The Impact of a Delayed SARS-CoV-2 Vaccine Dose Interval on the Immune System: B Cell Maturation and Antibody Neutralization

By

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ABSTRACT

Early in the COVID-19 vaccine rollout, Canada implemented a dose-sparing strategy by delaying second doses to increase first-dose coverage. A longer interval between doses was associated with improved antibody titres and inhibition of variants. These improvements may reflect enhanced B cell affinity maturation during the extended interval. This study examines the effects of an extended dose interval on B cell maturation after the primary dose series and following a booster dose in mRNA vaccine recipients from Manitoba.

Blood samples were collected at multiple timepoints from participants who received either a short or extended dose interval. Peripheral blood mononuclear cells were isolated and stained with fluorescent probes to detect and phenotype antigen-specific B cells over time using flow cytometry. A delayed dose interval led to a greater increase in Spike-specific and RBD-specific B cells and antigen-specific memory B cells 1-2 weeks after the second dose compared to a standard dose interval. Spike-specific and RBD-specific B cells in the standard dose interval participants increased to match the levels in the delayed dose interval participants 6 months after the second dose. The impact of a third dose in both groups did not result in differences in the proportions of SARS-CoV-2-specific B cells between participants with standard or extended dose intervals.

To investigate the impact of dose interval on antibody maturation over time, PBMCs from various post-vaccination timepoints were stimulated in vitro to differentiate memory B cells into antibody-secreting plasma cells. Antibodies derived from antibody-secreting plasma cells in participants with a delayed dose interval saw increased affinity and a wider breadth of inhibition for SARS-CoV-2 RBD 10-14 days following the second dose of the vaccine compared to standard dose interval participants. The third dose did not result in differences in the breadth of inhibition between participants with standard or extended dose intervals.

Dosing intervals affect B cell phenotypic proportions and antibody responses following vaccination. Extended intervals transiently increase SARS-CoV-2-specific B cell frequencies and antibody breadth, suggesting greater maturation, but shorter intervals eventually yield comparable responses. The study will shed light on whether the establishment of long-term memory B cells is also affected, with implications for future vaccine strategies.

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ABBREVIATIONS

Abbreviation	Definition
ACE2	Angiotensin-Converting Enzyme 2
ACIP	Advisory Committee on Immunization Practices
AID	Activation-Induced Cytidine Deaminase
APCs	Antigen Presenting Cells
ARDS	Acute Respiratory Distress Syndrome
BCRs	B Cell Receptor
CDC	Centers for Disease Control and Prevention
CLRs	C-Type Lection Receptors
COVID-19	Coronavirus Disease 2019
CSR	Class Switch Recombination
CVs	Coefficient of Variation
DCs	Dendritic Cells
DMSO	Dimeythl Sulfoxide
DZ	Dark Zone
E	Envelope Protein
Fab	Fragment Antigen-Binding Site
FBS	Fetal Bovine Serum
Fc	Fragment Crystallizable Site
FDCs	Follicular Dendritic Cells
GCs	Germinal Centres
HPV	Human Papillomavirus
IL	Interleukin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
LD/NIR	Live/Dead Fixable Near-IR Cell Stain
LNs	Lymph Nodes
LNPs	Lipid Nanoparticles
LZ	Light Zone
M	Membrane Protein
MHC	Major-Histocompatibility Complex
N	Nucleocapsid Protein
NACI	National Advisory Committee on Immunization
NHS	National Health Services
NSP	Non-Structural Protein
PAMPs	Pathogen Associated Molecular Patterns
PFA	Paraformaldehyde
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffer Saline
PHAC	Public Health Agency of Canada
PRRs	Pattern Recognition Receptors
R0	Reproduction Number
RAS	Renin-Angiotensin System
RBD	Receptor Binding Domain
RTC	Replication Transcription Complex
S	Spike Protein
scRNA-seq	Single Cell RNA Sequencing
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SHM	Somatic Hypermutation
SA	Streptavidin
TCRs	T Cell Receptors

T_{fh}	Follicular Helper T Cell
T_h1	T Helper 1 Cell
T_h2	T Helper 2 Cell
T_h17	T Helper 17 Cell
TLRs	Toll-Like Receptor
T_{reg}	Regulatory T Cell
VOC	Variant of Concern
VOI	Variant of Interest
VUM	Variant Under Monitoring
VZV	Varicella Zoster Virus
WHO	World Health Organization

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This thesis represents the culmination of two and a half years of research, data collection, frustrations, and successes. It would also not have been possible without the guidance and support of numerous individuals and institutions. First, I would like to start by acknowledging and thanking my supervisor, Dr. Catherine Card, who has undoubtedly been the most involved in following and developing the project alongside me. In 2022, I was at a crossroads, uncertain about my future direction yet eager to contribute. Dr. Card allowed me to step into a field I was passionate about, and her mentorship allowed me to grow professionally, develop critical skills and gain the confidence to pursue my aspirations. For that, I am extremely grateful.

