EXPOSURE-, INFECTION, AND VACCINE-INDUCED IMMUNE RESPONSES TO SARS-COV-2

Thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

Immunology & Infectious Diseases

Division of BioMedical Sciences, Faculty of Medicine

Memorial University of Newfoundland and Labrador

St. John's, Newfoundland and Labrador

May 2024

Abstract

There is tremendous diversity in the way individuals experience severe acute respiratory coronavirus 2 (SARS-CoV-2) infection. We analyzed immune responses to SARS-CoV-2 in the context of susceptibility to infection before and after vaccination. Within a cohort of subjects who were highly exposed to SARS-CoV-2 prior to vaccination and seronegative against the immunodominant spike (S) protein, there was evidence of cross-reactive immunoglobulin (Ig) G to nucleocapsid (N) from common β-coronavirus exposure and specific cellular immune responses against SARS-CoV-2 envelope (E), membrane (Mem), N, and S proteins. There was no evidence of underlying innate protection or natural immunity against SARS-CoV-2. Considering that the strength of the cellular immune responses correlated with time since exposure, we speculated that either rapid cellular immune responses or abortive infection resulted in infection being contained below the threshold for direct viral detection or generation of a humoral immune response.

We analyzed the characteristics and significance of circulating vaccine-induced IgA in a post-vaccination cohort with a relatively high incidence of breakthrough infection. Higher levels of vaccine-induced IgA were negatively associated with breakthrough infection. Breakthrough Omicron infection increased anti-ancestral S IgA responses more than booster vaccines. Longitudinal analysis of post-infection anti-S IgA decay showcased the durability of infection-induced responses. As reported for IgG responses, vaccination with ancestral SARS-CoV-2 S antigen-imposed imprinting on circulating IgA responses. This research addressees the variability in humoral and cellular immune responses to

SARS-CoV-2 and illustrates how the timing and nature of exposure to viral antigens impact the responses generated.

General Summary

The immune response is crucial for defending the body against viruses and other invading pathogens. The introduction of SARS-CoV-2 to the human population and the variability in exposure to viral antigens, either through vaccination, infection, or close contact without overt infection, create unique contexts in which to study viral immunity. This thesis explores aspects of susceptibility to SARS-CoV-2 infection and the underlying cellular and humoral immune responses before and after vaccination and infection. In distinct cohorts, some unvaccinated subjects appeared to clear SARS-CoV-2 infection before displaying overt symptoms of infection in the absence of vaccination. In another distinct cohort, vaccination against SARS-CoV-2 induced circulating IgA responses that could potentially be used as a marker to gauge susceptibility to breakthrough infection. Ongoing research regarding the immunological impacts of SARS-CoV-2 infection and vaccination will help to guide public health practices and further understanding of viral humoral and cellular immune responses.

Acknowledgments

First and foremost, thank you Dr. Michael Grant. It meant a great deal to be part of your lab and to have had your guidance. Throughout the past two years, your unwavering patience, profound knowledge, and steadfast support have been truly unparalleled. To my incredible colleagues in the Grant Lab; Danielle, Kayla, Keeley, Nazanin, Katie, Debbie, & David, thank you for your friendship, motivation, and the laughs. Thanks especially to Kayla for your invaluable help with laboratory techniques and experiment designs, and to Danielle for your understanding, compassion, and knack for creating joy every day.

I would like to thank my committee members, Dr. Deepak Kaushik and Dr. Rodney Russell, for their direction in helping me to become a better scientist, and Dr. Kensuke Hirasawa for challenging me to be a better leader and student. Thank you to the funding providers, Memorial University School of Graduate Studies and Research and Graduate Studies, the COVID Immunity Task Force and the Canadian Institute for Health Research.

I am immensely thankful for the love, support, and encouragement of family. Extraordinary friends make anything possible, and I am immeasurably grateful for Keeley, Rochelle, Danielle, Noah, Diana, Maddy, Cass, Bryn, and Jenna, and, of course, my favourite gal, Mabel. Joshua, your patience and optimism made it possible for me to pursue my dreams. Thank you for moving across the country (twice!) with me, I hope you know how appreciative I am to have you by my side.

I feel incredibly grateful and privileged to be part of the scientific community and know that I will forever be in awe of the passionate scientists around me.

This thesis is dedicated to my mother, Daniela, and my sisters Carolyn & Gina.

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List of Symbols & Abbreviations

©	Copyright
#	Number
%	Percent
®	Registered trademark symbol
≤	Less than, or equal to
≥	Greater than or equal to
◦**C**	Degrees Celsius
α	alpha
α-CD	anti-cluster of differentiation
α-IFN- γ	anti-interferon gamma
β	Beta
µL	Microlitre
µm	Micrometer
aa	Amino acid
Ab	Antibody(ies)
ACD	Acid-citrate-dextrose
ACE2	Angiotensin-converting enzyme 2
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
B.1.1.7	SARS-CoV-2 Alpha variant
BA.1	SARS-CoV-2 Omicron subvariant

BNT162b2	Pfizer BioNTech COVID-19 mRNA vaccine
BSA	Bovine serum albumin
CBC	Canadian broadcasting corporation
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
ChAdOx1	AstraZeneca SARS-CoV-2 vaccine
CO_2	Carbon dioxide
COVID-19	Coronavirus disease of 2019
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide
E	Envelope
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
F	Female
FBS	Fetal bovine serum
FLS	Full length spike
FSC	Forward scatter
H_2O	Water
H_2SO_4	Sulfuric acid
HCV	Hepatitis C virus
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSV-1	Herpes simplex-1
IFN-γ	Interferon gamma
IFN-α	Interferon alpha
Ig	Immunoglobulin
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-7	Interleukin 7
IQR	Interquartile range
IU	International units
LCMV	Lymphocytic choriomeningitis virus
LM	Lymphocyte medium
M	Molar
M	Male
Mem	Membrane
MHC I	Major histocompatibility complex class one
MHC II	Major histocompatibility complex class two
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
mRNA-1273	Moderna COVID-19 mRNA vaccine

N	Nucleocapsid
NaN_3	Sodium azide
ng	Nanogram
NK	Natural Killer
NL	Newfoundland and Labrador
nm	Nanometre
OD	Optical density
p	Probability
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
pH	Potential hydrogen
r	Correlation coefficient
RBD	Receptor binding domain
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
S	Spike
SA	Streptavidin
SARS-CoV-1	Severe-acute respiratory syndrome coronavirus 1
SARS-CoV-2	Severe-acute respiratory syndrome coronavirus 2
SD	Standard deviation
SIV	Simian immunodeficiency virus

SSC	Side scatter
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{H}	Helper T cell
™	Trademark
TMB	3,3’,5,5’-tetramethylbenzidine
T_{reg}	Regulatory T cell
T_{RM}	Tissue resident T cell
V	Variable region
WHO	World Health Organization
Wuhan-Hu-1	Ancestral strain of SARS-CoV-2

Chapter 1: Introduction and Overview

1.1 Immune Response

The immune response arises from a complex system of components that work together to defend the body from invading pathogens such as viruses, bacteria, parasites, and fungi. A functioning immune system is composed of many elements, but essential features underlying effective immunity include distinguishing between self and non-self-antigens, recognizing and responding to pathogens, establishing memory, and imposing appropriate regulation [1]. The human immune response derives from two linked systems, innate and adaptive. This thesis focuses on the adaptive immune response following exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens through exposure, infection, and/or vaccination.

1.2 Adaptive Immune Response & SARS-CoV-2

The adaptive immune response provides highly specialized defense against invading pathogens. Immunological memory that develops following antigenic exposure, clonal expansion, and cellular differentiation rests in retention of specialized cells that will rapidly respond to the same antigens upon subsequent exposure. This phenomenon can be exploited in development and administration of non-replicating vaccines to protect populations from severe infection by providing antigen exposure without the risk of infection. SARS-CoV-2 – a positive sense, single-stranded, enveloped RNA virus – was introduced to the human population in December 2019, and spread rapidly and widely. By March 2020, the World Health Organization (WHO) declared its associated disease, coronavirus disease (COVID-

19), a pandemic. As a respiratory virus, SARS-CoV-2 enters the body through respiratory droplets or aerosols in the upper respiratory tract. The S1 viral spike (S) protein subunit binds to host surface receptor angiotensin-converting enzyme 2 (ACE2), and following cleavage by host protease, the S2 viral protein subunit mediates fusion of the viral envelope with the host cell membrane. The virus then utilizes host machinery to produce and release progeny virus to infect neighbouring cells [2]–[4].

1.2.1 Vaccination

Vaccines provide controlled exposure to an antigen to stimulate an immune response. This allows adaptive immune responses to occur without infection, thereby playing a crucial role in protecting against diseases. Vaccine antigens are taken up and bound to B cells, or other antigen-presenting cells (APC), and presented to helper T cells, which in turn interact with B cells to drive their differentiation into plasma or memory B cells. Memory B cells are the basis of long-term humoral vaccine protection, allowing the individual to generate a rapid humoral response to subsequent antigen exposure, which can provide protection from infection or protection from illness if infection does occur [1]. Vaccines have shaped human history. Consider, for example, eradication of smallpox by vaccinia virus inoculation and establishment of annual flu shot recommendations after the 1918 Influenza Pandemic. The unprecedented global scientific effort to develop vaccines against SARS-CoV-2 highlights their pivotal role in combating infectious diseases. Varying vaccine types – for example, mRNA, viral vector, attenuated pathogen, or recombinant protein – will stimulate the production of specific antibodies and/or cell-mediated adaptive immune responses to aid in protection from infection.

1.2.2 Antibody Response

Antibodies (Ab), also called immunoglobulins (Ig), are a crucial part of the adaptive immune response, aiding in protection from pathogens through neutralization, opsonization, activation of complement, and triggering antibody-dependent cell-mediated cytotoxicity (ADCC). All classes of Ig are assembled from two basic subunits: heavy and light chains (Figure 1.1a). These chains can be further divided into two domains, variable and constant, which are responsible for binding to specific antigens and defining Ab effector function, respectively [1], [5] (Figure 1.1a). The humoral immune response is driven by B cells, which originate and mature in the bone marrow and circulate as mature naïve B lymphocytes until activated to produce specific Ig. Naïve B cells require receptor engagement for activation, achieved by direct interaction between lymphocyte receptors and invading pathogens, and differentiation through somatic hypermutation and isotype switching is driven by interaction with helper T cells. In the primary B cell response, newly activated antigen-specific B cells undergo clonal expansion and differentiate into IgM antibody-secreting plasma cells or memory B cells. The secondary B cell response occurs upon re-exposure to a previously seen antigen. The memory B cells are activated rapidly and under the influence of helper T cells, undergo genetic rearrangement for class switching to produce IgG, IgA and IgE Ab, and somatic hypermutation for affinity maturation [1]. Antigen-specific plasma cells and memory B cells, induced either through vaccination or infection, can survive for years following their initial activation [1], [6], [7]. Antibody-derived immunological memory, or humoral immunity, provides rapid defence against the same antigen, offering protection from severe disease, and in some cases, sterilizing

immunity that prevents infection altogether [8], [9]. Five types of Ig comprise the humoral response – IgG, IgM, IgA, IgD, and IgE – each with specific functions and distributions. This thesis focuses on IgM, IgG, and IgA responses due to their relevance in viral infection, as will be described later in this thesis.

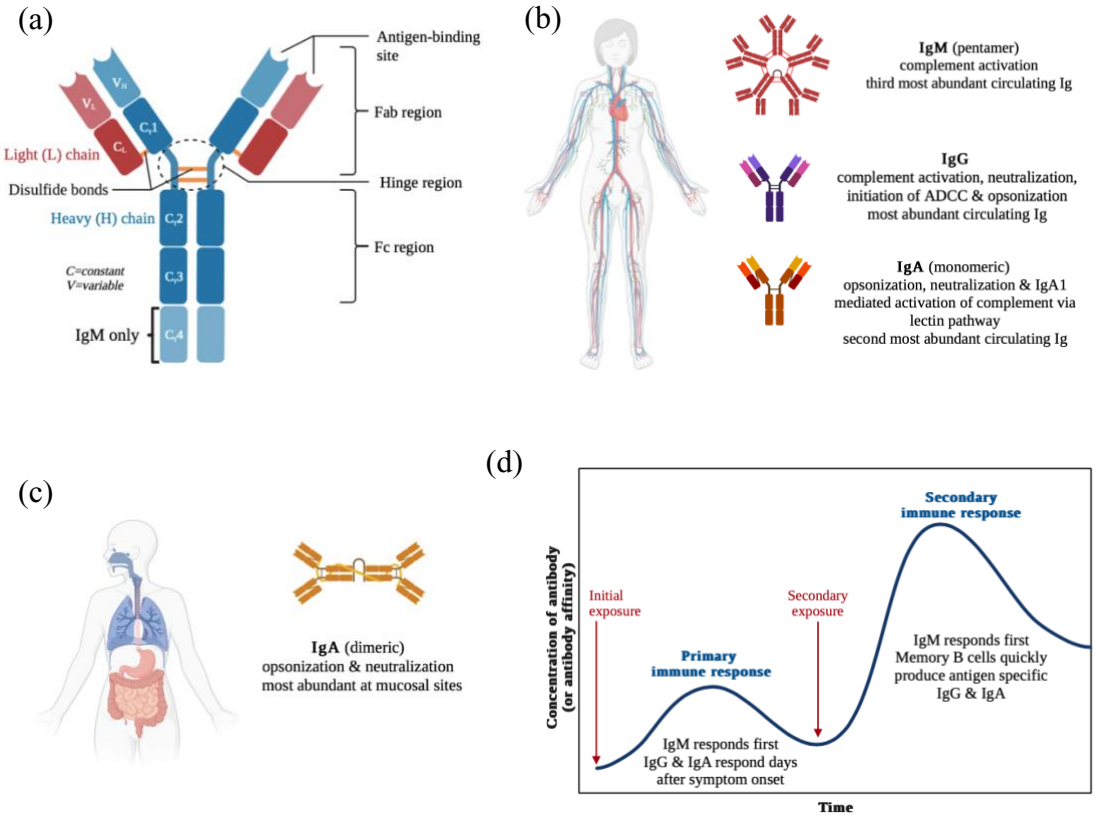

Figure 1.1 Immunoglobulin structure, location, and effector functions.
Immunoglobulin (Ig) G, M & A structure, typical location, and primary effector functions in the human immune response. (a) Ig are composed of heavy and light chain proteins that can be divided into constant and variable regions. (b) IgM, IgG, and IgA circulate in the body in plasma as well as in the lymphatic system. They provide protection through complement activation (IgM & IgG), opsonization (IgM, IgG, & IgA), neutralization (IgM weakly, IgG & IgA) and activation of ADCC (IgG). (c) At mucosal sites, dimeric IgA is the most abundant antibody and provides protection from infection through neutralization and opsonization to mark foreign pathogens for phagocytosis. (d) IgM is the first responder to invading foreign antigens, while secondary IgG and IgA ordinarily respond days to weeks after primary exposure. Subsequent exposures result in faster, more robust, and highly specialized Ig responses to antigen. Created using BioRender.com

1.2.2.1 Immunoglobulin M

Following maturation and initial B cell antigen stimulation, IgM is secreted as a pentamer. It is highly effective at recognizing pathogens with repeating surface antigens and in activating the complement system (Figure 1.1b) [1], [5], [10]. The low affinity, high avidity antibody responds quickly to invading pathogens, typically within 4-14 days, but the response is relatively weak and short-lived, often becoming undetectable within 6 weeks after SARS-CoV-2 infection [1], [4], [11]–[13]. Antibody production initially favours IgM because naïve B cells express IgM, and it is produced without T cell help, class switching or somatic hypermutation which will in turn lead to the cellular selection phase, and in turn, affinity maturation [14]. Following B cell activation and downstream signalling, the Ig genes will undergo somatic hypermutation to increase affinity for antigens and class switching, altering the constant region to an antibody with effector functions best suited to respond to the specific invading pathogen (Figure 1.1a) [1], [14].

1.2.2.2 Immunoglobulin G

The most abundant, longest lasting, and most studied antibody class is IgG. Monomeric IgG circulates in the blood, crosses the placenta, and spills over to mucosal surfaces for protection (Figure 1.1b) [1], [5], [15]. There are four human subclasses of IgG – IgG1, IgG2, IgG3, & IgG4 which differ in their constant region – each with variable functional preference – of which IgG1 is most abundant. While all four subclasses can neutralize pathogens, IgG3 is the most efficient due to its increased flexibility allowing it to exhibit more productive interactions with antigens, especially against human immunodeficiency virus (HIV) antigens [5], [15]–[17]. Similarly, IgG1 and IgG3 are

especially effective at neutralization of SARS-CoV-2 [18]–[20]. SARS-CoV-2 infection typically induces a detectable IgG response between 12 and 15 days after symptom onset (Figure 1.1d), which begins to decline in levels around six weeks later [21]–[27]. Considering its abundance, longevity, and rapid response, it is understandable that characterization of IgG responses has dominated research on humoral responses to SARS-CoV-2 infection and vaccination.

1.2.2.3 Immunoglobulin A

IgA is the dominant Ig at mucosal sites and second most abundant in blood (Figure 1.1b,c) [1], [5]. Circulating IgA is usually a monomer while mucosal, or secretory, IgA is typically a dimer (Figure 1.1b,c). Similar to IgG, IgA in humans is split further into subclasses, namely IgA1 and IgA2, where IgA1 constitutes the majority of circulating IgA, and IgA2 is dominant in secretory IgA [5]. IgA Ab are effective at neutralizing and opsonizing invading pathogens, however, they rarely activate complement, and do not elicit ADCC [1], [5], [28]–[30]. IgA can be transferred from mother to baby through breast milk, providing passive immune protection [29], [31], [32]. Viral infection, including SARS-CoV-2, induces the production of both secretory and circulating IgA responses [33]–[36], While there is conflicting evidence on the correlation between secretory and circulating IgA composition [37], [38], it is clear that both forms can aid in protection from viral infection. By elucidating the role of circulating IgA, we can better understand the dynamic interplay of humoral immune responses and utilize the information to better understand mechanisms of protection from SARS-CoV-2 or other viruses.

