associated with lower viral loads and less severe CoV-related disease. If there is suboptimal protection from heterotypic immunity, the subsequent CoV infection will result in higher viral loads and more severe disease symptoms. Image modeled after figure from (Bean and Sagar 2021).

Similar immunization strategies can be used to induce T cells against the non-structural proteins nsp12 and nsp13 (Dagotto et al. 2022). Mice can be vaccinated with either intact viral proteins or individual peptide targets and then subsequently challenged with a heterologous CoV (dos Santos Alves et al. 2024) (Illustration 6.3). To maintain human relevance, human HLAs will need to be expressed in the mice, and this will require careful consideration based on their coverage of and frequency in the human population (Bui et al. 2006). Individual peptide-based vaccines may be HLA dependent and would require testing the frequency of T cell responses to individual peptides, rather than a peptide pool. Alternatively, nsp12/nsp13 reactive T cells can be elicited, isolated, and then adoptively transferred to mice before heterologous CoV challenge. Disease associated with CoV infections in mice is highly variable and depends on the mouse model and infecting viral strains. Defining clinical manifestations and differences in CoV-related disease in the mice will require measurements of tissue damage and viral loads within the mucosal and lung tissue of the mice. These proposed mouse experiments could help elucidate the level of heterotypic immune protection offered by nsp12/nsp13 reactive T cells.

Depending on the success of the previous mouse experiments, the definitive experiment would be to test the proposed non-structural protein vaccine in humans. The non-structural protein vaccine targeting SARS-CoV-2 or eCoV nsp12/nsp13 would be administered to human volunteers and the vaccinated individuals would undergo repeated tests for SARS-CoV-2 and eCoV infections by CRP-PCR. Clinical assessment of the individuals would determine difference in asymptomatic and symptomatic infections. The non-structural protein vaccine could also be administered in combination with recent SARS-CoV-2 spike booster vaccines to determine if there is an additive or prolonged effect on the protection against CoV-related disease compared to the current recommended vaccine regimen. Alternatively instead of the longitudinal cohort study, especially since eCoV infections are seasonal and relatively uncommon outside of infection surges and outbreaks, human volunteers can be immunized with the non-structural protein vaccine, challenged with either SARS-CoV-2 or one of the eCoVs, and then monitored for clinical symptoms and viral and immune kinetics (Killingley et al. 2022; Callow et al. 1990; Lindeboom et al. 2024). Safety of the human volunteers in these proposed studies would be of utmost importance.

My work demonstrates the potential importance of heterotypic immunity in the prevention of CoV-related disease. Future pan-CoV vaccines should consider the addition of non-structural antigens to elicit broader CoV protection. Similar work and vaccination strategies should be considered in other viral systems as well, such as influenza, to address the role of both non-neutralizing antibody and non-structural protein T cell immunity in heterotypic immune protection. These proposed vaccines may not prevent

viral infection, but they could reduce viral loads and limit disease severity. As we prepare for the emergence of future HCoVs, pan-CoV vaccines and therapies will have to consider the importance of immune mechanisms beyond neutralizing antibodies.

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