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DEDICATIONS

To everyone who supported me along this journey

"They, looking back, all the eastern side beheld
Of Paradise, so late their happy seat,
Waved over by that flaming brand; the gate
With dreadful faces thronged, and fiery arms:
Some natural tears they dropt, but wiped them soon;
The world was all before them, where to choose
Their place of rest, and Providence their guide:
They, hand in hand, with wandering steps and slow,
Through Eden took their solitary way."
— John Milton, Paradise Lost

INTRODUCTION

1.1. The Immune System

1.1.1. The Innate Immune System

The immune system is a framework of tissues and cells responsible for eliminating pathogenic microorganisms and substances while ensuring that commensal organisms and self-tissues are not extensively damaged by either pathogenic or immune cellular processes. Nearly all organisms have some form of immune response against foreign agents, whether it be a rudimentary system that utilizes enzymes to clear viral infections such as in bacteria or more complicated systems in multicellular organisms[1,2]. The human immune system is divided into two categories: the innate immune system and the adaptive immune system. The innate (general) immune system is widely considered the first line of host defence found in all multicellular organisms and is evolutionarily older than the adaptive immune system[3,4]. The innate immune system is comprised of various components. These components can include physical barriers (skin junctions, mucosal surfaces), secretions (mucous, lysozyme, hydrochloric acid), immune cells (macrophages, monocytes, natural killer cells) and proteins (complement, lectins)[4]. These components are considered "innate" because they exhibit broad specificity and generally lack specific memory against any particular pathogen, although recent research has shown that the innate immune system does have some ability to retain memory after exposure to pathogens[5,6].

While physical barriers are effective, pathogens can still evade these components of the immune system and enter the body. Once this occurs, immune cells can remove pathogens through a process called phagocytosis, or the process of a cell ingesting a pathogen or other material[7]. These innate immune cells can phagocytize pathogens by recognizing specific components of microorganisms called pathogen-associated molecular patterns (PAMPs), including bacterial lipopolysaccharides or double-stranded RNA[8]. Pattern recognition receptors (PRRs) on the innate immune cells can recognize and bind PAMPs located on the pathogen, or they can recognize antibodies that will bind to the pathogens[8,9]. These PRRs include toll-like receptors (TLRs), which bind PAMPs and transmit signals for the cell to produce pro-inflammatory and antiviral factors[8]. Another type of PRR are the c-type lectin receptors (CLRs), which are responsible for activating the phagocytic pathways of innate immune cells[8]. The phagocytic cell will begin to activate an actin-myosin contractile system that sends arms of cytoplasm around the pathogen until it is completely enclosed within the phagocytic cell[8,9]. The pathogen is now surrounded in a membrane vesicle called a phagosome, which can then fuse

with vesicles that contain lysozymes, degrading and destroying the pathogen[8,9]. There are a wide range of cells, both phagocytic and nonphagocytic, that are considered part of the innate immune system. Neutrophils are the first cell line recruited at the site of infection and can recognize and phagocytize foreign microorganisms[4]. Eosinophils release inflammatory molecules such as histamine during infection and recognize and bind to larger pathogens, such as parasites[4,10]. Once activated, the eosinophils will release extracellular histamine, proteins, sulfatases and other chemotactic factors to damage the parasitic membrane[4,10]. Eosinophils also play an important role in the allergic processes and are primarily mediated by immunoglobulin E (IgE) antibodies[4,10]. Basophils and mast cells are predominantly responsible for releasing granules and cytokines that enhance the inflammatory process during early infection[4].

Antigen presentation is a crucial component of the adaptive immune response and is primarily carried out by cells of the innate immune system. Without antigen presentation, the cells of the adaptive immune system would not be able to recognize and respond to pathogens, generate immunological memory, or mount a targeted and effective immune response. Monocytes, or macrophages, specialize in phagocytosis and the production and release of pro-inflammatory cytokines[2,4]. Dendritic cells (DCs) are present in tissues in contact with the external environment and can migrate to lymph nodes to activate naïve T cells[2,4]. These two cell types are antigen-presenting cells (APCs) and can present antigen to adaptive immune cells after phagocytosis of antigenic molecules has occurred. Once the antigen has entered the APC, it can be tagged for proteolysis and is degraded into peptide fragments[11,12]. These peptides can be loaded onto major-histocompatibility complex (MHC) molecules and are presented on the cell surface to activate the T cells of the adaptive immune system. MHC class I molecules are present on all cell types and are responsible for presenting peptide fragments from endogenously-expressed proteins to CD8+ T cells[11,12]. MHC class II molecules are primarily expressed by the APCs and are responsible for presenting peptide fragments from exogenous proteins to CD4+ T cells[11,12]. The antigens are presented to T cells along with the required costimulatory molecules so that the T cells can become activated and carry out their respective functions[13].

1.1.2. The Adaptive Immune System

1.1.2.1. Cell-Mediated Immunity

Unlike the innate immune system, the cells of the adaptive immune system are antigen-specific. However, they can generate specific memory against particular pathogens, and the immune response improves after repeated contact with the pathogen[2]. The adaptive immune system has two main components: cell-mediated immunity and humoral immunity, which are made up of T and B cells[2]. Cell-mediated immunity comprises two cell types, CD4+ T cells and CD8+ T cells[2]. Their origins, roles and mechanisms will be discussed in more detail below.

T cells, or T-lymphocytes, are immune cells that develop in the bone marrow yet migrate to the thymus during development to complete their maturation process[14,15]. Mature T cells will then exit the thymus and migrate to secondary lymphoid sites as single positive T cells, expressing unique T cell receptors (TCRs) and either CD4 or CD8[14]. CD4+ T cells, or T-helper cells, mediate the immune response during infection by regulating and communicating with other immune cells[10]. CD4+ T cells are activated when the TCR complex binds to an antigen presented via an MHC II molecule[10]. When a CD4+ T cell recognizes the antigen, the TCR binds to the APC peptide-MHC complex[10,16,17]. CD4 also acts as a co-receptor during this interaction[17]. This is known as the primary signal between CD4+ T cells and APCs. A secondary signal is also necessary to ensure that the CD4+ T cell is recognizing a foreign antigen and not a self-peptide. This signal involves an interaction between the co-stimulatory molecule CD28 on the T cell and CD80 or CD86 on the APC[2,18]. Failure to acquire a secondary signal makes the cell anergic, or functionally inactive, and eventually apoptotic[2,19]. Once the signal between the two cells is received, the CD4+ T cell will begin to rapidly proliferate and differentiate into specialized T-helper subsets, defined by their transcriptional program, cytokines expressed and tissue homing potential. These different cell types produce and secrete different cytokines and influence different immune system components during an infection. A CD4+ T cell that differentiates into a Type 1 (T_h1) helper cell will secrete interleukin (IL) 2, interferon-γ and tumour necrosis factor-A and is responsible for activating macrophages and CD8+ T cells to kill intracellular pathogens[2,20]. A Type 2 (T_h2) helper cell will secrete cytokines such as IL-4, -5, -9, -10 and -13 and will defend the body against extracellular pathogens such as parasites or external bacteria[2,20]. Type 17 (T_h17) helper cells, once activated, primarily secr17, and are responsible for the induction of proinflammatory cytokines and chemokines[21,22]. T_h17 cells are also responsible for