1.2.3 Cellular Immune Responses

The uptake of foreign antigens by APC, in particular dendritic cells can result in antigen presentation to helper $CD4^+$ T cells (T_H). Cytotoxic $CD8^+$ T cells are primarily activated by antigenic peptides produced inside APC and once fully activated, can directly kill target cells. T_H cells help activate variable immune effector functions, depending on the specific T_H subset involved. T_H1 and T_H2 cells activate B cells to differentiate into plasma cells or become memory B cells. In addition, T_H1 cells can activate inflammatory and phagocytic responses and expand cytotoxic $CD8^+$ T cell responses through IL-2 and IFN-γ cytokine signalling [1], [39], [40]. Secretion of IL-17 by T_H17 cells can aid in T_H1 cell recruitment. Additionally, T_H17 cells can recruit neutrophils and aid in germinal center formation as well as antibody class switching [1], [41]. Regulatory T cells (T_{reg}) are important for immune system modulation, maintaining self-tolerance, preventing autoimmune diseases, and suppressing the activation and expansion of effector T cells [1], [42]. Many antigen-specific T cell progeny are short-lived, while some become long-lived memory T cells and persist as either central memory T cells (T_{CM}), effector memory T cells (T_{EM}), or tissue-resident memory T cells (T_{RM}). T_{EM} cells can regulate inflammation, encourage recruitment of phagocytic cells, and control T helper cell differentiation through their secreted cytokines, IL-2, IL-4, and IFN-γ. [1], [43]–[46].

1.2.4 Cross-Reactive Immune Responses

The immune system generates highly specialized and specific responses against invading pathogens, and an important aspect of immune responses is their breadth of activity against antigenically similar structures, even at a lower affinity. Immune

recognition of something distinct but related to the original antigen is termed cross-reactivity. A famous applied example of this is the administration of vaccina (cowpox) in order to elicit immune protection against variola (smallpox) [47]. Cross-reactivity can result in cross-protection when the clinical severity of a non-vaccine strain or variant infection is diminished by the ability of the immune system to recognize and generate effector functions to similar antigens [48]. Cross-reactive antibodies against SARS-CoV-2 have been found as a result of previous common coronavirus infections, SARS-CoV-1 infection, as well as in the ancestral vaccine- or infection-induced response reacting against SARS-CoV-2 variants [49]–[53]. Cross-reactivity is not limited to antibody responses as well conserved peptides in different strains or variants can encode cross-reactive T-cell epitopes [54]–[59]. The importance of cross-reactivity is evident in its ability to provide an adaptive immune response to a similarly structured but new antigen, therefore, enabling a rapid response to help slow infection and disease progression.

1.3 Special cases of immune responses to SARS-CoV-2

Considering the novel nature of SARS-CoV-2 and the infection rates globally, with over 676 million confirmed cases when John Hopkins University stopped collecting data as of March 10th, 2023 [60], there are many distinct situations in which persons are exposed to or infected with the virus, both before and after vaccination. Between late 2019 and the day this was written (August 7th, 2023), 207,049 articles referencing SARS-CoV-2 appear on PubMed, highlighting the substantial amount of research conducted to better understand the virology, immunology, and sociological impacts of COVID-19. The variability concerning SARS-CoV-2 immunology allowed us to explore different cases of exposure to

and infection with SARS-CoV-2 in distinct cohorts. Namely, we investigated (1) subjects who, prior to vaccination did not seroconvert or display signs of infection despite prolonged close contact with an active case of SARS-CoV-2 and (2) subjects who experienced a breakthrough SARS-CoV-2 infection following at least two Health Canada-approved vaccines, and (3) subjects who did not experience breakthrough infection.

1.3.1 Measurement of secretory antibodies

As previously discussed, IgG, IgM, and monomeric IgA exist in the blood at relatively high levels and, therefore, are easy to collect and analyze [1], [5], [10], [15]. All three antibodies play a role in the SARS-CoV-2 immune response and have been extensively studied in vaccine trials and vaccination regarding their potential cross-reactivity and induction following SARS-CoV-2 infection. However, analysis of mucosal antibody responses is lacking by comparison. Although SARS-CoV-2 begins as an upper respiratory tract infection and mucosal secretions (saliva, nasal fluid, tears) can be easily collected, the concentration of IgA in these samples is so low as to typically require ultra-sensitive quantification with the considerable variability between individuals making standardization difficult. Furthermore, the different collection methods themselves introduce variability and can impact measurement of total Ig concentrations [61]. It was generally thought that intramuscular vaccinations could not stimulate secretory antibody responses (Mestecky 1987), but recent reports have shown that intramuscular mRNA vaccines against SARS-CoV-2 generate local secretory IgA in saliva and nasal secretions [62]–[65]. Secretory antibodies in the nose and saliva are believed to play a crucial role in prevention of infection and transmission of SARS-CoV-2. Some research has indicated a

positive correlation between the levels and specificity of circulating and secretory IgA [38], [62], [66], while other research indicates there is no significant relationship between the two immune responses [37], [67], [68].

1.3.2 Imprinting

Immune imprinting, or original antigenic sin, occurs when the immune response favours activation of memory cells generated against a previously encountered antigen, rather than favouring *de novo* responses against the new and closely related antigen [69]. Initially used to describe the tendency of antibody responses to remain specific for the first influenza viral epitopes they were exposed to, imprinting diminishes naïve B cell responses to new viral variant epitopes, impacting both the immune responses to new infections and new vaccines [70], [71]. Immune imprinting is apparent with the increase of SARS-CoV-2 variants, where the antibody response from the initial exposure, either by vaccination or infection, selectively primes the initial response upon exposure to emerging variants, rather than allowing generation of more specific new responses (Figure 1.2) [72]. Immune imprinting is a type of cross-reactivity, molding the secondary viral infection of a variant onto the primary infection, thereby activating the ancestral antigen response, and ultimately preserving it as the more dominant response [73]. There is variability in the kinds of imprinting, and it is not unique to viral-induced humoral responses. For instance, pathological imprinting influenced by gut microbiota composition in early development can persist and exert long-lasting impacts into adulthood [74]. As well, there is evidence of $CD8^+$ T cell imprinting to the first infection strain of lymphocytic choriomeningitis virus (LCMV) rather than generating a new response to a second, and different, LCMV strain

exposure [1]. Further studies on immune imprinting can enhance our understanding of its impact and, in turn, improve our capacity to manipulate its occurrence to advance broadly neutralizing therapeutics.

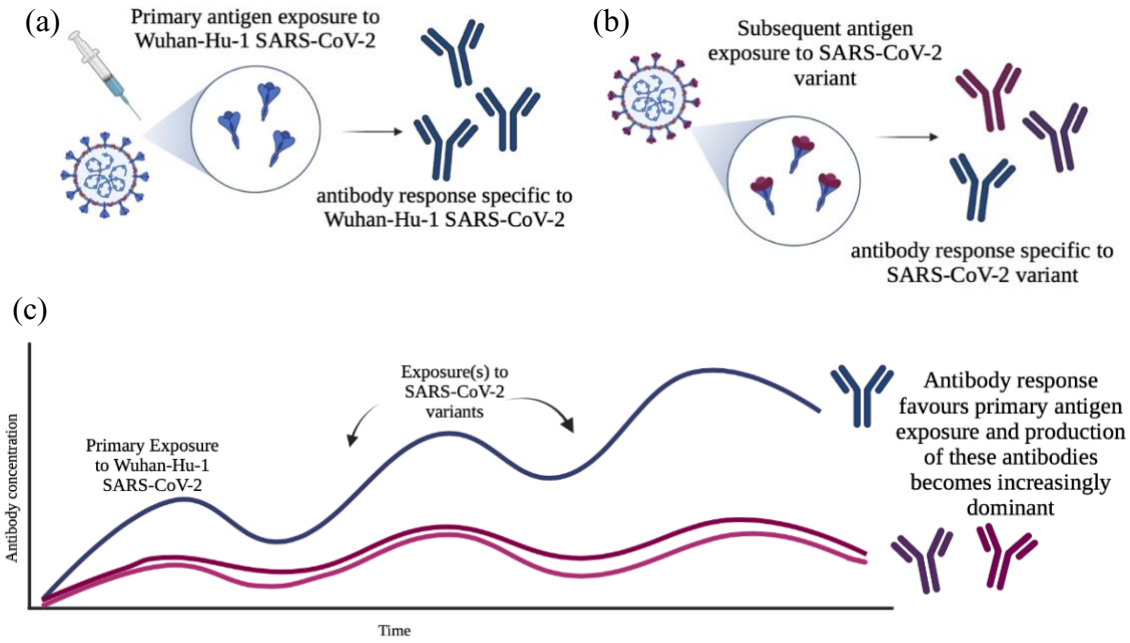

Figure 1.2 Humoral Immune Imprinting.

(a) Primary exposure to SARS-CoV-2 antigen, either via vaccination or infection results in production of antibodies and memory B cells specific to the particular virus represented. (b) Upon exposure to a variant of SARS-CoV-2, the antibody response should address variant antigens, some of which may be similar to the original SARS-CoV-2 antigen exposure. (c) Over time, repeated exposures to similar antigens will drive the antibody response to favour the original response generated, rather than favour development of variant-specific antibody responses. Created using BioRender.com

1.3.3 Uninfected despite viral exposure.

While the Ab response is typically measured to determine exposure and response to infection, there are presumed cases of cell-mediated immune responses aiding in the protection or clearance of invading pathogens before infection progresses to a level where specific Ab are produced. This phenomenon was documented over 30 years ago, first highlighted by mucosal immune responses following exposure to HIV and simian immunodeficiency virus (SIV) [75], [76], and later in the generation of $CD4^+$ and $CD8^+$ T cell responses against hepatitis C virus (HCV) in close household contacts and healthcare workers [77]–[79]. In the context of COVID-19, there were a large number of non-vaccinated health care workers highly exposed to SARS-CoV-2 who lacked detectable Ab responses yet had a virus-specific cellular immune response [80]–[83]. The accumulating evidence of the potential for rapid cell-mediated clearance in acute SARS-CoV-2 infection [84] can potentially inform COVID-19 vaccine design and has implications for rapid viral clearance in other infections.

1.3.3.1 Abortive Infection

One possible explanation for SARS-CoV-2 exposure without seroconversion or detectable infection may be abortive infection. In this circumstance, low viral load paired with induced death of infected cells results in no progeny virus released and, therefore, the virus is unable to spread by infecting neighbouring cells. Abortive infections occur quickly after viral entry, and as such, are difficult to evaluate. The death of infected cells before the release of virions suggests that some viral infections can be cleared through innate immune

mechanisms or by pre-existing, cross-reactive or rapidly developed cellular immunity before seroconversion or symptom manifestation.

1.4 Objective/Rationale

The overall objective of this thesis is to characterize the nature and significance of immune responses to SARS-CoV-2 following either exposure, infection, vaccination, or combinations thereof. The first aim is to provide insight into the potential immunological reasons for individuals remaining uninfected despite prolonged exposure to SARS-CoV-2 through analysis of SARS-CoV-2 specific humoral and cellular immune responses. The second aim is to investigate the circulating IgA responses following vaccination, examine its potential to serve as a marker for resistance to infection, and compare the effects of vaccine boosters to breakthrough infection on the strength and durability of the IgA responses to SARS-CoV-2.

Chapter 2: Cellular Immune Responses to SARS-CoV-2 in Exposed Seronegative Individuals

This manuscript was published with the following citation.

Norton, N.J.; Holder, K.A.; Ings, D.P.; Harnum, D.O.A.; Russell, R.S.; Grant, M.D. Cellular Immune Responses to SARS-CoV-2 in Exposed Seronegative Individuals. Viruses 2023, 15, 996. https://doi.org/10.3390/v15040996

* Note that reference numbers as well as the figure numbers from the published manuscript differ within the following chapter in accordance with the reference numbers and figures within thesis.

2.1 Co-Authorship Statement

In the following manuscript, I conducted and planned all the experiments as well as gathered and analyzed the data generated. As the primary author, I drafted the manuscript and produced all the figures used in its publication.

The following co-authors contributed to this manuscript. D.O.A.H: Recruited and consented subjects, scheduled appointments to collect blood samples. D.P.I: Processed incoming samples and managed data. R.S.R: Reviewed and revised manuscript drafts. K.A.H: Provided experiment guidance, supervision, and consulted on experiment planning. M.D.G: Conceptualized the project, reviewed data, and manuscript drafts, and supervised the project. Funding for this project was secured by M.D.G, R.S.R, and K.A.H.

2.2 Abstract

Some SARS-CoV-2-exposed individuals develop immunity without overt infection. We identified 11 individuals who were negative by nucleic acid testing during prolonged close contact and with no serological diagnosis of infection. As this could reflect natural immunity, cross-reactive immunity from previous coronavirus exposure, abortive infection due to de novo immune responses, or other factors, our objective was to characterize immunity against SARS-CoV-2 in these individuals. Blood was processed into plasma and peripheral blood mononuclear cells (PBMC) and screened for IgG, IgA, and IgM antibodies (Ab) against SARS-CoV-2 and common β-coronaviruses OC43 and HKU1. Receptor blocking activity and interferon-alpha (IFN-α) in plasma were also measured. Circulating T cells against SARS-CoV-2 were enumerated and $CD4^+$ and $CD8^+$ T cell responses discriminated after in vitro stimulation. Exposed uninfected individuals were seronegative against SARS-CoV-2 spike (S) and selectively reactive against OC43 nucleocapsid protein (N), suggesting common β-coronavirus exposure induced Ab cross-reactive against SARS-CoV-2 N. There was no evidence of protection from circulating angiotensin-converting enzyme (ACE2) or IFN-α. Six individuals had T cell responses against SARS-CoV-2, with four involving $CD4^+$ and $CD8^+$T cells. We found no evidence of protection from SARS-CoV-2 through innate immunity or immunity induced by common β-coronaviruses. Cellular immune responses against SARS-CoV-2 were associated with time since exposure, suggesting that rapid cellular responses may contain SARS-CoV-2 infection below the thresholds required for a humoral response.

2.3 Introduction

Since its introduction into the human population in late 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread widely and continues to circulate globally. As of March 2023, there have been over 675 million known cases of coronavirus disease (COVID-19) worldwide and almost 7 million related deaths. Canada alone has had over 4.5 million documented cases and nearly 50,000 related deaths [60]. It is estimated that more than 75% of the Canadian population has now been infected with SARS-CoV-2. Research over the last three years has increased the understanding of the virus and host immune response to infection and has helped to inform public health agencies on best practices to address the pandemic. As with other viruses, exposure to SARS-CoV-2 can occasionally occur with no overt signs of infection, negative nucleic acid-based testing, and no subsequent seroconversion. In a small fraction of cases, this is associated with detectable cellular immunity against SARS-CoV-2. Recent studies have explored the incidence of abortive infections, which occur when virally infected cells produce no progeny virus following exposure to SARS-CoV-2. A cohort of seronegative healthcare workers in the United Kingdom who were tested during the initial wave of COVID-19 (March 2020) had evidence of SARS-CoV-2-specific T cell responses [83]. A similar phenomenon of specific T cell responses in the absence of seroconversion was previously documented with exposure to hepatitis C virus (HCV) [78], [79]. This suggests that in rare cases, viral infections can be curtailed prior to seroconversion by either pre-existing or rapidly developing cellular immunity.

Pre-existing cellular immunity against SARS-CoV-2 could result from exposure to common coronaviruses that share T cell epitopes with SARS-CoV-2 [55], [85], [86]. Antibodies induced by circulating endemic α- and β-coronaviruses, NL63 and 229E, and OC43 and HKU1, respectively, which typically cause mild respiratory illness [87], cross-react with SARS-CoV-2 proteins [88]–[90]. Common coronavirus antibody cross-reactivity was also noted during the SARS-CoV-1 outbreak [91]. Infection with a common coronavirus prior to infection with SARS-CoV-2 can lessen COVID-19 disease severity [92]; however, it is unclear whether cross-reactive antibodies or other forms of immunity induced by infection with common coronaviruses provide protection against SARS-CoV-2 infection or against severe COVID-19 [88], [89], [92].

In this study, we investigated immune responses against SARS-CoV-2 of individuals who were in prolonged close contact to an active case of COVID-19 yet were seemingly uninfected. These individuals showed no evidence of viral replication by reverse transcriptase polymerase chain reaction (RT-PCR) testing, had no self-reported symptoms, and remained seronegative against the immunodominant SARS-CoV-2 spike (S) protein.

2.4 Materials and Methods

2.4.1 Selection of Study Participants and Sample Collection

This study was approved by the Newfoundland and Labrador Health Research Ethics Authority and carried out in accordance with the recommendations of the Canadian Tri- Council Policy Statement: Ethical Conduct for Research Involving Humans. Study subjects are nested within a cohort established for an ongoing study at the Memorial

University of Newfoundland and Labrador, where 263 participants were recruited based on previous RT PCR-confirmed or suspected SARS-CoV-2 infection [93]. Written informed consent was obtained for whole blood collection in accordance with the Declaration of Helsinki and subjects completed a questionnaire at study intake on SARS-CoV-2 exposure, testing, and symptom history. Through purposive sampling, individuals who reported close prolonged contact, either through a spouse or family member, to an active case of SARS-CoV-2 yet did not test positive for COVID-19 via PCR, were selected for further testing. Prolonged close contact included such things as caring for a partner throughout their illness, shared sleeping arrangements, shared eating and washroom facilities, exercise partners, ride sharing, and household proximity throughout the presumed infectious period of 5 days or more. All subjects identified as fitting this criterion were included. This was designed as an observational study without sample size calculation to assess the requirement for a valid estimate of overall frequency of such cases in the population. Whole blood was drawn by forearm venipuncture into acid-citrate-dextrose (ACD) preserved vacutainers and plasma was collected after centrifuging whole blood for 10 minutes at 500 *g*. Plasma was stored immediately in small aliquots at -80°C until analysis. Peripheral blood mononuclear cells (PBMC) were isolated from the cellular fraction of blood following the consensus protocol established by the Canadian Autoimmunity Standardization Core procedure [94]. Isolated PBMC were cryopreserved in 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA), 90% fetal bovine serum (FBS, HyCloneTM, GE Healthcare Life Sciences, Logan, UT, USA) at $\leq 2.0 \times 10^7$/mL by cooling to −80°C in a Frosty™ freezing container overnight before transfer to LN_2.