mediating defensive mechanisms against extracellular bacterial infections[21,22]. However, they are also responsible for the pathogenesis of many autoimmune diseases[21,22]. Regulatory CD4+ T (T_{reg}) are responsible for the maintenance of immunological tolerance to self-antigen[23]. T_{reg} cells also regulate the immune response after the pathogen has been cleared, thereby protecting the host against allergic reactions, inflammation and immunopathological conditions[23]. Lastly, follicular helper (T_{fh}) T cells are T cells that help B cell development. T_{fh} reside in lymph node tissues and other lymphoid structures while T_h1, T_h2 and T_h17 leave lymphoid tissues and traffic to sites of infection or inflammation in addition to residing in lymphoid structures[24]. T_{fh} migrate to a region in the lymph nodes called the T cell-B cell border and secrete IL-21 required for B cell proliferation and differentiation during memory B cell maturation[24]. T_{fh} are also responsible for the selection of maturing B cells that have increased affinity for the pathogen peptides presented by the T_{fh} cells, which will be explained in further detail below[24].

While CD4+ T cells regulate the immune response, CD8+ T cells, or cytotoxic T cells, can destroy intracellular pathogens by killing infected cells[25]. CD8+ T cells promote cytotoxicity of infected cells and can induce cell death to curb intracellular infections and eliminate tumour growths[26,27]. CD8+ T cells are activated by MHC I molecules, which are found on all nucleated cells[10]. While MHC II molecules present antigen fragments from external pathogens broken down in phagocytic APCs, MHC I molecules present endogenous antigens from internal infection, such as a virus[10]. DCs can also present exogenous antigen fragments on MHC I molecules through a process called cross-presentation, which can activate cytotoxic CD8+ T cells against pathogens that do not infect APCs[28]. The activated APC, such as a DC, can travel to the lymphoid sites where naïve T cells are primarily located, and the MHC-peptide complex will interact with a CD8+ T cell that can recognize the foreign antigen present on the MHC I molecule[10,29]. Once activated by the MHC-peptide complex and the secondary signal, CD8+ T cells will rapidly proliferate, travel to the site of infection and trigger apoptosis of their cellular target through a wide variety of cytotoxic mechanisms[10,27]. Generally, organelles within the CD8+ T cell containing cytotoxic mediators are released after activation into the synaptic cleft, which is the space between the T cell and the target cell[25,30]. The cytotoxic mediators include granzymes and a pore-forming protein called perforin which allows the proteases to enter the infected cell[25]. These granzymes enter the cell through pores formed by perforin and trigger cell apoptosis by activating caspase-dependent and caspase-independent pathways[25].

1.1.2.2. HUMORAL IMMUNITY

The other branch of adaptive immunity is known as humoral immunity and involves cells that, rather than destroy infected cells through cytotoxicity, produce and rely on immunoglobulins to carry out immune system functions[10]. Cells that produce antibodies and that are part of the humoral immune system are known as B cells[10]. B cells play a pivotal role in the humoral immune system, producing antibodies as plasma cells and as memory B cells[31]. Unlike T cells, B cells develop exclusively in the bone marrow from hematopoietic stem cells throughout life[31,32]. During this development, B cells undergo a negative selection process to prevent the development of B cells with autoimmunity[31,32]. The surviving B cells will circulate to secondary lymphoid organs, awaiting foreign antigens[31,32]. B cells can be activated either through T cell-dependent or independent pathways, depending on the nature of the antigen. T cell-independent activation occurs in response to non-protein antigens, such as polysaccharides from bacterial capsules[31]. T cell-dependent activation occurs when B cells recognize protein antigens binding to the B cell receptor (BCR) and present antigen fragments to CD4 T cells via the MHC II molecules[31]. Similar to the T cells, B cells express various molecules on their surface at different stages of maturation or activation. These molecules include the BCR, the membrane-bound immunoglobulins described in later sections. The BCR will interact with free antigen or antigen presented by APCs such as macrophages or DCs and induce a signalling cascade that leads to B cell activation and proliferation[31]. When a B cell has a BCR that recognizes an antigen, it will bind the antigen and undergo endocytosis of the BCR-antigen complex[31]. B cells also express MHC II molecules, which are loaded with processed antigenic peptides and transported to the B cell surface, allowing them to present antigens to CD4 T cells[31]. This results in CD4 T cell activation, which provides co-stimulatory signals and cytokines that promote B cell proliferation and differentiation, such as CD40-CD40L interactions and the secretion of IL-4 and IL-21[31]. Regardless of how the B cell is activated, the recognition of an antigen by the BCR will cause the B cell to undergo rapid changes, resulting in two distinct types of B cells: plasma cells or memory B cells.

The immunoglobulins produced by B cells are known as antibodies, which can have vastly different structures and functions (Figure. 1.1-1)[33]. Broadly speaking, these molecules serve two main functions: either as a BCR localized to the cell membrane that permits cell signalling and activation or a soluble molecule that can recognize, bind and neutralize antigens[33].

Antibodies consist of two heavy chains and two light chains, with each chain made up of one N-terminal variable region and 1 C-terminal constant region in the light chain and 3 or 4 constant regions in the heavy chains[33]. The variable regions of the light and heavy chains, as well as the constant region of the light chains and the first constant region of the heavy chains, make up the fragment antigen-binding site (Fab)[27,33]. This region of the antibody is where antigen interactions occur between the paratope, the site on the antibody that recognizes and binds to the antigen, and the epitope, the binding site on the antigen[33]. The remaining constant regions of the heavy chain are called the fragment crystallizable site (Fc)[27,33]. The Fc region determines the isotype of the antibody being produced by the B cell and is responsible for linking the antibody to the effector cells that interact with antibodies[33]. Different antibody classes have distinct Fc regions, which determine their functional properties and enable the antibodies to activate various components of the immune system[27].

Antibodies have numerous functions against pathogens that can occur independently or dependently of immune cells. Binding to the epitope of a foreign antigen that is required for attachment to the host can prevent that antigen from recognizing and interacting with host cells and tissues, thus preventing infection, in a process known as neutralization[34]. This can include binding to pathogen ligands essential for attachment or adhesion to the host, as well as inhibiting fusion of viruses to host membranes[34]. Some antibody types have been shown to bind to multiple antigens, causing agglutination of the pathogen and allowing for more efficient uptake of the antigen by phagocytic cells[34]. Antibodies can also destabilize foreign organisms by mimicking the cell receptor that the virus recognizes, inducing virion conformational changes without binding to the cell surface[34]. However, much of the biological activity of the antibodies is through interactions with immune cells and Fc receptors[34]. Fc receptors link the humoral immune component with cellular immunity, and engagement of Fc receptors with the Fc region of the antibody will lead to several effects depending on the type of Fc receptor or the isotype of antibody. Interactions between Fc regions on an antibody with an infected cell or pathogen bound to the Fab region and Fc receptors on effector cells can result in outcomes such as antibody-dependent cellular cytotoxicity or phagocytosis, which can cause apoptosis of the infected cell or phagocytosis of the pathogen by the effector cell[27,33] The binding of the antibody-antigen complex to Fc receptors also regulates the immune response. The activation of Fc receptors on macrophages and neutrophils induces phagocytosis but also results in the release of

pro-inflammatory cytokines, which promote the recruitment of additional immune cells[35]. Fc receptors on DCs, when bound to antibody-antigen complexes, can facilitate antigen uptake and enhance antigen presentation to T cells, which strengthens the adaptive immune response[35]. B cells also have inhibitory Fc receptors that can dampen activation signals to help resolve inflammation and maintain immune tolerance[35].

1.1.3. B Cell Activation and Somatic Hypermutation

1.1.3.1. Lymph Node Structure and Initial Activation

A B cell will be activated once the BCR recognizes and binds foreign antigens. For this to occur, the immune cells and the antigen need to be brought into close contact with each other. This process primarily occurs in secondary lymphoid tissue structures known as lymph nodes (LNs), which filter lymphatic fluid containing cells and antigens from the blood[36]. LNs, in addition to filtering through fluids, recruit immune cells through lymphocyte-binding endothelial cells[36]. These tissues allow for the concentration of antigens, APCs and T- and B-cells within

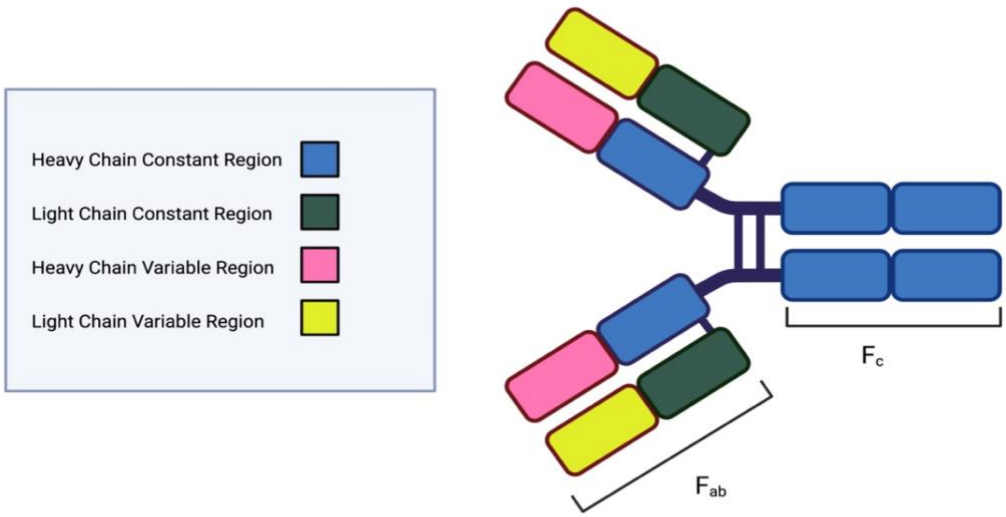


Figure 1.1-1: Structure of Immunoglobulin Monomer. The variable and constant regions, heavy and light chains, and Fab and Fc regions are shown. Made in BioRender.