2.4.2 Serological Testing

Plasma was diluted in phosphate buffered saline (PBS) containing 0.05% Tween 20 (Fisher Bioreagents, Thermo Fisher Scientific, Rochester, NY, USA) and 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and tested for anti-SARS-CoV-2 antibodies by enzyme-linked immunosorbent assay (ELISA) using recombinant proteins as antigens. Proteins were coated overnight (4°C) at 50 ng/well in 50 µL Dulbecco's PBS (Corning, Mediatech, Inc., Manassa, VA, USA) onto 96-well Immununlon-2 HB (Thermo Fisher Scientific, Waltham, MA, USA) ELISA plates to test for antibodies against SARS-CoV-2 Wuhan-Hu-1 receptor binding domain (RBD; Sino Biological, Wayne, PA, USA), nucleocapsid (N) proteins (Sino Biological) and full-length spike (FLS, SMT1-1 National Research Council of Canada), and β-coronavirus N proteins from OC43 and HKU1 (Sino Biological). Plates were washed 4 times after coating and 6 times between all subsequent steps with 300 µL/well PBS + 0.05% Tween® 20. Plates were blocked with 200 µL 1% BSA in PBS for 1 hour, after which 100 µL diluted plasma was added for 1.5 hours, and 100 µL diluted goat-anti human IgG, IgA, or IgM horseradish peroxidase (HRP) conjugated detection antibodies (IgG and IgA Jackson ImmunoResearch, Baltimore Pike, West Grove, PA, USA; IgM NCI Biological Resources Branch, Frederick National Library, Fredrick, MD, USA) were added to the wells for 1 hour. Colour was developed using 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB, T8665, Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes and the reaction was stopped by adding 100 µL of 1 Mol H_2SO_4. Optical density (OD) was read at 450 nm on a BioTek Synergy HT plate reader. Plasma was diluted 1:100 to test for IgG antibodies and 1:50 to

test for IgM and IgA antibodies. The anti-IgG*HRP and anti- IgM*HRP conjugates were diluted 1:50,000 and 1:25,000, respectively. The anti-IgA*HRP was also diluted 1:25,000. A set of 40 control serum samples collected before October 2019 was used to establish cut-off OD values for IgG seropositivity against SARS-CoV-2 S and RBD [93]. Any sample producing an OD more than 2 standard deviations (SD) above the mean OD of the 40 control samples was considered seropositive.

2.4.3 SARS-CoV-2 Pseudo-Neutralization ELISA

Immulon-2 96 well ELISA plates were coated with 100 ng of SARS-CoV-2 FLS protein in 50 µL PBS overnight at 4˚C. The plates were then washed 4 times with 300 µL/well of PBS + 0.05% Tween 20, blocked with 200 µL 1% BSA in PBS for 1 hour, and then washed 4 more times. Plasma was diluted 1:100 in 0.1% BSA in PBS + 0.05% Tween 20 (diluent) and 100 µL was added to the respective wells for 1.5 hours, then washed 6 times. Biotinylated ACE2 (RayBiotech Life, Inc., Peachtree Corners, GA, USA), made up in diluent, was added in 100 µL at 40 ng/well for 1 hour and then the plate was washed 6 times. Next, streptavidin (SA)-HRP (Jackson ImmunoResearch, Baltimore Pike, West Grove, PA, USA) diluted 1:50,000 was added to the wells for 1 hour and the plates were washed another 6 times. The enzymatic colour reaction was developed using 100 µL TMB per well for 20 minutes and stopped with 100 µL 1 Mol H_2SO_4. Optical density (OD) was read at 450 nm on a BioTek Synergy HT plate reader. Percent neutralization was calculated using the following equation:

Equation 2.1. Percent neutralization

$$\% \text{ Inhibition} = (1 - \left({}^{OD450nm\ of\ sample}/_{OD450nm\ of\ negative\ control}\right) x\ 100$$

2.4.4 Measurement of Interferon-α

Plasma interferon alpha (IFN-α) levels were measured using the RayBio® Human IFN-α kit (RayBiotech Life, Inc., Peachtree Corners, GA, USA) following the manufacturer's instructions. Briefly, all reagents, standards, and samples were brought to room temperature before use. Samples and standards (100 µL) were added to the respective wells for 2.5 hours with gentle shaking at room temperature. After 4 300 µL washes, 100 µL of biotinylated detection antibody was added to wells for 1 hour with gentle shaking, then plates were washed. Next, 100 µL SA-HRP was added for 45 minutes, again with gentle shaking. Following another wash, 100 µL TMB was added, colour developed for 20 minutes in the dark, and the reaction stopped with 50 µL stop solution. Plates were read at 450 nm on a BioTek Synergy HT plate reader. The calculation of the IFN-α concentration in samples was performed based on the standard curve generated.

2.4.5 ELISpot Assay

Peripheral blood mononuclear cells were recovered by rapid thawing in a 37˚C water bath and added to 9 mL of lymphocyte medium (LM; RPMI-1640, 10% FBS [HyClone™], 200 IU/mL penicillin/streptomycin [Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA], 0.01 Mol HEPES [Invitrogen], and 2×10^{-5} Mol 2-mercaptoethanol [Sigma-Aldrich]). Cells were then centrifuged at 450× *g* for 5 minutes, resuspended in 5

mL of CTL medium (CTL-Test™ Medium, CTL ImmunoSpot®, Shaker Heights, OH, USA) with 1% L-glutamate, and maintained overnight at 37˚C, 5% CO_2 prior to use on ELISpot. Recovered cells were added in duplicate at 2.0×10^5/well to 96-well pre-coated IFN-γ ELISpot plates (CTL ImmunoSpot®). The cells were stimulated with peptide pools of SARS-CoV-2 spike, nucleocapsid, membrane, and envelope proteins with the final concentration of each peptide at 1 μg/mL. PepTivator® SARS-CoV-2 ProtS complete peptide pool mainly consisted of 15-mer amino acid (aa) sequences with 11 aa overlaps (Miltenyi Biotec, San Diego, CA, USA) resuspended in endotoxin-free ultra-pure water (H_2O, Millipore, Sigma-Aldrich). All other SARS-CoV-2 protein peptide pools were pooled from BEI resources peptide sets (Nucleocapsid NR-52419, Envelope NR-52405, Membrane NR-52403) and consisted of 17-mer aa sequences with 10–11 aa overlap to cover the whole protein of interest resuspended in dimethyl sulfoxide (DMSO) and diluted in unsupplemented RPMI 1640. The membrane and envelope peptides were combined into a single pool. Cells and peptides, along with their respective vehicle controls (H_2O or DMSO) and anti-CD3 as a positive control (OKT3, ATCC, CRL-8001), were incubated for 24 hours at 37˚C, 5% CO_2. After 24 hours, the plate was washed twice with 200 μL/well PBS and another 2 times with 200 μL/well PBS + 0.05% Tween 20. Anti-human IFN-γ detection antibody was diluted in diluent B, filtered through a 0.1 μm filter, and 80 μL was added to each well. Following a 2-hour incubation at room temperature, the plate was washed 3 times with 200 μL/well PBS + 0.05% Tween 20. Next, 80 μL of a tertiary solution (SA-HRP) in diluent B was added to wells for 30 minutes at room temperature, then plates were washed twice with 200 μL/well PBS + 0.05% Tween 20 and then two more times

with 200 µL/well dH_2O. Colour was developed using 80 µL of developer solution in the dark at room temperature for 15 minutes and the reaction was stopped by gently rinsing the plate with tap water 3 times. The plate was airdried overnight, then scanned and counted on a CTL ImmunoSpot® S6 Universal Analyzer (CTL Analyzers, Shaker Heights, OH, USA). Subjects who had ≥ 50 IFN-γ producing T cells/10^6 PBMC above the vehicle control background following stimulation with at least one of the peptide pools were considered to have a specific cellular immune response against SARS-CoV-2. Results are shown with the vehicle control background subtracted.

2.4.6 In Vitro Stimulation with SARS-CoV-2 Peptides

Recovered cells from samples used in ELISpot assays that yielded ≥ 50 IFN-γ producing T cells/10^6 PBMC in response to at least one of the peptide pools were then stimulated for 7 days with the same peptide pool(s) in vitro as previously described [95]. Depending on availability, from 2×10^6 to 5×10^6 total PBMC were pelleted and stimulated in small volumes for 1 hour at 37°C, 5% CO_2 with SARS-CoV-2 spike, nucleocapsid, or membrane and envelope combination peptide pools (1 µg each individual peptide). After 1 hour, the culture volume was increased to 1 mL using LM supplemented with 25 ng/mL interleukin (IL)-7 (National Cancer Institute, Frederick, MD, USA). These cells were then incubated for 7 days at 37°C, 5% CO_2, adding LM when needed to support their growth.

2.4.7 Flow Cytometry

After 7 days' in vitro stimulation at 37°C, 5% CO_2, responder cells were analyzed by short-term restimulation, and flow cytometry as previously described [95]. Briefly, 5.0 × 10^5 cells were restimulated in a final volume of 500 µL with the SARS-CoV-2 peptide pool of interest at 1 µg/mL for each peptide or a matching volume of vehicle control, with Brefeldin A (Sigma-Aldrich) added to a final concentration of 10 µg/mL. After 5 hours, the cells were washed with flow cytometry buffer (1 X PBS, 5 mMol EDTA, 0.2% NaN_3, 0.5% FBS) and stained with the following fluorochrome conjugated antibodies for 20 minutes in the dark: αCD3 (VioGreen™, REAfinity™ Clone REA613, Miltenyi Biotec), αCD4 (APC-Vio® 770, REAfinity™ Clone REA623, Miltenyi Biotec), and αCD8 (PerCP, Clone HIT8a, BioLegend, San Diego, CA, USA). The cells were washed again using flow cytometry buffer and intracellular IFN-γ stained following the MACS Miltenyi Biotec intracellular staining of eukaryotic cells procedure and kit. Briefly, the cells were fixed in a final volume of 500 µL using equal amounts of Inside Fix and buffer (PBS pH 7.2, 0.5% BSA, and 2 mMol EDTA) for 20 minutes in the dark, centrifuged, then washed using flow cytometry buffer. Next, anti-IFN-γ (PE, eBioscience™ Clone 4S.B3, Invitrogen, Thermo Fisher Scientific), diluted in Inside Perm to a final volume of 100 µL, was added and incubated at room temperature for 10 minutes. Next, 1 mL of Inside Perm was added to each sample and the samples were centrifuged, decanted, and resuspended in the remaining liquid prior to analysis on a Beckman Coulter CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). At least 100,000 events were collected for each sample stimulation condition. We gated on PBMC, distinguished $CD3^+$ cells and then gated

separately on $CD3^+CD4^+$ and $CD3^+CD8^+$ cells to analyze IFN-γ expression by each T cell subset. The background from unstimulated conditions was subtracted from the percentage of IFN-γ producing cells in test conditions to calculate the percentage of $CD4^+$ or $CD8^+$ T cells producing IFN-γ in response to SARS-CoV-2 peptides. Data were analyzed and visualized using Kaluza Version 2.1 (Beckman Coulter).

2.4.8 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism Version 9.5.0. Significance values, where applicable, are shown above lines spanning the groups compared. The following statistical tests were conducted for data analysis in this study, as specified in the relevant figure captions: Mann-Whitney test; Wilcoxon signed rank test; Spearman correlation.

2.5 Results

2.5.1 Selection of SARS-CoV-2 Exposed Uninfected Persons

For a study initiated in March 2020, we recruited individuals with confirmed COVID-19, suspected COVID-19, and contacts of persons with confirmed COVID-19 into a study of immune responses against SARS-CoV-2. Prior to the widespread introduction of COVID-19 vaccines, we identified 11 non-immunocompromised individuals (Table 2.1) defined as discordant cases who were in prolonged close contact through their spouse or family member(s) with active cases of COVID-19. Of these, 7 were exposed to the ancestral Wuhan-Hu-1 strain of SARS-CoV-2 between March 15th and April 4th, 2020, and 4 were exposed to the SARS-CoV-2 Alpha variant (B.1.1.7) between February 10th and February 15th, 2021. Despite prolonged close contact with one or more confirmed cases of COVID-19, these 11 exposed individuals had negative RT-PCR test results at the time and reported no symptoms of infection throughout or shortly after the course of their close contact. No other exposures or signs of infection were noted before their sample collection dates. All samples used in this study were collected prior to any instance of COVID-19 vaccination, COVID-19 infection, or documented infection with another coronavirus.

Table 2.1 Demographics of discordant case subjects and information on exposure to SARS-CoV-2.

Subject ID	Sex	Age	Date of Contact's Confirmatory Test	Contact's Symptoms	SARS-CoV-2 Exposure Strain	Days from Exposure to Sample Collection
1185	F	68	25 March 2020	Severe	Wuhan-Hu-1	187
1212	M	63	30 March 2020	Moderate	Wuhan-Hu-1	211
1257	M	26	31 March 2020 to 4 April 2020 [1]	Moderate (×1) Mild (×3) [1]	Wuhan-Hu-1	227
1282	M	76	3 April 2020	Moderate	Wuhan-Hu-1	241
1340	F	56	15 March 2020	Severe	Wuhan-Hu-1	325
1383	F	54	31 March 2020	Moderate	Wuhan-Hu-1	337
1418	F	66	24 March 2020	Moderate	Wuhan-Hu-1	356
1559	F	57	10 February 2021 [1]	Moderate (×2) [1]	B.1.1.7	77
1568	M	49	11 February 2021	Moderate	B.1.1.7	84
1637	F	39	15 February 2021 [2]	Mild [2]	B.1.1.7	197
1638	M	68			B.1.1.7	197

[1]–Multiple family members tested positive over multiple days for SARS-CoV-2 infection with varying symptoms. [2]–Exposed to the same family member.

2.5.2 Anti-SARS-CoV-2 Serology

Although infection with SARS-CoV-2 can occur without seroconversion, especially in mild or asymptomatic cases, we investigated antibody responses against SARS-CoV-2 in the discordant individuals to corroborate the absence of overt infection indicated by negative PCR tests. All 11 discordant individuals were seronegative for IgG antibodies against SARS-CoV-2 RBD and FLS (Figure 2.1a). All discordant individuals were also seronegative for IgM antibodies against FLS (Figure 2.1b). Several studies reported detection of IgA antibodies against SARS-CoV-2 S either before, or in the absence of IgG antibodies, therefore, we also measured IgA antibodies against FLS. All discordant individuals were seronegative for IgA antibodies against FLS (Figure 2.1c). To test the possibility that the immune system of the discordant case individuals was primed to respond to vaccination, similar to what occurs in previously infected individuals, we compared the IgG anti-S response after one dose of the Pfizer BioNTech (BNT162b2) mRNA vaccine of 5 discordant cases for whom we had post-vaccination samples, to that of age, sex and days-post vaccination matched previously infected individuals and non-exposed individuals (Figure 2.1d). The discordant cases had an IgG anti-FLS antibody response to vaccination similar to non-exposed individuals, while the previously infected individuals had a significantly greater IgG response. Thus, we found no evidence of a humoral response against SARS-CoV-2 S in the exposed uninfected individuals, nor of occult priming for a humoral response to S-based SARS-CoV-2 vaccination.

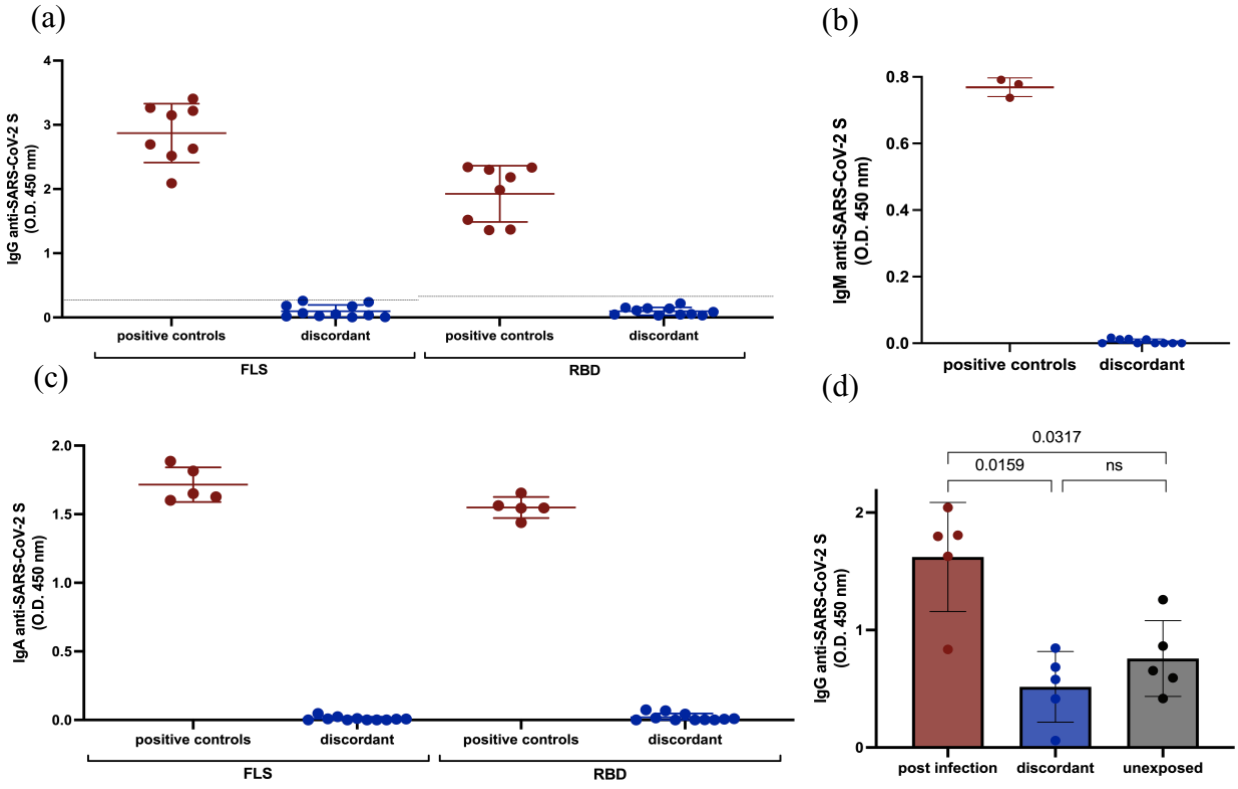

Figure 2.1 Serological responses of discordant case subjects.
Antibody responses of discordant subjects against SARS-CoV-2 S measured by ELISA. (a) IgG antibody responses to SARS-CoV-2 S – FLS and RBD with plasma samples from previously infected subjects included as positive controls. The lines represent cut-off values for positivity against SARS-CoV-2 FLS (Y = 0.265) and RBD (Y = 0.327) as was previously described in [93]. (b) IgM antibody response of discordant subjects with plasma samples from previously infected subjects included as positive controls against SARS-CoV-2 FLS. (c) IgA antibody response of discordant subjects with plasma samples from previously infected subjects included as positive controls against SARS-CoV-2 FLS and RBD. (d) Comparison of IgG antibody responses against SARS-CoV-2 FLS protein following one dose of BNT162b2 mRNA vaccination between previously infected, non-exposed, and 5 discordant individuals, (1185, 1340, 1383, 1418, 1637) for whom post-vaccine 1 samples were available. The probability of a significant difference between groups was calculated using the Mann-Whitney test with *p* values shown above lines spanning the groups compared.

2.5.3 Innate Immunity against SARS-CoV-2

To investigate whether any of these discordant individuals had evidence of intrinsic interferon responses against SARS-CoV-2, we tested for receptor blocking activity and measured IFN-α in their plasma. There was no significant plasma-mediated inhibition of SARS-CoV-2 spike binding to angiotensin converting enzyme 2 (ACE2) in any of the 11 discordant cases (Figure 2.2a) and no significant difference in circulating IFN- α levels for the 11 discordant individuals compared to matched controls (Figure 2.2b).

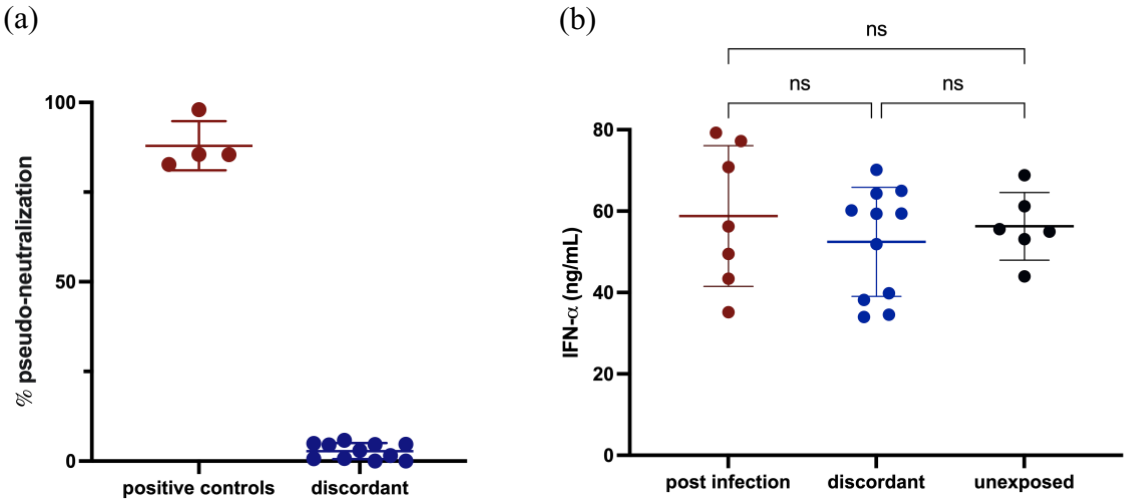

Figure 2.2 Presence of underlying and innate immune protection.
Assessment of potential for innate protection against SARS-CoV-2 infection measured by ELISA. (a**)** The ability of plasma from discordant subjects to inhibit the SARS-CoV-2 S interaction with ACE2 was tested with plasma from previously infected subjects included as positive controls. (b) Circulating IFN-α levels in plasma from discordant subjects and age and sex matched previously infected and unexposed individuals were measured and compared. The probability of a significant difference between groups was calculated using the Mann-Whitney test with *p* values shown above lines spanning the groups compared.

2.5.4 Cross-Reactive Immunity with Common β-Coronaviruses

In a previous study, we found that some individuals seronegative for antibodies against SARS-CoV-2 S have cross-reactive antibodies against SARS-CoV-2 N protein resulting from infection with common β-coronaviruses [93]. To investigate cross-reactive immunity against common β-coronaviruses in the discordant individuals, we measured plasma IgG anti-N antibodies against SARS-CoV-2, OC43 and HKU1 β-coronaviruses. Relatively low, but detectable levels of IgG reactivity against SARS-CoV-2 N protein were present in plasma from the 11 discordant individuals (Figure 2.3). Antibody activity was significantly greater against HKU1 and OC43 nucleocapsid proteins compared to SARS-CoV-2, suggesting that previous infection with these common β-coronaviruses underlay the presence of antibodies against SARS-CoV-2 N protein. Since exposure to the common β-coronaviruses was ubiquitous prior to the emergence of COVID-19, we compared IgG anti-SARS-CoV-2, OC43, and HKU1 N levels in the 11 discordant individuals' plasma samples to levels in plasma from a set of age and sex matched individuals collected before October 2019. There was no significant difference in anti-N antibody level against any N protein between the groups (Figure 2.3), indicating that development of cross-reactive antibodies against SARS-CoV-2 N from previous exposure to common β-coronaviruses was not a distinguishing feature of the 11 discordant individuals we identified.

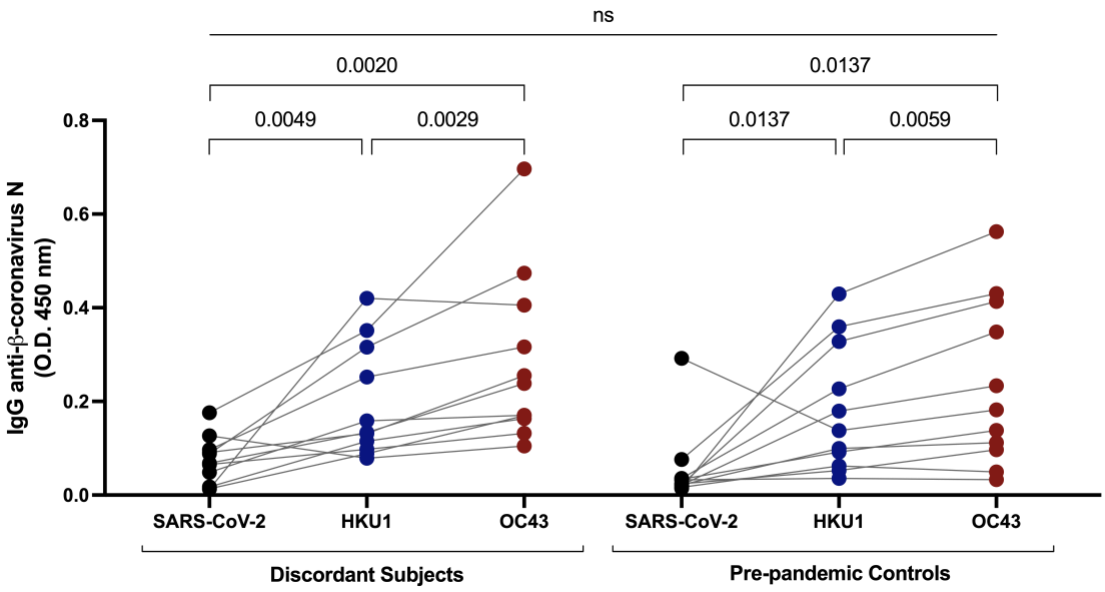

Figure 2.3 Measurement of cross-reactive antibodies against β-coronaviruses.
IgG responses against N antibodies against SARS-CoV-2, and common β-coronavirus HKU1 and OC43 in plasma samples from the discordant cases were measured and compared to pre-pandemic plasma samples from age and sex matched controls. The probability of a significant difference between groups was calculated by Mann-Whitney test and responses to different N proteins compared by Wilcoxon signed rank test with *p* values above lines spanning the groups compared.

2.5.5 T Cell Responses to SARS-CoV-2

Antigen specific T cell responses have previously been reported in individuals who tested negative by PCR and remained seronegative following exposure to SARS-CoV-2 proteins. We tested for cellular immune responses in our discordant case cohort using three peptide pools spanning SARS-CoV-2 S, N, and envelope/membrane (E/M) proteins. Six of the eleven discordant individuals tested had ≥ 50 IFN-γ producing T cells/10^6 PBMC above background in response to at least one of the peptide pools and were deemed responders on this basis (Figure 2.4a). Of note, subject 1185 had the greatest response with 475 IFN-γ producing S-specific T cells/10^6 PBMC and subject 1637 had greater than 100 IFN-γ producing T cells/10^6 PBMC in response to all 3 peptide pool stimulations. Thus, more than half of the discordant cases showed evidence of T cell immunity either from previous infection with common coronaviruses or from exposure to SARS-CoV-2 through close personal contact with one or more infected family members. The magnitude of the IFN-γ responses correlated significantly with time between sample collection and SARS-CoV-2 exposure (Figure 2.4b). Comparison of time between exposure and sample collection for ELISpot responders versus non-responders indicated that the responders as a group had significantly less time between SARS-CoV-2 exposure and sample collection (Figure 2.4c). While the T cell responses could reflect responses to cross-reactive epitopes in common coronaviruses, the inverse correlation between time since exposure and magnitude of the T cell response suggests a specific cellular response induced by exposure to SARS-CoV-2. This relationship with time since exposure also suggests that additional cellular immune responses against SARS-CoV-2 in the exposed seronegative individuals might have been

detected if we had collected samples at earlier time points post exposure. Time since exposure was the only parameter that significantly correlated with strength of the cellular immune response against SARS-CoV-2. No other immunological parameters we measured correlated significantly, including IgG responses against coronavirus N proteins, and IgG, IgA and IgM responses against SARS-CoV-2 S protein. Age can also play a role in reducing the strength of immune responses induced following exposure to antigens and reducing their durability, but we found no significant difference in age between responders and non-responders (Figure 2.4d).

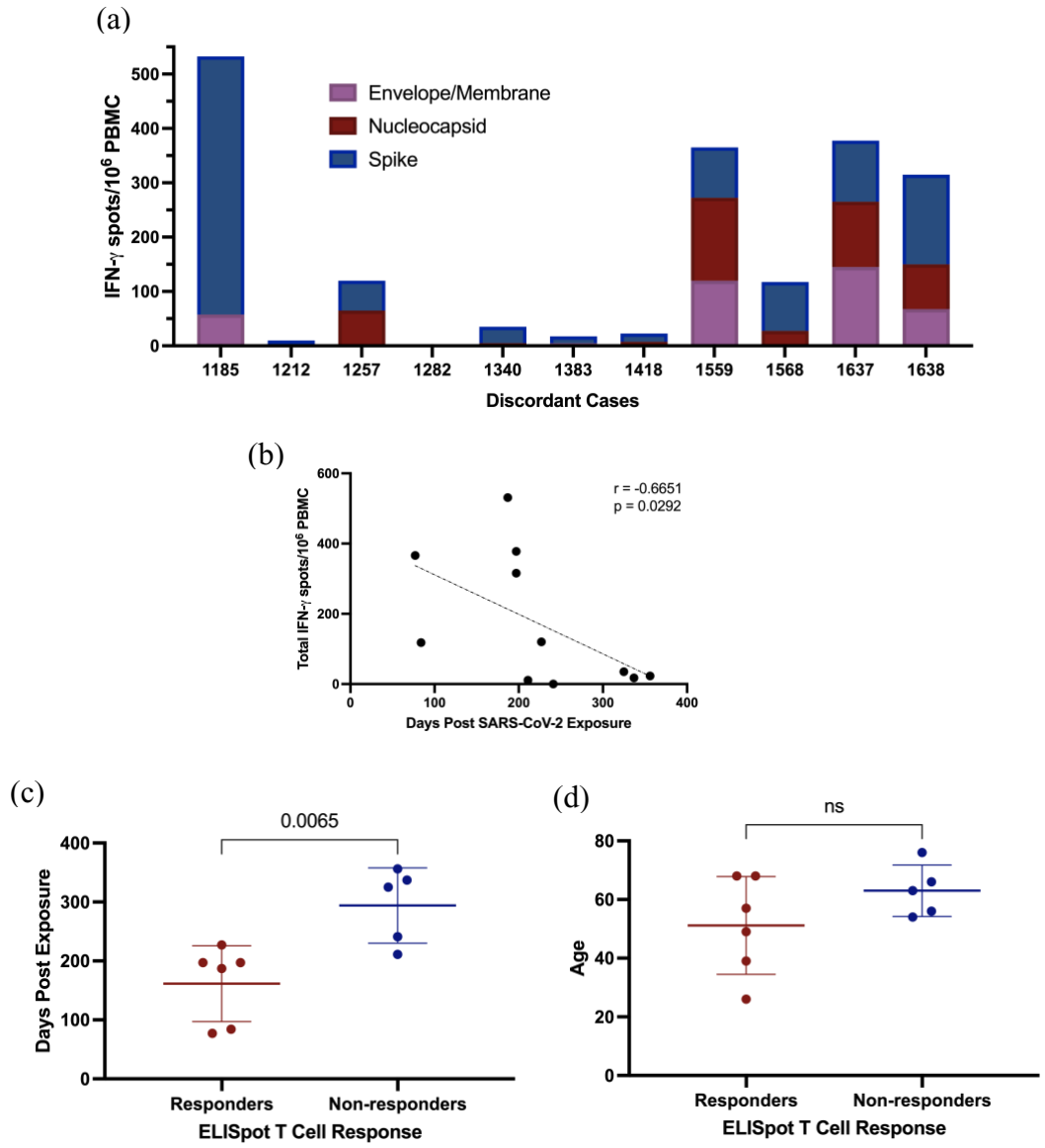

Figure 2.4 Specific cellular immune responses in discordant case subjects.
Production of IFN-γ by PBMC from discordant case subjects following stimulation with SARS-CoV-2 protein overlapping peptide pools. (a) IFN-γ producing T cells/10^6 PBMC of discordant case subjects following 24-hour stimulation with SARS-CoV-2 E/Mem, N, and S protein peptide pools. (b) Scatterplot with line of best fit showing the relationship between total IFN-γ producing T cells detected by ELISpot following stimulation and the number of days post exposure. Spearman correlation was done to assess the significance of correlation with correlation coefficient (r) and *p* value shown within graph plot. (c) Days post exposure and (d) age were compared between responders and non-responders on ELISpot. The probability of a significant difference between groups was calculated using the Mann-Whitney test with *p* values shown above lines spanning the groups compared.

To discriminate $CD4^+$ and $CD8^+$ T cell responses against SARS-CoV-2 in these individuals, PBMC of responders from the same sample time point used for ELISpot assays were stimulated with SARS-CoV-2 peptide pools for 7-days *in vitro* and then analyzed for IFN-γ production following 5-hour restimulation (Figure 2.5a-f). Of the six responders by ELISpot, four had both $CD4^+$ and $CD8^+$ T cell responses to the SARS-CoV-2 peptide pools following *in vitro* restimulation (Figure 2.5g, h). Notably, subject 1185 had 4.8% of their $CD8^+$ T cell population responding against S and subject 1638 had 8.7% of their $CD8^+$ T cell population responding against the E/Mem peptide pool combination. Four individuals in our discordant cohort had robust SARS-CoV-2 specific T cell responses between 2-6 months following exposure despite testing PCR negative, remaining seronegative, and having no overt symptoms of infection. This may indicate that these individuals experienced viral replication but cleared all viral progeny before seroconversion through either rapidly developing immunity, or pre-existing cross-reactive immunity.