LNs rather than attempting to locate foreign antigens outside of the LNs that may be present at low frequencies within the host[36]. The LNs are divided into three regions, known as the cortex, paracortex and medulla[36–39]. The cortex consists of the outermost layer of the LN and is comprised of niches that contain B cells and follicular dendritic cells (FDCs) called B cell follicles[36]. The inner region is called the paracortex and contains T cells[36]. The medulla contains the efferent lymphatic vessels that transport the fluids that have already been filtered by the LNs back to the blood circulation[36]. Lymph that is formed in local tissues will enter LNs through afferent lymphatic vessels, travel through subcapsular and medullary sinuses and exit through the efferent lymphatic vessels to return to blood circulation[36,37]. This allows for antigens, APCs, and B cells to be delivered to the LN[36,37]. LN-resident macrophages located in the sinuses capture antigen located in the lymph, which is relayed to B cell follicles in the cortex[36,37]. Non-specific B cells can transfer antigens captured by macrophages to the FDCs located in the B cell follicle via complement receptors 1 and 2[40,41]. FDCs have the unique ability to retain antigen for long periods of time, while protecting the antigen from degradation and concentrating the antigen in the follicle where B cells reside.[40,41] B cells that are able to recognize the antigen presented on FDCs or macrophages will migrate to the T-B border, the region between the cortex and paracortex, where T_{fh} and CD4 T cells provide survival and differentiation signals to the B cells[37]. The activated B cells migrate to the T-B border by upregulating chemokine receptors, particularly CCR7, which directs them toward the T cells in the LN via a chemokine gradient, where they present antigen to T_{fh} cells via MHCII molecules[39]. The co-stimulatory signals between the T_{fh} cells and B cells induce a proliferative response in B cells, along with the upregulation of Bcl6, a transcriptional repressor that downregulates chemokine receptors that kept B cells at the border, resulting in the B cells migrating to the B cell follicles to begin maturation[39]. T_{fh} cells also undergo upregulation of Bc16 and will migrate to the B cell follicles to aid in B cell maturation[39]. In addition to migrating to the B cell follicles, immature B cells have two other fates following interactions with T cells (Figure 1.1-2). B cells can differentiate into short-lived plasma cells called plasmablasts that can rapidly produce antibodies against the

pathogen[42]. These antibodies are produced by cells with a short lifespan that have not undergone affinity maturation, and as a result, have lower affinity for the pathogen[42]. Immature B cells can also differentiate into memory B cells independently of the maturation process[42]. These cells provide an immediate, short-lived response to control the initial infection, but B cells selected for

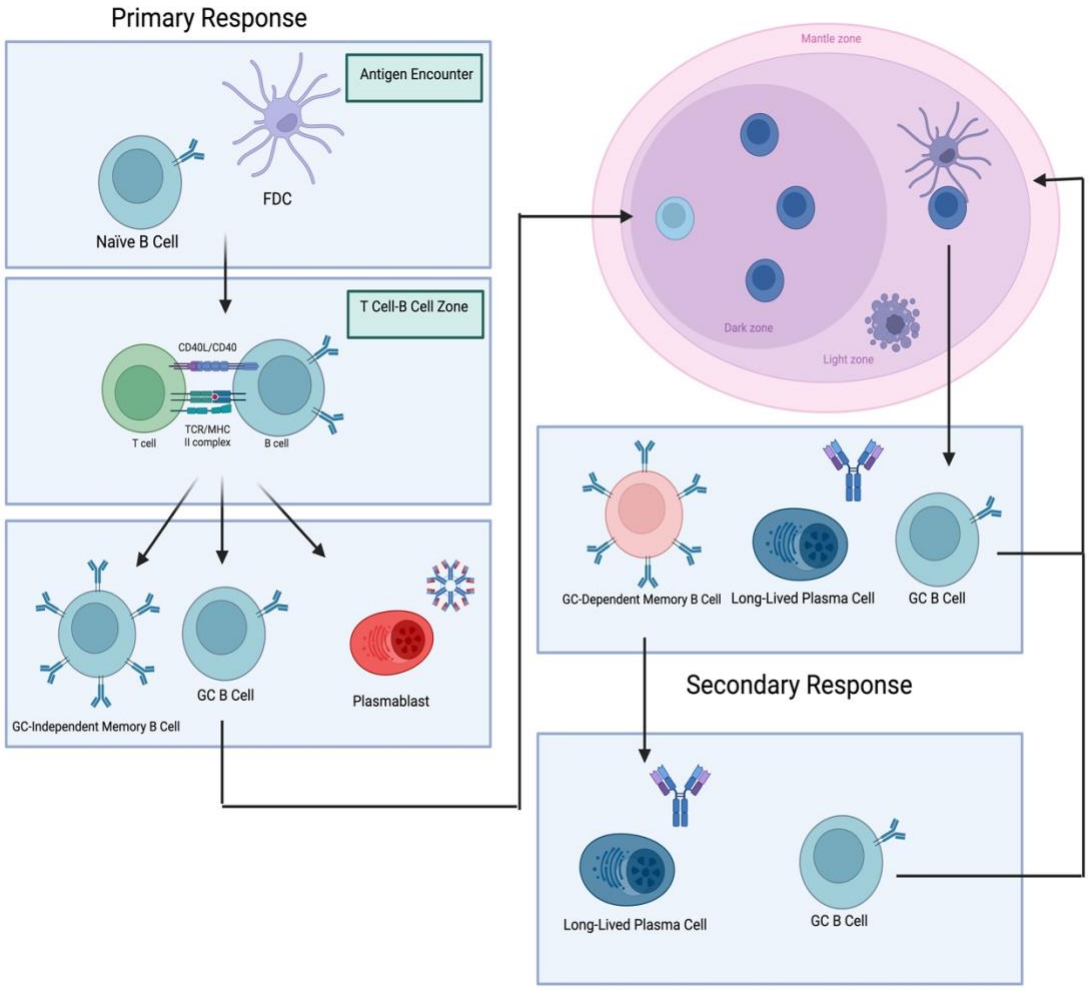


Figure 1.1-2: Diagram of Somatic Hypermutation within a Germinal Centre. Naïve B cells will encounter antigens in the secondary lymphoid organs. Upon activation by T-cell interaction, the B cell will either develop into a GC-independent memory B cell, a short-lived plasmablast, or a GC B cell. The GC B cells, Tfh cells and FDCs will migrate to the follicles to form the GC, where GC B cells will begin to proliferate and undergo SHM in the DZ rapidly. The LZ will have FDCs and Tfh cells that interact with the GC B cells, and B cells that can recognize and bind to the antigen will either develop into plasma cells, memory B cells or will re-enter the GC reaction to further mutate the immunoglobulin genes. B cells that do not recognize the antigen will undergo apoptosis. Made in BioRender.

affinity maturation will contribute to long-lived B cell immunity through maturation processes that will be discussed below.