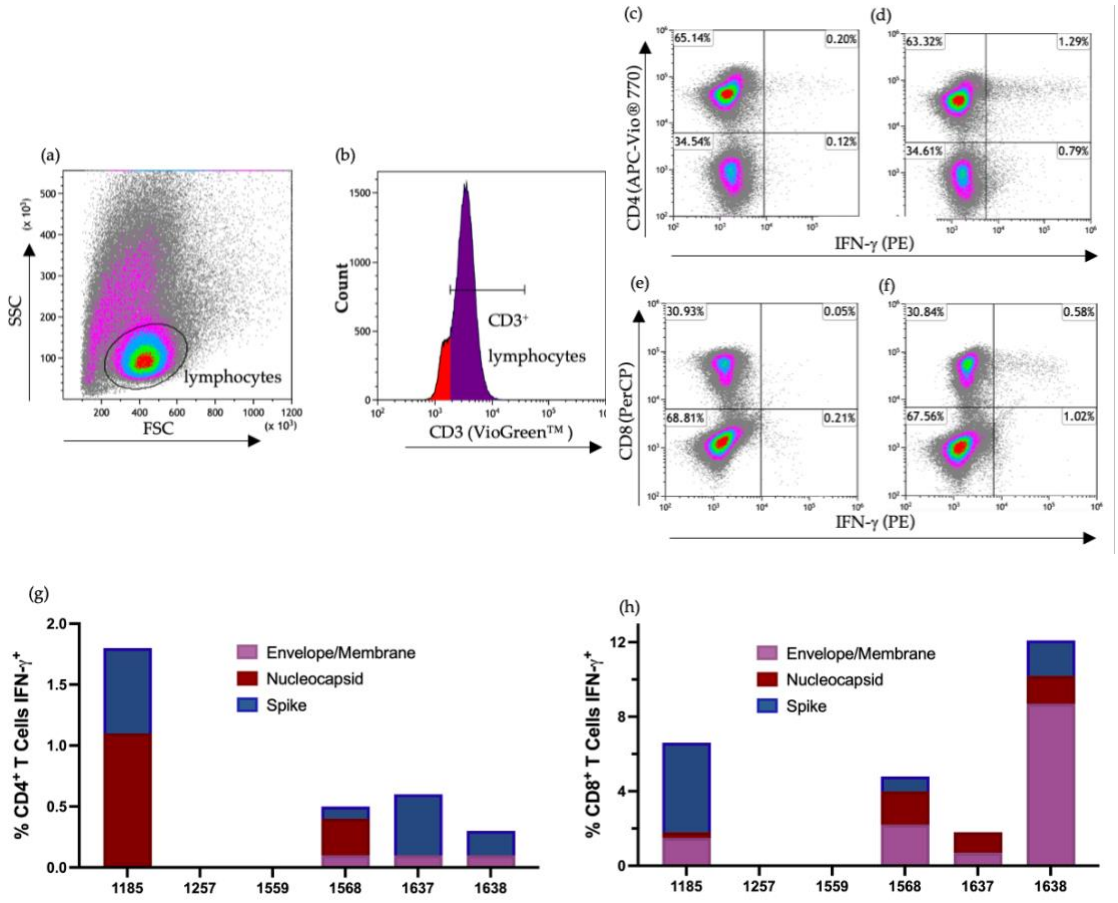

Figure 2.5 Quantification of T cell responses in discordant case subjects.
Flow cytometry gating strategy to discriminate SARS-CoV-2 specific $CD4^+$ and $CD8^+$ T-cell responses. Following 7-day stimulation, cells were stained for extracellular CD3, CD4 and CD8 to discriminate T-cell populations and for intracellular IFN-γ to identify SARS-CoV-2 specific T cells. (a) Gating on lymphocytes following 7-day *in vitro* stimulation. (b) Gating on $CD3^+$ lymphocytes. (c) Non-restimulated $CD4^+$IFN-γ^+ cells and (d) $CD4^+$ IFN-γ^+ cells after 5-hour stimulation with SARS-CoV-2 N protein peptide pool. (e) Non-restimulated $CD8^+$IFN-γ^+ cells and (f) $CD8^+$IFN-γ^+ cells after 5-hour stimulation with SARS-CoV-2 S protein peptide pool. Data were analyzed and visualized using Kaluza Version 2.1 (Beckman Coulter). Total percentages of the (g) $CD4^+$ and (h) $CD8^+$ T cell populations responding to SARS-CoV-2 protein peptide pools with IFN-γ production after 7-day *in vitro* stimulation.

2.6 Discussion and conclusion

In this this study, we assessed immunity against SARS-CoV-2 in individuals who experienced prolonged close contact with COVID-19 but showed no overt signs of infection. As these samples were collected prior to the widespread availability of COVID-19 vaccines, this cohort of discordant cases all lacked detectable IgG responses to SARS-CoV-2 S protein. Seronegativity for SARS-CoV-2 S clearly distinguished them from their infected close contacts and from the vast majority of individuals with RT-PCR-confirmed SARS-CoV-2 infection. Within the discordant cohort, there was also no evidence of IgM or IgA antibodies specific for SARS-CoV-2 S protein. Anti-SARS-CoV-2 N IgG responses were present within the cohort, but by comparing them with IgG responses against two common β-coronavirus (OC43 and HKU1) N proteins, we concluded that the response against SARS-CoV-2 most likely represented cross-reactive antibodies due to previous immunogenic exposure(s) to common β-coronaviruses. Similar results showing cross-reactive antibodies against SARS-CoV-2 from common coronavirus infection have previously been reported [89], [90]. Comparison to pre-pandemic IgG antibody responses against SARS-CoV-2, OC43, and HKU1 in age- and sex-matched controls revealed that cross-reactive antibodies against SARS-CoV-2 N were no more prominent in the discordant case cohort than in the general population. While we can infer that common β-coronaviruses, specifically OC43, circulated in Newfoundland and Labrador and induced antibodies cross-reactive against SARS-CoV-2, there was no evidence that more repeated or more recent exposure was responsible for an apparent resistance to SARS-CoV-2 infection in the exposed uninfected individuals whom we identified. Given their

widespread circulation, especially amongst younger, school-aged individuals, we would expect the majority of the population has been exposed to common coronaviruses, either in Newfoundland and Labrador or elsewhere while travelling, and, thus, have circulating IgG antibodies cross-reactive against SARS-CoV-2 N protein.

A previous suggestion that elevated levels of circulating ACE2 provide some protection from severe COVID-19 [96] led us to consider the possibility that higher-than-normal levels of circulating ACE2 could also protect against infection by inhibiting SARS-CoV-2 attachment to membrane-bound ACE2. However, within this cohort, we saw no evidence of meaningful receptor blocking by circulating ACE2. Constitutively elevated levels of circulating IFN-α can provide non-specific protection from viral infection [97], but there was no evidence of higher plasma IFN-α levels in the discordant cohort compared to matched control groups.

The detection of T cell responses against SARS-CoV-2 in seronegative individuals varies quite widely based on the method used, with proliferation and expression of markers of immune activation more licentious than IFN-γ ELISpot. We tested against peptides representing only a small fraction of the SARS-CoV-2 genome and 6 of the 11 discordant cases that we identified had circulating SARS-CoV-2-specific T cells detectable by IFN-γ ELISpot. The 5 exposed individuals whom we identified that did not have detectable T cell responses in our ELISpot assay were tested after a significantly longer interval from exposure than those with T cell responses, suggesting responses may have waned below detectable levels in some of these 5 cases. It is likely that the T cell responses elicited from exposure without seroconversion are less durable than those elicited by PCR-confirmed

infection and especially less durable than those elicited by severe infection [95], [98], [99]. Due to the waning of responses, the true incidence of induction of cellular immunity against SARS-CoV-2 from exposure without seroconversion may be underestimated when testing is delayed. When testing only includes a small subset of potential T cell epitopes from SARS-CoV-2, this also increases the possibility of underestimating the incidence of cellular immunity.

Further investigation of the T cell response to SARS-CoV-2 following a 7-day in vitro stimulation showed that 4 of the 6 ELISpot responders within the discordant case cohort had both $CD4^+$ and $CD8^+$ T cell responses to the same SARS-CoV-2 peptide pools, indicative of some level of viral replication in host cells. This type of robust virus-specific T cell response in the absence of seroconversion has also been reported in some cases of asymptomatic COVID-19 [80], in populations at high risk for hepatitis C virus (HCV) infection, and in populations at high risk for human immunodeficiency virus (HIV) infection [100]–[103]. The 2 cases with SARS-CoV-2-specific T cell responses detected on ELISpot with no evidence of in vitro expansion of SARS-CoV-2-specific T cells following the 7-day stimulation could be attributed to collateral activation by cytokines from T cells responding to a non-SARS-CoV-2 antigen or acute IFN-γ production by unstable memory T cells [73], [103]–[105].

While the relationship between time since exposure and a detectable T cell response offers evidence that exposure to SARS-CoV-2 underlies these responses, several previous studies showed that SARS-CoV-2-specific T cell responses in exposed uninfected individuals can result from cross-reactive responses against epitopes shared with common

β-coronaviruses [89], [106]. Of 100 potential antigenic S peptides identified among SARS-CoV-2 and 4 common coronaviruses, NL63, 229E, OC43, and HKU1, 8 have ≥ 67% aa identity, indicating possible cross-reactivity [107]. Delineation of the specific epitopes that elicited responses by T cells in the exposed uninfected individuals and their comparison across SARS-CoV-2, OC43, and HKU1 could resolve this issue; however, we were limited by the availability of PBMC collected prior to vaccination from the 11 discordant case individuals. Given the relationship between detection of the responses and time since exposure to SARS-CoV-2 and similar examples with other viruses, we favour the possibility that the SARS-CoV-2-specific T cell response reflects an acute response to SARS-CoV-2 exposure rather than long-term cross-reactive T cell memory formed through previous infection with common β-coronaviruses. Considering the small sample size and other confounding factors, this remains a speculative assumption.

If responses observed in the exposed uninfected individuals reflect exposure to SARS-CoV-2 in a setting that allows *de novo* T cell responses in the absence of seroconversion, this likely relates to individual variability in the nature of exposure. Expansion of both $CD4^+$ and $CD8^+$ T cells in 4 of 6 responders following in vitro stimulation with SARS-CoV-2 peptides indicates exposure to low amounts of replicating virus that can stimulate T cell responses in the absence of seroconversion. This phenomenon was illustrated three decades ago in immunological studies following mucosal exposure to HIV and in simian immunodeficiency virus (SIV) vaccination experiments [75], [76]. It is possible that within our discordant cohort, low levels of viral replication resulted in T cell memory development, but this short-lived acute infection was cleared by

the T cells themselves or by other factors before a detectable antibody response could develop. There was no evidence that the humoral response to COVID-19 vaccination was primed through whatever exposure elicited the T cell responses. While this study was carried out with a small number of subjects, the fact that 11 discordant cases were identified within a relatively small number of subjects screened suggests that this is not a rare phenomenon. The overall significance of antiviral cellular immunity developing in the absence of seroconversion is unknown as it remains an open question whether its rapid development played a key role in viral containment and whether determining the fine specificity of these responses can inform better vaccine strategies. It will be important to investigate this phenomenon in larger, more controlled studies to determine if and how pre-existing or rapidly developing cellular immunity can abrogate SARS-CoV-2 infection.

This investigation was limited by the rarity of exposed uninfected individuals recruited into our study. In light of this small sample size, the results reported may not extend beyond the group studied and not apply to the general population. Conclusive results on the presence or absence of cellular immune responses against SARS-CoV-2 would require samples from earlier time points and testing against the entire SARS-CoV-2 peptidome, which was not possible due to limiting cell numbers and the availability of SARS-CoV-2 peptide sets. Therefore, we cannot definitively exclude the possibility of cellular immune responses against SARS-CoV-2 being present in the individuals categorized as non-responders based on our ELISpot assays. We were also limited in the ability to conduct confirmatory or follow-up testing by a lack of additional samples

collected closer to the time of exposure and prior to vaccination or subsequent COVID-19 infection.

Chapter 3: Characteristics of Systemic Vaccine and Infection-induced anti-SARS-CoV-2 Spike IgA Responses

This manuscript was published with the following citation.

Norton, N.J.; Ings, D.P.; Fifield, K.E.; Barnes, D.A.; Barnable, K.A.; Harnum, D.O.A.; Holder, K.A.; Russell, R.S.; Grant, M.D. Characteristics of Vaccine- and Infection-Induced Systemic IgA Anti-SARS-CoV-2 Spike Responses. *Vaccines* 2023,*11*,1462. https:// doi.org/10.3390/vaccines11091462

* Note that reference numbers as well as the figure numbers from the published manuscript differ within the following chapter in accordance with the reference numbers and figures within thesis.

3.1 Co-authorship statement

In the following manuscript, I conceptualized, conducted, designed, and planned all the experiments as well as gathered, analyzed, and reviewed the data generated. As the primary author, I wrote the manuscript draft and constructed the figures within. The following co-authors contributed to the generation of this manuscript. D.O.A.H: Recruited and consented subjects, scheduled appointments to collect blood samples. D.P.I: Processed incoming samples and managed data. K.E.F, K.A.B & D.A.B: Processed incoming samples. R.S.R: Reviewed manuscript drafts. K.A.H: Provided experimental guidance. M.D.G: Consulted on experiment planning, aided in data analysis and interpretation, reviewed data and manuscript drafts. Funding was secured by M.D.G, R.S.R, and K.A.H.

3.2 Abstract

Mucosal IgA is widely accepted as providing protection against respiratory infections, but stimulation of mucosal immunity, collection of mucosal samples and measurement of mucosal IgA can be problematic. The relationship between mucosal and circulating IgA responses is unclear, however, whole blood is readily collected and circulating antigen-specific IgA easily measured. We measured circulating IgA against SARS-CoV-2 spike (S) to investigate vaccine- and infection-induced production and correlation with protection. Circulating IgA against ancestral (Wuhan-Hu-1) and Omicron (BA.1) S proteins was measured at different time points in a total of 143 subjects with varied backgrounds of vaccination and infection. Intramuscular vaccination induced circulating anti-SARS-CoV-2 S IgA. Subjects with higher levels of vaccine-induced IgA against SARS-CoV-2 S (p = 0.0333) or receptor binding domain (RBD) (p = 0.0266) were less likely to experience an Omicron breakthrough infection. The same associations did not hold for circulating IgG anti-SARS-CoV-2 S levels. Breakthrough infection following two vaccinations generated stronger IgA anti-SARS-CoV-2 S responses (p = 0.0002) than third vaccinations but did not selectively increase circulating IgA against Omicron over ancestral S, indicating immune imprinting of circulating IgA responses. Circulating IgA against SARS-CoV-2 S following breakthrough infection remained higher than vaccine-induced levels for over 150 days. In conclusion, intramuscular mRNA vaccination induces circulating IgA against SARS-CoV-2 S, and higher levels are associated with protection from breakthrough infection. Vaccination with ancestral S enacts imprinting within circulating IgA responses that become apparent after breakthrough infection with Omicron. Breakthrough infection

generates stronger and more durable circulating IgA responses against SARS-CoV-2 S than vaccination alone.

3.3 Introduction

The COVID-19 pandemic inspired massive scientific and clinical research efforts that introduced and distributed vaccines against SARS-CoV-2 within a uniquely accelerated time frame. As of July 2023, more than 13 billion vaccine doses have been administered globally, over 98 million of which were administered within Canada [60], [108]. While these vaccines continue to protect against severe illness, they fail to provide sterilizing immunity against emerging SARS-CoV-2 variants [22], as clearly illustrated by the widespread occurrence of Omicron breakthrough infections. Ongoing diversification of SARS-CoV-2 raised concerns around the ability of ancestral Wuhan-Hu-1 based SARS-CoV-2 vaccines to continue providing protection against illness, prompting introduction of bivalent mRNA vaccines encoding both ancestral and Omicron spike (S) antigens.

While IgG subclass antibodies (Ab) reach the highest levels in circulation, IgA Ab dominate at mucosal sites, where they may play a more significant role in protection from respiratory and other mucosal infections. Unfortunately, systemically administered, non-replicating vaccines are conspicuously poor at inducing mucosal IgA responses. Following initial antigenic exposure, responding B cells secrete immunoglobulin (Ig) M subclass Ab with relatively low affinity and poor tissue penetration. With T cell help, proliferating B cells in newly formed germinal centres undergo somatic hypermutation and isotype switching, differentiate into plasmablasts, and potentially become plasma cells. Isotype

switching to IgG predominates systemically, while isotype switching to IgA predominates mucosally. Most IgA produced mucosally is secreted as a dimer into mucosal fluids lining the oronasal, esophageal, lower respiratory, gastrointestinal, and urogenital tracts, which are sites of entry and/or replication for many pathogens. Monomeric IgA circulates in the blood stream and there are conflicting reports on the relationship between mucosal and plasma IgA responses [37], [38]. Mucosal IgG composition reflects spillover from the circulation, but commonalities in origin between systemic and mucosal IgA remain undefined [64], [109].

While mucosal IgA is most likely to contribute protection from respiratory infections, for ease and simplicity, vaccine-induced Ab production studies primarily focus on circulating IgG responses and their capacity to neutralize SARS-CoV-2 variants [23]–[27]. Vaccine-induced anti-SARS-CoV-2 IgG responses are optimized with sufficient time intervals between doses yet fall short of responses seen with hybrid immunity [95], [110]–[112]. Furthermore, subjects who experience infection with SARS-CoV-2 variants following vaccination with ancestral S-based vaccines continue to display preferential IgG responses against ancestral S. This favouring of an immune response towards previously encountered, closely related versions of extant antigen is known as original antigenic sin, or immune imprinting [69]. Such a preference to reactivate existing memory B cells at the expense of *de novo* B cell activation potentially reduces the ability to neutralize emerging variants [69]. While imprinting is well described for circulating IgG, there has been less study of this phenomenon with circulating or mucosal IgA.

In this study, we investigated circulating IgA anti-SARS-CoV-2 S responses after vaccination and after breakthrough infection (SARS-CoV-2 infection after receiving at least 2 COVID-19 vaccines) to better understand the impact of breakthrough infection relative to vaccination on anti-SARS-CoV-2 S IgA levels. As intramuscular vaccination delivers antigens differently than infection and does not favour IgA production, we also assessed whether vaccination with ancestral S caused imprinting of the IgA response in the context of Omicron breakthrough infection. By comparing circulating anti-SARS-CoV-2 S IgA levels in subjects who became infected after vaccination to levels in those who remained uninfected, we tested whether circulating IgA anti-SARS-CoV-2 S or IgA anti-SARS-CoV-2 S receptor binding domain (RBD) levels can predict protection from infection.

3.4 Methods & Materials

3.4.1 Study participants and sample processing

This study was approved by the Newfoundland and Labrador Health Research Ethics Board and carried out in accordance with the recommendations of the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. Study subjects are nested within an ongoing study cohort at Memorial University of Newfoundland and Labrador, where participants were recruited into the study based on previous reverse-transcriptase polymerase chain reaction (RT-PCR) confirmed or suspected SARS-CoV-2 infection [93]. Written informed consent was obtained for whole blood collection in accordance with the Declaration of Helsinki. Subjects completed a questionnaire at study intake on SARS-CoV-2 exposure, testing and symptom history. Individuals who self-reported Omicron infections – between February and August 2022 – based on RT-PCR or rapid test results following receipt of at least two Health Canada approved anti-SARS-CoV-2 vaccinations were identified and selected for further study. Previous infection was confirmed by detection of antibodies selective for SARS-CoV-2 nucleocapsid (N) [93]. Whole blood was drawn by forearm venipuncture into acid-citrate-dextrose preserved vacutainers and plasma collected after centrifuging 10 minutes at 500 *g*. Plasma was stored immediately at -80°C until testing.

3.4.2 Assessment of circulating IgG and IgA anti-SARS-CoV-2 S levels by ELISA

Plasma was thawed on ice, diluted in phosphate buffered saline (PBS) containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO,

USA) and tested for anti-SARS-CoV-2 Ab by enzyme-linked immunosorbent assay (ELISA) using recombinant proteins as antigens. Proteins were coated overnight at 4°C at 50 ng/well in 50 μL Dulbecco's PBS (Corning, Mediatech, Inc., Manassa VA, USA) onto 96-well Immulon-2 HB (Thermo Fisher Scientific, Rochester, NY, USA) ELISA plates to detect Ab against SARS-CoV-2 Wuhan-Hu-1 full-length spike (FLS, SMT1-1 Wuhan-Hu-1, National Research Council of Canada), and RBD (Sino Biological, Wayne, PA, USA) and SARS-CoV-2 Omicron BA.1 FLS (SMT1-1 Omicron BA.1, National Research Council of Canada) and RBD (Omicron BA.1, ACROBiosystems, Newark, DE, USA). Plates were washed 4 times after overnight coating, and 6 times between all subsequent steps, with 300 μL/well PBS plus 0.05% Tween 20. Plates were blocked with 200 μL 1% BSA in PBS for 1 hour after which 100 μL diluted plasma was added for 1.5 hours, and 100 μL diluted goat-anti human IgG or IgA horseradish peroxidase (HRP)-conjugated detection antibodies, (Jackson ImmunoResearch, Baltimore Pike, West Grove, PA, USA) was added to the wells for 1 hour. Colour was developed using 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB, BD Biosciences, OptEIA™ Substrate Reagents A & B, San Diego, CA, USA) for 20 minutes and the reaction stopped by adding 100 μL 1 M H_2SO_4 (Sigma-Aldrich). Optical density (OD) was read at 450 nm on BioTek Synergy HT plate reader. Plasma was diluted 1:100 to assess IgG Ab levels and 1:50 to assess IgA Ab levels. The anti-IgG*HRP conjugate was diluted 1:50,000 and anti-IgA*HRP was diluted 1:25,000.

3.4.3 Statistical analysis

All statistical analyses were done using GraphPad Prism Version 9.5.1. Significance values, where applicable, are expressed above lines spanning the groups being compared. As not all data were normally distributed, non-parametric Mann-Whitney, Wilcoxon signed rank, and Spearman correlation tests were used to compare medians between groups, assess differences in paired data and assess correlation respectively.

3.5 Results

3.5.1 Study cohort

For an ongoing study of immunity against SARS-CoV-2 initiated in March 2020, individuals in Newfoundland and Labrador were recruited based on confirmed or suspected SARS-CoV-2 infection. Subjects provided whole blood samples every 3 months throughout their course of vaccinations against COVID-19 and potential exposures to SARS-CoV-2. We selected non-immunocompromised individuals with documented evidence of infection following at least two doses of Canadian Public Health Agency approved vaccines, Pfizer BioNTech (BNT162b2), Moderna (mRNA-1273), and AstraZeneca (ChAdOx1). These individuals self-reported positive SARS-CoV-2 antigen rapid test or RT-PCR results between February 21st and August 8th, 2022. All had increases in IgG anti-SARS-CoV-2 N protein levels indicative of infection, and in most cases, experienced symptoms of COVID-19. Humoral immune responses of this group of subjects identified as having breakthrough infections were compared to groups of subjects with at least 2 COVID-19 vaccinations that did not experience breakthrough infection. The first confirmed case of the SARS-CoV-2 Omicron variant was detected in Canada on November 29th, 2021 [113] and in Newfoundland & Labrador (NL) on December 15th, 2021 [114]. Omicron quickly became the dominant strain in NL and across Canada. Although our subjects' infections were not typed for SARS-CoV-2 variants, based on the time since first case in NL, we assumed the majority of infections occurring over the study period were with a SARS-CoV-2 Omicron variant or subvariant (Table 3.1). We further separated subjects into comparison groups based on the number of COVID-19 vaccinations received.

Group size, subject sex, and age, together with information on vaccine types and number of days after vaccination or infection that samples were collected are summarized in Table 3.1.

Table 3.1 General demographics and vaccine types received by subjects grouped by number of vaccines and incidence of SARS-CoV-2 breakthrough infection.

	2 Vaccines	**2 Vaccines + Omicron**	**3 Vaccines**	**3 Vaccines + Omicron**	**4 Vaccines**
n	13	12	49	47	22
Sex (M/F)	6 / 7	6 / 6	20 / 29	16 / 31	10 / 13
Age (mean, range)	41 (16-65)	40 (15-65)	55 (25-72)	53 (22-75)	63 (37-74)
[a]**Days Post Vaccine** (mean)	61 days	79 days	57 days	66 days	59 days
[b]**Vaccine Types** (mRNA-1273 / BNT162b2 / ChAdOx1)	1 / 10 / 2 2 / 11 / 0	2 / 11 / 0 3 / 10 / 0	5 / 38 / 6 13 / 36 / 0 22 / 27 / 0	0 / 39 / 8 4 / 41 / 2 21 / 26 / 0	2 / 18 / 2 8 / 14 / 0 9 / 13 / 0 9 / 13 / 0
[c]**Days Post Infection** (mean)	-	40 days	-	52 days	-
Number of Immunogenic Exposures	2	3	3	4	4

[a]-Number of days between last vaccination and sample collection.

[b]-Number of people in each subgroup who received each type of licensed COVID-19 vaccine (mRNA-1273 / BNT162b2 / ChAdOx1) for vaccinations 1 through 4.

[c]-Number of days between documentation of infection and sample collection

3.5.2 Intramuscular vaccination induces circulating IgA

Plasma samples from all subjects were tested by ELISA for IgA anti-SARS-CoV-2 S and RBD responses after intramuscular vaccination. Our data confirm that intramuscular vaccination alone generates readily detectable circulating IgA anti-SARS-CoV-2 Wuhan-Hu-1 FLS (Figure 3.1a). Longitudinal assessment of plasma samples from 22 subjects indicated a significant increase in vaccine-induced circulating IgA responses following third vaccine doses (median OD IgA anti-SARS-CoV-2 FLS ± interquartile range (IQR) 0.36, 0.21 - 0.55 versus 0.57, 0.30-0.83, $p = 0.0006$), however, IgA anti-SARS-CoV-2 S levels in plasma were significantly lower (0.57, 0.30 - 0.83 versus 0.39, 0.30 - 0.65, $p = 0.0053$) after a fourth vaccination at time of measurement (Figure 3.1b).

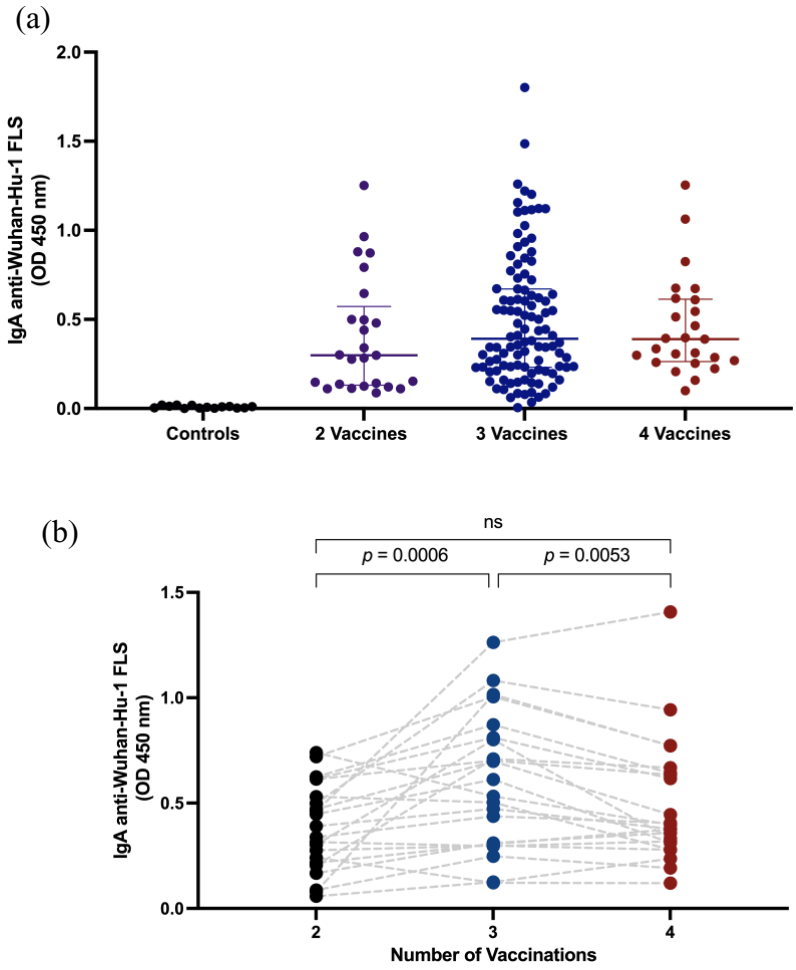

Figure 3.1 Intramuscular vaccines induce circulating IgA antibodies.
Circulating intramuscular vaccine-induced SARS-CoV-2 S-specific IgA. Plasma IgA OD levels against SARS-CoV-2 Wuhan-Hu-1 FLS were measured in pre-pandemic controls and study subjects grouped by number of vaccines received (a). Plasma IgA levels against SARS-CoV-2 Wuhan-Hu-1 FLS were measured longitudinally in subjects following second, third and fourth vaccines (b). Horizontal lines within the grouped data points (a) represent median plus or minus interquartile range (IQR). Comparison of median values between groups was performed using the Wilcoxon signed rank test (b). When significant, probability values for differences between groups are shown above lines spanning the groups compared.

3.5.3 Plasma IgA anti-SARS-CoV-2 S levels and breakthrough infection

We compared levels of IgA anti-SARS-CoV-2 FLS and RBD in 59 subjects who experienced breakthrough infections to levels in 84 subjects who remained uninfected. Mean duration between second and third vaccines is 192 days and between third and fourth vaccines is 238 days. Subjects provided blood samples an average of 72, 44, and 58 days post vaccines 2, 3, and 4, respectively. Following two vaccinations, there was no significant difference in the amount of plasma IgA or IgG anti-SARS-CoV-2 S (Figure 3.2a, b) between groups (Figure 3.2c). The paucity of subjects remaining who had recived only two vaccines limited the strength of this comparison. The subjects with two vaccines were roughly ten years younger than those receiving three vaccines and almost twenty years younger than those receiving four. Following three vaccinations, the group who remained uninfected had stronger vaccine-induced plasma IgA responses against FLS (median OD ± IQR 0.50, 0.31 - 0.76 versus 0.28, 0.17 - 0.61, $p = 0.0471$) and RBD (0.24, 0.13 - 0.46 versus 0.13, 0.05 - 0.46, $p = 0.0266$) compared to subjects who later experienced breakthrough infections (Figure 3.2a, b). There was no significant difference in vaccine-induced anti-SARS-CoV-2 FLS IgG between the same groups (Figure 3.2c). In subjects who experienced breakthrough infections after three vaccines, there was no significant correlation between either vaccine induced anti-FLS (Figure 3.2d) or anti-RBD (Figure 3.2e) IgA levels and days between vaccination and infection.

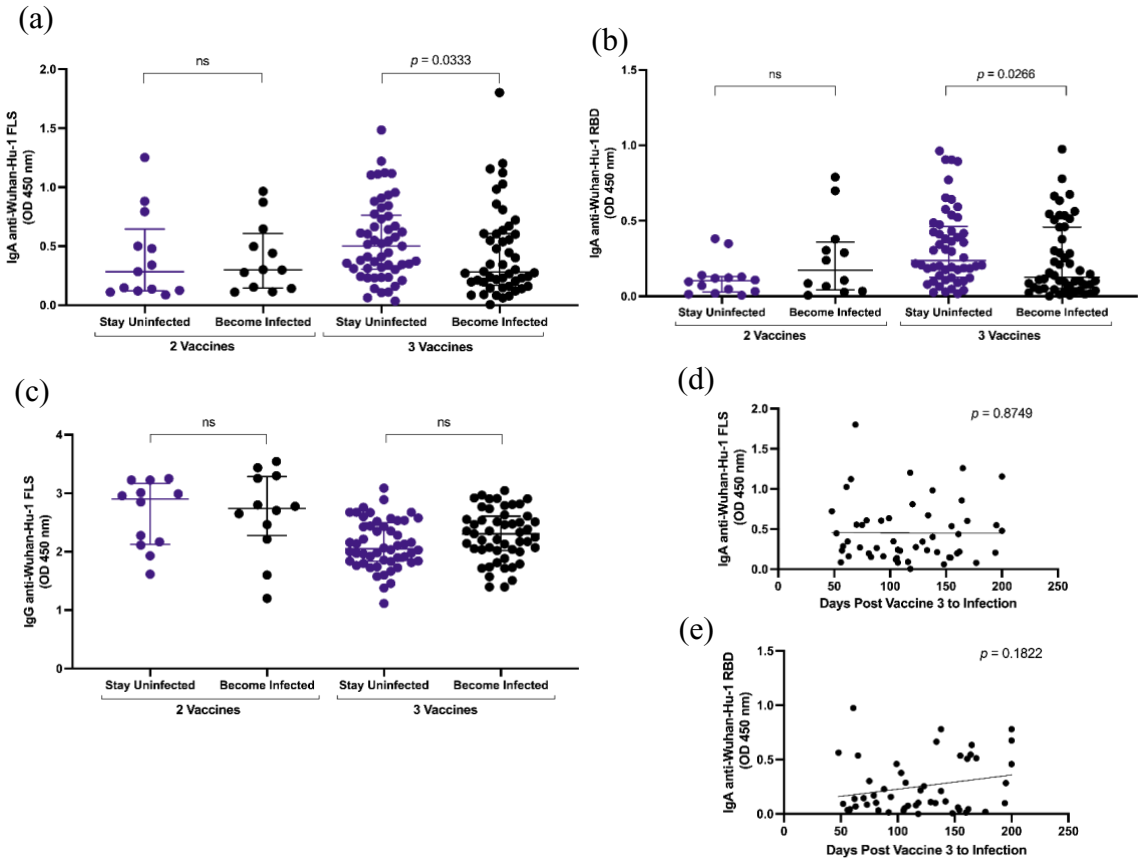

Figure 3.2 Vaccine-induced circulating IgA and IgG responses.
Comparison of plasma IgA and IgG anti-SARS-CoV-2 Wuhan-Hu-1 FLS and RBD in subjects that remained uninfected versus subjects experiencing breakthrough infection. Plasma IgA OD against Wuhan-Hu-1 FLS (a) and RBD (b) in subjects after 2 or 3 vaccines. Plasma IgG OD against Wuhan-Hu-1 FLS in subjects after 2 or 3 vaccines (c). Horizontal lines within the grouped data points represent median plus or minus interquartile range (IQR). Relationship between IgA levels against Wuhan-Hu-1 FLS (d), RBD (e) and days between vaccination and infection was assessed by Spearman correlation. Comparison of median values between groups was done using the Wilcoxon signed rank test. When significant, probability values for differences between groups are shown above lines spanning the compared.

3.5.4 Effect of Omicron breakthrough infection on plasma IgA

Within this study, the term immunogenic exposure refers to the total number of times a subject was exposed to SARS-CoV-2 antigens through either vaccination or infection. We compared IgA anti-SARS-CoV-2 FLS responses between subjects with the same number of immunogenic exposures, with and without SARS-CoV-2 infection. Breakthrough infection after two vaccines produced more robust circulating IgA responses than a third vaccine dose (median OD ± IQR, 1.22, 1.11 - 1.41 versus 0.63, 0.34 - 1.06, $p = 0.0002$, Figure 3.3a). Breakthrough infection after three vaccines resulted in stronger circulating IgA responses than a fourth vaccine dose (median OD ± IQR, 1.20, 0.89 - 1.71 versus 0.39, 0.26 - 0.61, $p < 0.0001$, Figure 3.3a). Longitudinal assessment of circulating IgA levels against SARS-CoV-2 Wuhan-Hu-1 (Figure 3.3b) and Omicron BA.1 FLS (Figure 3.3c) after two vaccines and breakthrough infection and after three vaccines and Omicron breakthrough infection (Figure 3.3d, e) showed that post-infection levels remained significantly greater over 150 days post infection.