1.1.3.2. Class-Switch Recombination and Antibody Classes

Class switch recombination (CSR) is a mechanism by which B cells change the structure and isotype of the antibody that will be secreted during the immune response[43]. This process happens during the initial interactions between T cells and B cells at the T-B border of the LN before GC formation and declines as somatic hypermutation (SHM) occurs[44]. Once activation at the T-B border begins, the B cells selected for affinity maturation will undergo CSR through recombination between different regions located upstream of the immunoglobulin genes that code for the constant region of the antibody[43]. This process is mediated by activation-induced cytidine deaminase (AID), which promotes double-strand breaks in the regions between the genes that code for the different constant regions of the antibody isotypes[43]. AID can deaminate deoxycytosines in the upstream and downstream regions of the areas set to undergo recombination, yielding deoxyuracils[43]. The presence of deoxyuracils in the gene results in the insertion of double-stranded DNA breaks in the upstream and downstream regions[43]. These breaks are repaired via non-homologous end joining or alternative end joining, which can lead to the deletion of genetic material and the recombination of a new constant region adjacent to the variable region genes, resulting in a different antibody isotype being produced by the B cell[43].

There are various antibody isotypes, each with a unique structure and function (Figure 1.1-3). Immunoglobulin D (IgD) remains one of the least understood immunoglobulin isotypes[33]. IgD is one of the first antibody isotypes expressed on the surface of B cells as a receptor[45,46]. After B cell activation, IgD is downregulated, and other immunoglobulin isotype genes are upregulated to diversify their repertoire and tailor the humoral response to whatever type of pathogen is present in the host[45,46]. While secreted IgD in monomeric form has been detected, its primary role as a secreted molecule remains largely unknown. Recent research has found that IgD interacts with basophils and mast cells, triggering the release of pro-inflammatory cytokines and contributing to mucosal immunity[47]. Immunoglobulin M (IgM) exists in the serum as a pentameric structure and is the first type of antibody secreted by the humoral immune system in response to a foreign antigen[33]. Alongside IgD, IgM is the other antibody that serves as the BCR for B cells that have not yet been activated by an antigen[48]. Upon antigen recognition, the pentameric IgM is secreted by plasmablasts to neutralize pathogens. Since IgM is the first

isotype produced, it has lower affinities for foreign antigens than isotypes that are produced by mature B cells[48]. However, the pentameric structure provides IgM with a higher avidity for binding antigens due to the 10 Fab regions per IgM molecule[48]. The increased avidity is helpful for binding and neutralizing pathogens with repetitive epitopes, such as on a bacterial capsule[48]. The polymeric structure allows a single IgM molecule to bind numerous antigens together, making them easier to target for processes such as phagocytosis[33,48]. IgM molecules can also activate the classical complement system, a key component of the innate immune system, by binding the Fc region to C1q protein[48].

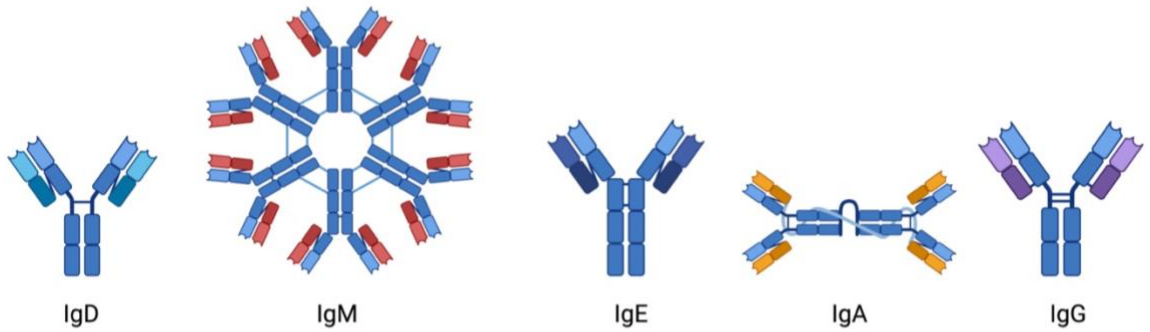


Figure 1.1-3: Antibody Isotypes. The five antibody classes (IgD, IgM, IgE, IgA and IgG) and their structures are outlined. Made in Biorender.

After class switching has occurred, naïve B cells that had expressed IgM and IgD as a BCR will eventually be able to express mature BCRs without altering the specificity for the antigen[43]. Immunoglobulin G (IgG) is among the most abundant proteins in the human serum, accounting for 10-20% of all plasma protein[49]. It is the primary class of the five immunoglobulin isotypes, and it is the most abundant antibody class in human serum[49]. Like other antibody isotypes, IgG antibodies can bind and neutralize toxins and pathogens, as well as bind multiple antigens together via agglutination[49,50]. However, IgG antibodies are secreted as monomers that are small in size and connected with a flexible hinge region not seen in IgM, which provides IgG antibodies with a greater ability to neutralize antigens, interact with Fc receptors as well as enter

tissues from the bloodstream[48,51–53]. The smaller size also allows IgG to diffuse across tissues and cross the placenta to provide immunity to fetuses[54]. Additionally, IgG is generated in the later stages of the immune response from B cells that have had time to undergo CSR, providing greater specificity and neutralization strength than other antibodies produced earlier in the immune response, such as IgM[48,55,56]. IgG also has a half-life of 21 days on average, which is far greater than other antibody types, allowing for a longer immune response to a pathogen[57,58]. IgG can be divided into 4 subclasses in order of decreasing abundance IgG1, IgG2, IgG3 and IgG4[49]. Although they are more than 90% identical on the amino acid level, the subclasses of IgG each have unique roles in the humoral immune system[49]. IgG1 is the main subclass of IgG and mainly recognizes protein antigens[59]. IgG2 is primarily responsible for recognizing and binding bacterial capsule polysaccharide antigens[49]. IgG3 is a potent pro-inflammatory antibody, as it is effective in the induction of effector functions and complement proteins and has an extremely short half-life to limit the potential of excessive inflammatory responses[49]. Lastly, IgG4 responses are typically seen against allergens in non-infectious settings, or against parasites such as helminths[49]. IgE is present at the lowest serum concentration and has the shortest half-life[33]. It is associated with hypersensitivity, allergic reactions, and parasitic infections[33]. Immunoglobulin A (IgA) is the second most abundant immunoglobulin type found in the body, and IgA production is greater than all other immunoglobulin subtypes due to its important role in mucosal secretions, binding and neutralizing pathogens that attempt to cross the mucosal barrier[60]. IgA is present in a monomeric form when in the serum, but IgA in external mucosal secretions of the respiratory, gastrointestinal and genitourinary tracts is predominantly dimeric, comprised of two immunoglobulin molecules linked through disulphide bridges at the C-terminal constant regions[61].

1.1.3.3. Somatic Hypermutation

SHM is a process during memory B cell maturation where mutations are introduced into the immunoglobulin variable region genes of B cells. (Fig. 1.1-4)[42,62]. This process occurs in a structure called a germinal centre (GC), a structure that forms in the B cell follicles of the LNs where activated B cells begin to rapidly proliferate and form tight clusters in the centre of the follicle[39,63]. After B cells interact with T cells at the T-B border, the GC is formed out of T_{fh} cells, activated B cells and FDCs[39,63]. The first signs of GC formation appear around 4-8 days after antigen exposure, typically formed by 50-200 activated B cells[55]. Once established, the GC

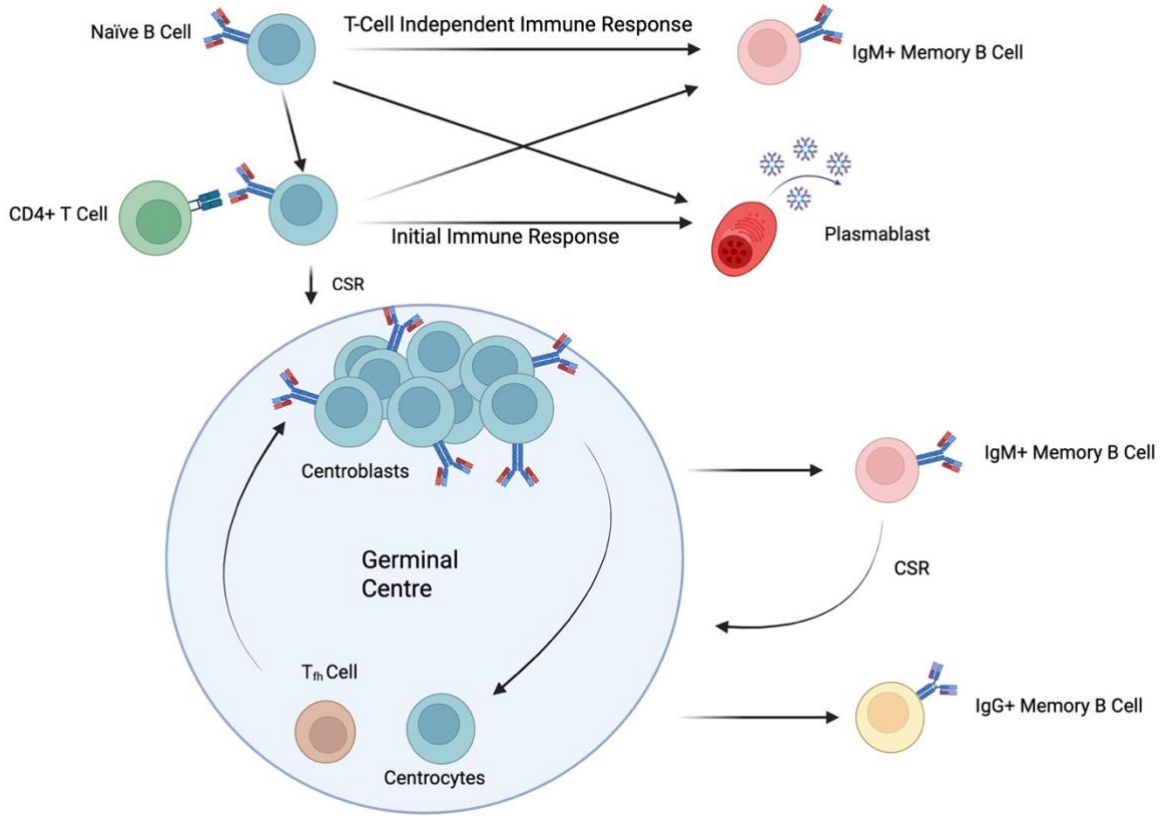


Figure 1.1-4: Overview of the Different Developmental Pathways of Memory B cells. Naïve B cells express IgM and IgD as their BCR. Upon activation by a T cell, the B cell will either enter the GC reaction or develop into a short-lived plasmablast or an IgM+/IgD+ memory B cell with unmutated immunoglobulin genes. Both cell types will secrete IgM+ when activated. Class switch recombination will commence before the GC reaction occurs. Cells that exit the GC reaction early will be IgM+/IgD+ memory B cells and can re-enter the GC reaction. Fully mature memory B cells will express IgG or IgA antibodies. Made in BioRender.