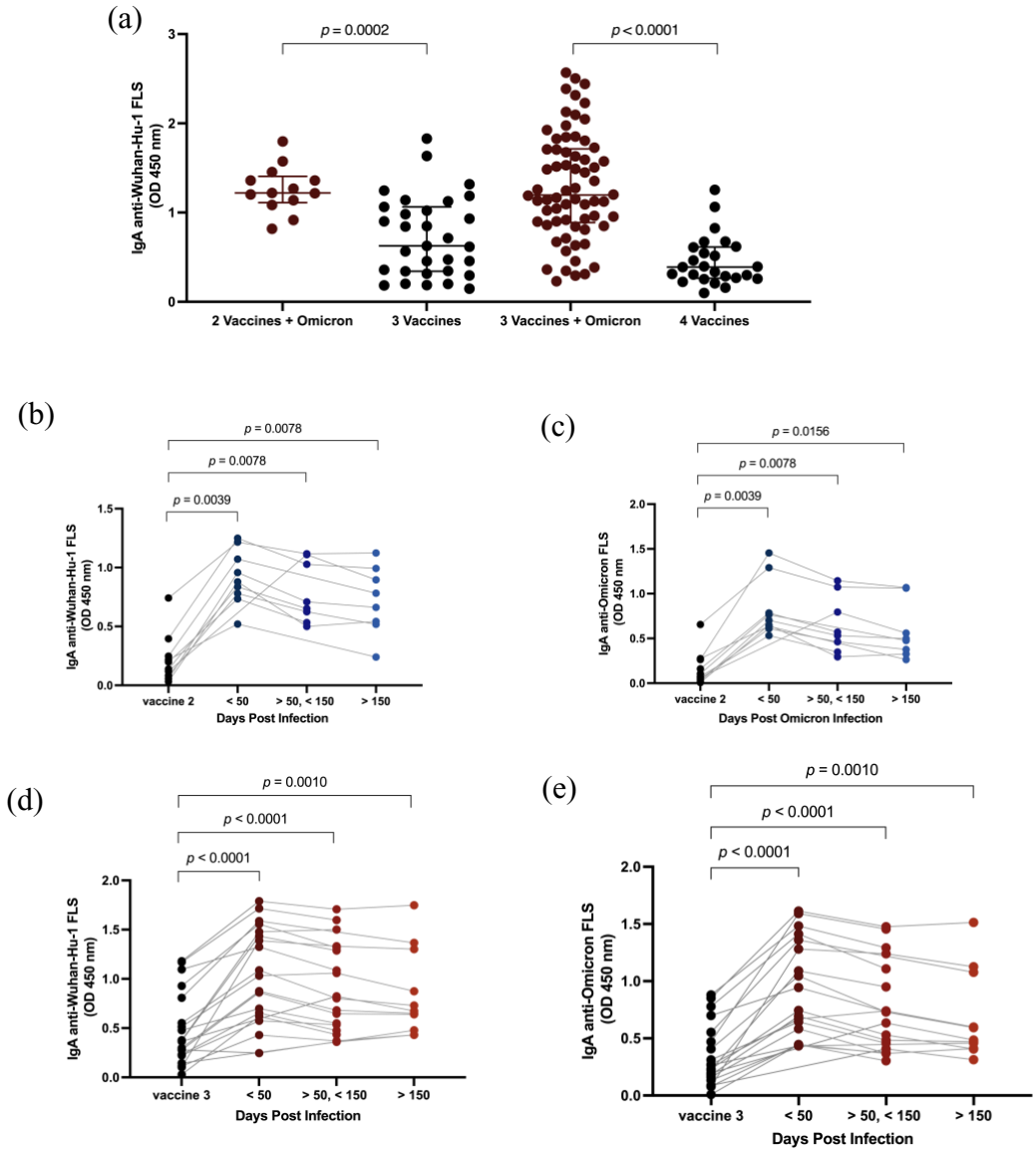

Figure 3.3 Breakthrough infection impact on circulating IgA responses.
Effect of breakthrough infection on plasma IgA levels against SARS-CoV-2. Vaccination versus infection was compared in groups of subjects with 3 or 4 exposures to SARS-CoV-2 S (a). Longitudinal analysis of IgA anti-SARS-CoV-2 Wuhan-Hu-1 (b, d) and Omicron FLS (c, e) in subjects after 2 vaccines and Omicron infection (b, c) and 3 vaccines followed by Omicron infection (d, e). Horizontal lines within the grouped data points (a) represent median plus or minus interquartile range (IQR). Comparison of median values between groups was done using Mann-Whitney test (a) and Wilcoxon signed rank test (b-e). When significant, probability values for differences between groups are shown above lines spanning the groups compared.

3.5.5 Selective recognition of Wuhan-Hu-1 S is preserved despite Omicron breakthrough infection

To investigate whether imprinting of systemic humoral responses against SARS-CoV-2 affects IgA responses similarly to IgG responses, we compared response levels against ancestral Wuhan-Hu-1 FLS versus Omicron BA.1 FLS after vaccination and again following Omicron breakthrough infection. After receiving vaccines based on ancestral Wuhan-Hu-1 S, persons vaccinated three (median OD ± IQR, 0.59, 0.31 - 1.03 0.35, versus 0.63, 0.17 - 0.70, $p < 0.0001$) or four times (0.39, 0.26 - 0.61 versus 0.23, 0.14 - 0.49, $p < 0.0001$) had significantly stronger anti-FLS IgA responses against Wuhan-Hu-1 than against Omicron BA.1 (Figure 3.4a), as expected. However, circulating IgA responses remained stronger against Wuhan-Hu-1 FLS even after Omicron breakthrough infection following two (median OD ± IQR, 1.22, 1.10 - 1.43 versus 0.98, 0.87 - 1.25, $p = 0.0010$) or three (0.87, 0.61 - 1.42 versus 0.67, 0.48 – 1.23, $p = 0.0003$) vaccinations (Figure 3.4a). Favoured recognition of ancestral Wuhan-Hu-1 FLS over Omicron BA.1 FLS response was confirmed for circulating IgG after three vaccines (median OD ± IQR, 2.03, 1.78 - 2.44 versus 1.77, 1.69 - 1.85, $p = 0.0038$), four vaccines (2.06, 2.03 - 2.36 versus 1.51, 1.43 - 1.64, $p < 0.0001$), two vaccines followed by Omicron infection (2.21, 1.89 - 2.42 versus 1.76, 1.72 - 1.83, $p = 0.0005$) and three vaccines (2.33, 2.04 - 2.46 versus 1.62, 1.54 - 1.67, $p < 0.0001$) followed by Omicron infection (Figure 3.4b). Thus, immune imprinting from vaccination impacts SARS-CoV-2 FLS IgA and IgG responses.

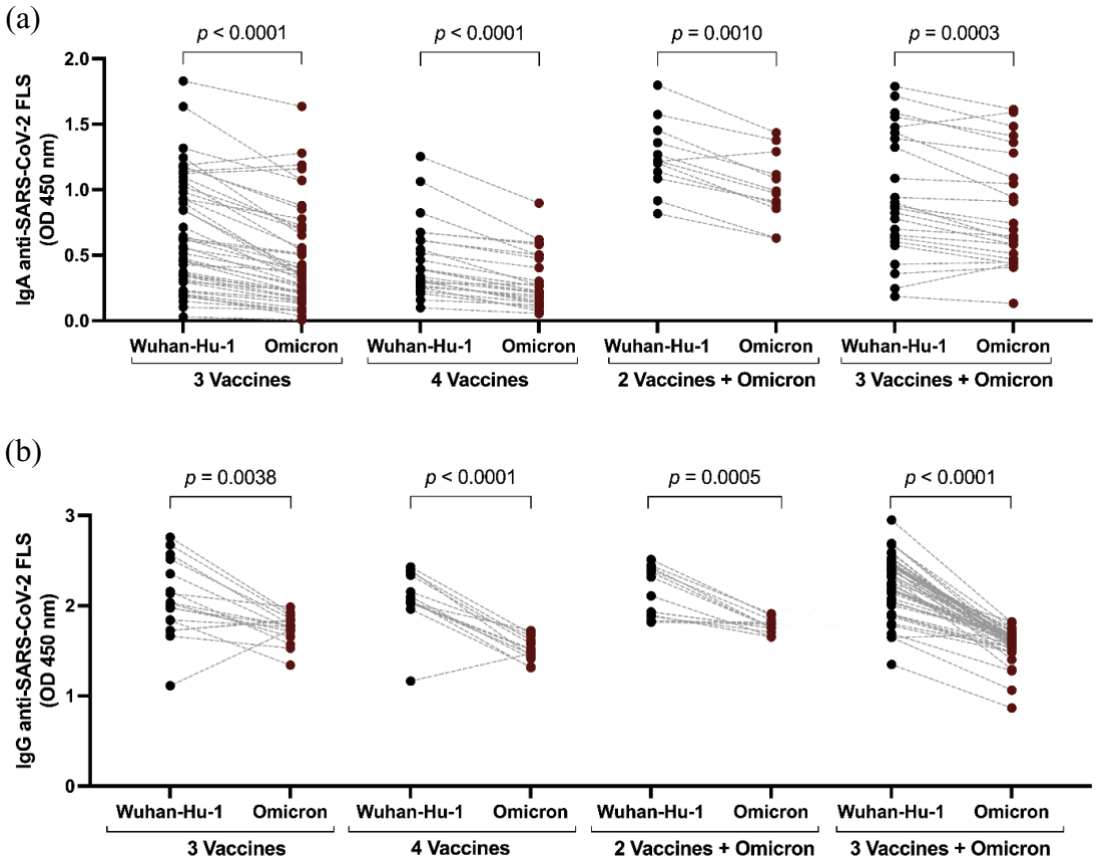

Figure 3.4 Circulating IgA response against SARS-CoV-2 ancestral and Omicron S. Comparison of plasma IgA and IgG Ab responses against SARS-CoV-2 Wuhan-Hu-1 versus Omicron BA.1 FLS after vaccination and after breakthrough infection. IgA (a) and IgG (b) responses against SARS-CoV-2 Wuhan-Hu-1 and Omicron BA.1 FLS were compared in vaccine recipients before and after Omicron breakthrough infection. Comparison of median values between groups was by Wilcoxon signed rank tests with values for the probability of significant differences between groups shown above lines spanning the groups compared.

3.6 Discussion

In this study, we investigated the impact of multiple vaccinations and breakthrough infection on SARS-CoV-2 S specific circulating IgA. We confirmed that two intramuscular vaccinations with mRNA-based vaccines induce systemic IgA anti-FLS and anti-RBD responses against SARS-CoV-2 and that a third immunization boosts this response. However, the increase in circulating anti-SARS-CoV-2 S IgA from a third vaccination was lesser in both magnitude and durability compared to the increase observed from a breakthrough infection following two vaccinations. Persons experiencing breakthrough infections after two or three vaccines had higher levels of circulating IgA anti-SARS-CoV-2 S than persons receiving third or fourth vaccines. Fourth vaccinations had no significant effect on SARS-CoV-2 S-specific circulating IgA, however it is worth noting that the subjects in the four-vaccine subgroup are on average ten years older than the rest of the subgroups, and age is a known factor contributing to a decrease in antibody binding [115]. Higher levels of vaccine-induced circulating anti-SARS-CoV-2 S IgA after three vaccinations were associated with a reduced risk of breakthrough infection, despite no similar association with vaccine-induced anti-SARS-CoV-2 S IgG levels. As with circulating IgG responses, vaccination with an ancestral SARS-CoV-2 S antigen im- posed immunological imprinting on IgA responses with preferred recognition of ancestral SARS-CoV-2 S protein over Omicron SARS-CoV-2 S protein persisting following Omicron breakthrough infection.

Intramuscular vaccines, like the mRNA-based vaccines approved for use against SARS-CoV-2, are taken up by antigen-presenting cells such as dendritic cells in the muscle

and trafficked to the draining lymph node for presentation to T cells. Since the mRNA encoding SARS-CoV-2 S protein is translated inside host cells and the newly synthesized S protein is released for uptake by other cells, antigen presentation through MHC class I and II molecules occurs to prime both $CD4^+$ and $CD8^+$ T cells. Binding antigenic epitopes of SARS-CoV-2 S protein and interacting with $CD4^+$ follicular helper T cells within lymph node germinal centres encourages B cells to undergo proliferation, somatic hypermutation and isotype class switching as they differentiate into Ig-secreting plasma cells or memory B cells. With boosting, the mRNA vaccines generate robust circulating anti-SARS-CoV- 2 S Ab responses, where IgG reaches the highest levels and is most relevant for virus neutralization [28], [112], [116]–[119]. Circulating IgA against SARS-CoV-2 S also rises with booster vaccination and can contribute to neutralization, but IgA anti-SARS-CoV-2 S plateaus at lower levels than IgG. Although not administered in a manner to effectively induce mucosal immunity, intramuscular mRNA vaccines do generate local salivary responses, where IgA levels dominate over IgG [64], [120]. The mucosal anti-SARS-CoV-2 S response can potentially block viral binding to host cells in the oral cavity, nasal and upper respiratory tract, thereby acting directly at sites of exposure to prevent infection. Induction of both serum and mucosal IgA responses after vaccination has also been described with oral rotavirus live attenuated vaccines and intramuscular replication defective lentivirus vaccines [121], [122].

While there is general agreement mucosal IgA provides protection against respiratory infections, there is considerable controversy around the relevance of circulating antiviral IgA. Since IgA is present at easily measurable levels in plasma and serum, it would

be valuable to know how levels and specificity relate to mucosal IgA activity in terms of protection from infection and protection from severe illness. Several studies reported that systemic IgA anti-SARS-CoV-2 is often detectable earlier than either IgM or IgG and that higher systemic levels of SARS-CoV-2-specific IgA detectable early in the course of infection are associated with severe illness [34], [123], [124]. Conversely, other studies found early systemic IgA levels were unrelated to disease severity or even associated with asymptomatic infections [28], [125]. Discrepant results and interpretations of the significance of anti-SARS-CoV-2 IgA in these studies likely reflect variation in the study designs, subject populations (hospitalized versus community) and timing of Ab measurements (acute or convalescent). Our study was no designed or powered to compare the immunogenicity of different mRNA vaccines.

The earlier appearance of IgA anti-SARS-CoV-2 at higher levels than IgM or IgG systemically early after infection would not be predicted, based on the time required for transition from a primary to secondary response and the greater frequency of isotype switching to IgG compared to IgA. One possible explanation is that the early phase systemic IgA response in these cases reflects production of natural antibodies from activation of B1 B cells expressing IgA [126]. Activation of B1 B cells was previously associated with failure to clear acute hepatitis C virus infection, so it may signify failure to rapidly activate potent antiviral adaptive immunity [127], [128]. In contrast, higher IgA levels during convalescence might signify development of robust humoral immunity, enhanced viral clearance and some level of protection against future infection. Both our results and those of Sheikh-Mohamed *et al.,* 2022 [64] indicate that higher levels of

vaccine-induced circulating IgA, but not IgG anti-SARS-CoV-2 S Ab, are associated with some aspect of protection from SARS-CoV-2 infection.

As there are conflicting reports regarding correlation between circulating and mucosal IgA Ab levels, the relationship between circulating and mucosal IgA responses is uncertain [37], [38], [62], [66], [68]. Although circulating IgA is not itself protective at the site of infection, if levels and specificity of mucosal and circulating IgA are closely related, it could serve as an easily measured marker for mucosal antiviral IgA activity. Robust mucosal and systemic responses might be induced independently, or there could be mutual spillover of IgG and IgA between mucosal and systemic sites. Comparison of variable (V) region sequences between circulating IgG and IgA anti-SARS-CoV-2 S and between mucosal and circulating anti-SARS-CoV-2 S-specific B cells is required to address the origin of circulating SARS-CoV-2 S-specific IgA and the nature of its relationship to mucosal IgA. The association between plasma IgA anti-SARS-CoV-2 S levels and protection from infection that we and others observed [64], which was not found for IgG anti-SARS-CoV-2 S levels, suggests that circulating IgA is not simply a fractional representation of the IgG responses reflecting the lower frequency of systemic isotype switching to IgA.

Despite breakthrough infection after 2 vaccinations having a greater impact on circulating anti-SARS-CoV-2 IgA levels and durability than a third vaccination, we observed vaccine-induced imprinting of the IgA response to SARS-CoV-2 S antigen. Following infection with Omicron, Ab reactivity against the ancestral Wuhan-Hu-1 S

protein represented in the vaccine remained higher than reactivity against the S protein of the infecting variant. Despite remaining lower than reactivity against vaccine-encoded S in all but a few cases, IgA reactivity against Omicron S was increased by infection, illustrating the value of boosting humoral responses to higher levels, even with imprinting. A similar level of imprinting for IgG and IgA anti-SARS-CoV-2 S Ab suggests significant overlap in B cell clones selected through mRNA vaccination for affinity maturation and isotype switching to IgG and IgA. Whether imprinting of IgA responses through repeated exposure to ancestral antigens occurs similarly to that of IgG responses through variable isotype switching of overlapping B cell clones or through a separate pathway of B cell activation and maturation remains to be demonstrated via V region analysis. Likewise, whether spillover of IgA between systemic and mucosal sites occurs requires further analysis. Given that SARS- CoV-2 enters through the respiratory tract and that the respiratory tract is at least initially its primary site of replication, the impact of infection on mucosal IgA anti-SARS-CoV-2 S levels would likely be much greater than that of an intramuscular vaccine. The greater impact on circulating IgA anti-SARS-CoV-2 S may also reflect greater antigen exposure and/or spillover from IgA induced at mucosal sites; however, no saliva or other mucosal fluids were collected for this study to test this possibility.

While more information is required on the relationship between mucosal and circulating IgA to clearly establish the relevance of circulating IgA in protection against respiratory viruses such as SARS-CoV-2, several factors illustrate value in its measurement. Intramuscular mRNA vaccination induces a circulating IgA response that

generally parallels the IgG response in specificity at lower, but easily measured levels. Our study corroborated a previously reported association between higher levels of circulating anti-SARS-CoV-2 S IgA and protection from breakthrough infection, illustrating a direct or indirect relationship to protective immune features [64]. The relevance that circulating IgA responses have towards protection from infection and/or towards limiting the duration and severity of infection warrants further study.

3.7 Conclusion

This study examined the effects of vaccination and breakthrough infection on circulating IgA responses against SARS-CoV-2 S. Intramuscular mRNA vaccines induced systemic IgA responses that were boosted by third vaccinations and higher IgA anti-SARS-CoV-2 S levels were associated with protection from breakthrough infection. However, a break- through infection caused a more robust increase in IgA responses compared to vaccination. Immunological imprinting of IgA responses occurred from vaccines encoding ancestral S in that recognition of ancestral SARS-CoV-2 S protein was favoured over recognition of the Omicron S protein even after an Omicron breakthrough infection. Elucidating a relation- ship between circulating and mucosal anti-SARS-CoV-2 IgA will be important to further document value in measuring circulating vaccine- and infection-induced IgA responses.

Chapter 4: Thesis Discussion & Conclusion

This thesis explores humoral and cell-mediated immune responses in individuals following exposure to SARS-CoV-2 antigens. Our goal was to extend information on the diversity of immune responses mounted against SARS-CoV-2 following exposure, infection, vaccination, and combinations thereof.

In the first part of this study, we analyzed the immune responses of a cohort of subjects who experienced prolonged close contact with SARS-CoV-2 prior to vaccination. These subjects remained uninfected as indicated by molecular testing, had no serological evidence of infection, and reported no overt symptoms of infection. The ability of T cells to aid in protection from infection by generation and maintenance of cellular immunity without seroconversion has been studied in SARS-CoV-2, HCV, and HIV exposures [75]–[81], [83], [129], [130]. Based on our findings, we suggest that our study subjects likely resisted infection and seroconversion either through pre-existing cross-reactive immunity, rapid cellular immune responses, or abortive infections. Both rapid cellular responses and abortive infection occur swiftly, making it difficult to detect, measure, and interpret relevant responses occurring within an individual. Considering this, we speculate that rapid cellular immune responses or abortive infections could be at least partially distinguished based on persistence of either $CD4^+$ or $CD8^+$ T cell responses.

Repeated coronavirus exposures may prime the immune system to favour a rapid cross-reactive cellular immune response. Considering the coordinated activation required

for a complete immune response alongside the greater promiscuity of $CD4^+$ T cells, we suspect that rapid cellular responses favour stimulation of $CD4^+$ T cells. The activated $CD4^+$ T cells can orchestrate and regulate a robust and co-ordinated immune response, activating phagocytic cells, releasing pro-inflammatory cytokines, facilitating generation of cytotoxic $CD8^+$ T cells and occasionally, exerting direct cytolytic functions. In almost all subject cases there was IgG antibody cross-reactivity to common β-coronaviruses, indicating exposure and the potential presence of memory T cells that could aid in elimination of infection through rapid cellular function. The T cell receptor (TCR) will recognize and bind to conserved regions of coronavirus epitopes presented during various exposures, initiating a memory response. This expansive and coordinated response suggests that the clearance of viral infection by rapid cellular responses is primarily driven by $CD4^+$ T cells. Due to the limited availability of samples and products as well as time constraints, we did not determine if the epitopes from SARS-CoV-2 recognized by T cells from the exposed uninfected individuals were from conserved common coronaviruses epitopes. However, we speculate that individuals who previously experienced a common coronavirus infection and generated a response against conserved T cell epitopes are more likely to clear SARS-CoV-2 infection through the activation of cross-reactive $CD4^+$ T cells and the subsequent rapid mobilization of the immune response.

Abortive infections, defined here as clearance before the production of viral progeny from the initially infected cell, potentially trigger $CD8^+$ T cell activation, which relies on presentation of foreign peptides via major histocompatibility complex class I

(MHC I) molecules. Given the rapid clearance of infected cells in abortive infections, there would be little time to activate $CD8^+$ T cells and elicit their effector functions to clear infected cells. Bearing this in mind, we suspect that innate immune mechanisms (or cells) are responsible for the effective clearance of infected cells while the activation of $CD8^+$ T cells occurs fortuitously. For example, macrophages and NK cells could eradicate infected cells through phagocytosis or direct cytotoxicity, while the transiently infected host cell simultaneously present viral peptides on MHC I molecules to activate virus specific $CD8^+$ T cell responses. This activation and establishment of $CD8^+$ T cell memory responses could, therefore, be a coincidental measure of abortive infections cleared by innate immunity. Bacterial studies with *Listeria monocytogenes* [131] and *Burkholderia pseudomallei* [132] have shown that rapid clearance of infection – the hallmark of abortive infections – is associated with rapid and dominant $CD8^+$ T cell responses. We suggest that innate-induced abortive infections can result in activation of $CD8^+$ T cell responses that could potentially be used as a marker of abortive infections.

Accurately measuring virus exposure and distinguishing the immediate immune response triggered rapidly by cross reactive epitopes or the generation of *de novo* responses in an abortive infection is exceedingly difficult and would likely rely on human challenge studies. Nevertheless, in a hypothetical scenario of analyzing subjects' T cell responses by flow cytometry following SARS-CoV-2 exposure along with a lack of seroconversion or overt display of symptoms, we anticipate that individuals who cleared infection through rapid cellular responses via activation of cross-reactive memory responses would likely possess a greater abundance of virus-specific $CD4^+$ T cells and that those who underwent

abortive infection would exhibit a selective presence of SARS-CoV-2 specific $CD8^+$ T cells.

There is a possibility that subjects may have avoided infection despite exposure due to genetic resistance. Genetic resistance to viral infections can be found when a key viral receptor is not expressed. This has been studied in the loss of CCR5 and ACE2 resulting in resistance from infection with HIV and SARS-CoV-2, respectively [133], [134]. Considering the small sample size in this cohort analysis, it is unlikely – but not impossible – that subjects within the group had protection from infection due to a loss of ACE2 expression. Microarray analysis, flow cytometry and immunofluorescence could be used to assess ACE2 expression in the study subjects but lack of ACE2 expression was not considered likely. Another unlikely, but not impossible, reason for their resistance could be a currently undescribed form of genetic resistance to SARS-CoV-2, but identifying this would require whole genome sequencing and analysis which was not feasible for this investigation. The newly described association between HLA-B*15:01 and asymptomatic infection [135]may also be a plausible explanation, however this study was published after the completion of the first part of this study.

In the second part of this study, we analyzed humoral immune responses within our cohort after vaccination against, and in some cases, after breakthrough infection with SARS-CoV-2. We investigated the historically understudied circulating IgA responses to assess the potential for association with mucosal protection against SARS-CoV-2 infection. We found the combination of vaccination with breakthrough infection generated a more

robust and longer-lasting IgA response than what was observed by vaccination alone. We showed that vaccine-induced circulating IgA responses can serve as a marker for resistance to infection and that multiple exposures to the ancestral SARS-CoV-2 strain via vaccination can induce humoral imprinting for IgA.

Our breakthrough infection results were similar to those described in hybrid immunity, demonstrating that the combination of infection and vaccination in a sequence-independent manner generates a more robust immune response than vaccination alone [136]. SARS-CoV-2 infection and replication increases the overall load and variety of viral antigens, which produces a more durable and comprehensive Ab response. The broader antigenic stimulation with infection involves activation of different immune cell subsets in different locations and results in a more powerful humoral response. Vaccines, in contrast, contain a limited number of antigens resulting in a relatively weaker and narrower immune response. Nonetheless, vaccination provides a more controlled stimulation of the Ab response, offering the potential for protection against infection without the risks of severe illness and other complications associated with natural infection.

Some subjects in our study had much higher levels of vaccine-induced anti-SARS-CoV-2 S IgA responses than their counterparts with similar antigenic experience. As a group, those with higher levels of vaccine-induced anti-S IgA responses were more likely to avoid Omicron breakthrough infection than those with lower vaccine-induced IgA responses. Although IgA is the second most abundant circulating Ab, it constitutes only ~15% of serum antibodies, while IgG makes up ~80% of serum antibodies [137]. The

specific proportions of IgG and IgA antibodies in circulation can vary depending on the specific immune response and differ among individuals, but typically retain an approximate 80/20 ratio. In respiratory infections like SARS-CoV-2, both IgG and IgA responses can be detected in circulation. IgA antibodies play a key role in protection at mucosal sites, while the relatively lesser abundance of circulating IgA antibodies makes it difficult to credit them as protecting against infection. Considering this, the levels of circulating IgA may be an indirect marker of mucosal IgA protection. While the correlation between mucosal and circulating IgA responses is debated in the literature [37], [38], [62], [66]–[68], our findings offer support for the responses being related. Exploration of possible correlation between mucosal and circulating IgA fine specificity and levels could include ultra-sensitive quantification of both saliva and serum IgA responses, as well as clonotyping and memory B cell receptor (BCR) sequencing to determine if the responses are generated independently or at least partially are the result of spillover. Additional analysis of vaccine-induced circulating IgA responses in larger cohorts, will be required to further verify our findings.

We observed humoral immune imprinting in the circulating IgA and IgG responses, as has been previously described [72], [138], [139]. We characterized imprinting as the humoral immune response favouring recognition of ancestral SARS-CoV-2 S protein, even after Omicron breakthrough infection. Following Omicron breakthrough infection, the anti-SARS-CoV-2 ancestral S IgG and IgA response was clearly favoured over Omicron. The subjects in our analysis had at least two intramuscular vaccines and their memory B cells have presumably undergone somatic hypermutation to increase the B cell affinity for

ancestral SARS-CoV-2 S antigen. The strong and highly specific interaction between the antigenic epitope and Ab paratope can nonetheless engender cross-reactive responses, through which it appears to suppress the generation of new Ab responses specific to variant strains of SARS-CoV-2. Despite the humoral immune response cross-reacting against the new variants, inhibition of a new Ab response against the Omicron variant and boosting of Ab responses favouring the ancestral response may result in lesser neutralization and a higher chance of re-infection with subsequent SARS-CoV-2 variants.

Analysis of humoral and cellular responses to SARS-CoV-2 has helped in the design and manufacturing of vaccines and assessment of their effectiveness. Antibody research is crucial in understanding the humoral immune responses to SARS-CoV-2 vaccination and our findings confirm that intramuscular vaccination generates both IgG and IgA antibody responses. Additionally, by comparing the immune response induced by the vaccines in individuals who remained uninfected to those who later experienced breakthrough infections, we identified circulating IgA as a marker for protection from Omicron breakthrough infection. A thorough understanding of the humoral responses to both vaccination and infection with SARS-CoV-2 and its variants can inform future vaccine design to better induce the IgA response as well as the overall development, composition, and dosing regimens of immunizations.

The results of this study have implications for better understanding immune responses following vaccination and infection as well as offering insight into control of disease severity and effective protection against SARS-CoV-2 variants. Moreover, the

results potentially contribute to advancement of vaccine development and better understanding the role of circulating IgA in humoral immune responses. We demonstrated that there are cross-reactive IgG antibody responses from previous exposure to common β-coronaviruses against the ancestral SARS-CoV-2 strain, as well as cross-reactivity from vaccine-induced IgA and IgG against the SARS-CoV-2 Omicron variant. Cross-reactivity can reduce the risk of infection and severity of symptoms through cross-protection. However, as previously mentioned, reliance on cross-reactive responses can also restrict the development of new humoral immune responses against SARS-CoV-2 variants. Considering that many viruses adapt and mutate over time, the favouring of an ancestral immune response could result in a potential loss of protection as the virus continues to evolve away from its original form. Understanding the implications of cross-reactivity in immunity is essential for identifying the advantages and limitations in the context of infectious diseases and informing vaccine development strategies.

Through this analysis, we aimed to provide insight into unique circumstances where individuals have been exposed to SARS-CoV-2. Our first investigation had a small sample size, therefore, our findings may not translate to the greater population, reducing our ability to make general conclusions. As well, in chapters two and three, we were limited by the inclusion criteria for our cohorts; unvaccinated, seronegative close contacts to SARS-CoV-2, as well as, vaccinated, and vaccinated with a SARS-CoV-2 Omicron breakthrough infection between February and August 2022. These limitations restricted the number of subject samples available for our analyses, and it is a challenge to predict how the definition

of these cohorts might have affected the implications and general applicability of our research.

The individuals included in this thesis lack diversity. The majority of subjects within the cohort are Caucasian with no self-reported co-morbidities, who reside in an urban setting with steady and reliable sources of income along with secure and consistent housing, all of whom present as cisgendered. Generally, this demographic group has better access to healthcare services, faster vaccine distribution and are less likely to experience healthcare inequities compared to marginalized or minority groups, individuals without secure housing, or individuals residing in rural environments. Furthermore, the longitudinal study demographic pool within which both investigation cohorts were selected lacks pediatric subjects and consists primarily of middle-aged individuals overrepresented with females. However, the immune system's basic structure and function are highly conserved. The shared characteristics can have broad implications for understanding and addressing immunity in geographically and racially diverse populations, yet the universal applications rely on subjects having healthy, functional immune responses, without underlying co-morbidities. Therefore, the conclusions drawn from our analysis are not necessarily applicable to subjects unrepresented in this study and the sample sizes of our cohorts may not be large enough to represent the general populations experience with SARS-CoV-2.

Future analyses should encompass broader and more diverse populations. Replicating these experiments across multiple and larger distinct groups could significantly contribute to validating and generalizability of our findings. Ideally, further investigations

would also include subjects with no exposure, either by vaccination or infection, to SARS-CoV-2 as well as subjects who have only been infected and have not had any form of vaccination. While these cohorts may be difficult to assemble in the context of SARS-CoV-2, it would be relevant to include them in other viral or infectious disease investigations. Furthermore, the long-term durability of humoral and cellular responses from vaccination and infection with SARS-CoV-2 could provide insight into optimal vaccine regimens and contribute to a greater overall understanding of the humoral immune responses.

Another area deserving further investigation is the impact of bivalent vaccines. Bivalent mRNA vaccines including both ancestral and Omicron S sequences were introduced to the population following the rise of SARS-CoV-2 Omicron and its subvariant infections. Recent publications have shown that vaccination with bivalent vaccines increases Ab responses to both Omicron and ancestral SARS-CoV-2 S, although the Ab response following bivalent vaccines is still stronger against the ancestral protein [140], [141]. With a larger cohort and more longitudinal samples, the IgA and IgG antibody responses in subjects with ancestral vaccines, vaccines and breakthrough infections, and ancestral and bivalent vaccines could be compared. Despite the favouring of ancestral responses, the vaccine-induced immunity provided by a bivalent booster offered more protection against infection and severe disease than previous ancestral-based monovalent vaccines alone [142]. As an active area of research, continued analysis of the impact of bivalent vaccines on antibody responses needs to be and is being conducted. Furthermore, humoral immunity resulting from of tri-, quad-, and multivalent mRNA vaccines should be investigated – the designs of which could include complete strain antigens. For example,

inclusion of all known β-coronavirus spike proteins in a multivalent mRNA vaccine could result in broadly protective responses. Recent administration of a multivalent mRNA influenza vaccine in mice and ferrets resulted in immune responses against all 20-encoded influenza A and B viral lineages [143].

Pfizer and BioNTech have recently developed a new monovalent Omicron vaccine; Pfizer-BioNTech-X.B.B.1.5-adapted monovalent COVID-19 vaccine [144]. We suspect that exclusion of the ancestral SARS-CoV-2 S antigen in the vaccine will enable generation of a stronger variant-specific Ig response, however, considering this is the first sole Omicron-specific vaccine dose, the newly induced Ig will not likely outcompete the ancestral Ig responses. Longitudinal analysis to determine if bivalent or monovalent Omicron vaccine recipients are better protected from SARS-CoV-2 variant breakthrough infection than individuals with only ancestral-monovalent vaccines or subjects who have already had a breakthrough infection and are at risk of a re-infection would provide more insight on the importance of vaccine evolution.

As previously mentioned, the Ab response resulting from infection is variable, although the relative abundance of Ig classes remains consistent based on their expected concentrations and need for specific effector functions. Our study confirms that SARS-CoV-2, an enveloped viral respiratory pathogen, induces circulating IgA and IgG responses. An area that would benefit from further analysis would involve comparing Ig variable regions following infection. By sequencing the variable region of SARS-CoV-2

specific IgA and IgG responses within an individual, we could gain insight into whether there is the potential of a shared parental B cell clone producing both IgG and IgA responses or if the production of IgG and IgA responses occurred independently. Subjects who have only received vaccination may be more likely to have conserved variable regions compared to those who have been infected due to the constraint of antigen availability. Ideally, the analysis would assess similarities or differences between Ig variable regions among vaccinated individuals, those who received a combination of vaccination and infection, and those who have been solely infected. It would also be worthwhile to investigate whether non-enveloped and/or non-respiratory viruses elicit similar Ab responses and if mRNA vaccines are more prone to activating and selecting similar variable regions between Ig.

Intramuscular vaccines are capable of inducing mucosal IgA responses and our data suggests that circulating IgA levels may be indicative of mucosal protection, which could potentially aid in a more thorough understanding of the overall IgA response. This possibility could be assessed by collecting both circulating and salivary IgA responses from the same individual and comparing the relative levels of responding antigen-specific IgA in serum and saliva through standard and chemiluminescence ELISA, respectively. The implications of our findings could inform the need to develop intranasal vaccines or aid in the development of more robust mucosal activation via intramuscular vaccines. Concurrently with their development, it would be worthwhile to investigate if intranasal vaccines are capable of producing a circulating antibody response and determine if the levels of mucosal IgA responses are indicative of circulating IgA.

Future research should explore the long-term durability of humoral and cellular immune responses from vaccination and infection with SARS-CoV-2 to gain insight into waning immune protection. Further investigation regarding immune imprinting, B cell exhaustion, and a more comprehensive understanding of the relationship between secretory and circulating IgA responses would increase our knowledge regarding vaccine and infection-induced immune memory, thereby informing our ability to optimize immune protection. By addressing these future directions, we can not only enhance our understanding of SARS-CoV-2 but also support and improve public health strategies.

The research conducted in this thesis on humoral and cellular immunity to SARS-CoV-2 has contributed to our knowledge and understanding of the immune response to the virus, vaccine development, and long-term COVID-19 management. By studying the immune responses generated in close-contact exposures to, vaccinations against, and breakthrough infections with SARS-CoV-2, we have gained practical information on the variability of immune responses in individuals and determinants of their strength and variability. Ongoing research on the immunological impact of SARS-CoV-2 infection and vaccination will continue to further our comprehension of antiviral humoral and cellular immune responses, enhancing our ability to inform public health strategies in future infectious disease outbreaks.

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Appendix

Health Research Ethics Approval

From: do-not-reply-mun@researchservicesoffice.com <do-not-reply-mun@researchservicesoffice.com>
Sent: Thursday, July 13, 2023 10:33 PM
To: Harnum Debbie(Key Contact) <dharnum@mun.ca>
Cc: Grant, Michael <mgrant@mun.ca>; administrator@hrea.ca
Subject: HREB - Approval of Ethics Renewal 20210347

Researcher Portal File #: 20210347

Dear Dr. Michael Grant:

This e-mail serves as notification that your ethics renewal for study HREB # 2020.085 – Characterization of Cellular and Humoral Immunity Against SARS-CoV-2 – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from 16 Jul 2023 to 16 Jul 2024.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal will be reported to the Health Research Ethics Board at their meeting dated 27 Jul 2023.
Thank you,

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