differentiate into plasma cells[64].undergoes spatial organization into two distinct compartments. The dark zone (DZ) is populated by rapidly dividing B cells known as centroblasts, which express high levels of CXCR4, directing them toward the CXCL12-rich environment of the DZ[39,55,63]. Centroblasts undergo intense proliferation while undergoing SHM, a process mediated by AID, the enzyme from CSR[55,65]. AID introduces point mutations in the variable region of the immunoglobulin genes through an error-prone mutation process by converting cytosines to uracil, which results in a diverse pool of BCR variants with the potential for higher or lower antigen affinity[65]. This diversity is what contributes to the breadth of the humoral immune

response, which refers to the ability of antibodies or B cells to recognize a wide range of distinct antigenic variants (i.e. the number of unique epitopes targeted)[66]. After several rounds of division and SHM, centroblasts transition into centrocytes and migrate to the light zone (LZ), the second compartment of the GC[39,55,63]. Centrocytes downregulate CXCR4 but express high levels of CXCR5 and CD83, which guides them to the CXCL13-rich environment of the LZ[39,55,63]. The centrocytes are exposed to antigens displayed on FDCs and compete for survival with other centrocytes[39,55,63]. B cells with higher affinity BCRs developed during SMH will successfully recognize and capture those antigens[39,55,63]. These B cells will present the captured antigen via MHC II molecules to T_{fh} cells, which provide several survival signals and cytokines for B cell development such as CD40L, IL-4 and IL-21[39,55,63]. Centrocytes that do not have BCRs with high affinity for the antigen will not interact with the FDCs and will not receive the stimulatory signals from the T_{fh} cells, eventually undergoing apoptosis[39,55,63]. Centrocytes that have high affinity for the antigen will begin upregulation of the transcription factor Myc, which protects cells from apoptosis, but also triggers cell cycle activation and transition from the LZ to the DZ, where the high-affinity B cells will undergo SHM again[39,55,63]. This cycle results in antigen-specific memory B cells and plasma cells within 1 week after antigen encounter[67]. At the same time, the overall GC reaction has been shown to last several months after initial antigen detection[68].

1.1.4. Memory B Cells

1.1.4.1. B Cell Subsets

After SHM and selection, B cells will differentiate into long-lived plasma cells or memory B cells, depending on the signals they receive. Generally, B cells that will differentiate into plasma cells are restricted to higher-affinity GC B cells in the LZ due to signals received via antigen engagement and T_{fh} cells[55]. Conversely, B cells that will differentiate into memory B cells have a lower affinity for the antigen in the LZ, which results in a weaker signal due to fewer antigen and T_{fh} interactions[55]. Memory B cells can be further separated into unique subsets defined by cell surface markers. IgG+ memory B cells produce IgG antibodies when activated and have undergone CSR[64,69]. Nearly all IgG+ memory B cells have undergone SHM and are faster and stronger activated than naïve B cells[64,69]. IgA+ memory B cells are similar to IgG+ memory B cells, in that the vast majority have undergone SHM[64,69]. IgA+ memory B cells are generated in the intestine and mucosal tissues and produce IgA antibodies to protect mucosal

surfaces[64,69]. IgE+ memory B cells are scarce and represent another form of class-switched memory B cell that produces IgE antibodies against parasites[64,69]. Unlike the class-switched memory B cells (IgG+, IgA+, IgE+), another distinct subset of memory B cells includes IgD/IgM memory B cells. These include IgD+/IgM+ memory B cells that are generated after initial activation by the antigen or through T-cell independent means and have not undergone CSR and SHM[64,69]. It is important to note that while CSR and SHM result in mature B cells that express IgG+, IgA+ or IgE+, SHM can still produce IgD+/IgM+ memory B cells that have a typically earlier exit from the GC reaction, which leads to a lack of class-switching to an IgG+/IgA+/IgE+ phenotype[64]. Some IgD+/IgM+ memory B cells have mutated regions in their variable region genes, which indicates some amount of SMH occurring, but not as much as class-switched memory B cells[64]. Regardless, these cells can re-enter the GC reaction upon further activation due to a pathogen, unlike mature IgG+ memory B cells, which mainly differentiate into plasma cells[64]. IgD-/IgM+ memory B cells show similar BCRs and expressed genes as IgD+/IgM+ memory B cells, with the only difference being the downregulation of the IgD BCR[64]. However, IgM-only B cells have slightly higher variable region gene mutation frequencies than IgD+/IgM+ memory B cells and may indicate cells that have developed later in the GC response, although not before the cells were able to begin CSR due to the loss of IgD[64,69]. IgD+/IgM- memory B cells are a small fraction of B cells that have undergone an atypical CSR event and may represent the origin of heavily mutated IgD+ multiple myeloma and may also be enriched for autoreactivity, although IgD-only memory B cells do require further investigation[64,69]. The emergence of specific memory B cell subsets generally follows a sequential timeline that reflects their developmental pathways. Generally speaking, memory B cells begin to appear in the blood 7-10 days after vaccination, alongside the appearance of plasmablasts[70,71]. As germinal centres begin to form after a week following exposure to a pathogen, the memory B cell population at this time consists of unswitched memory B cells that produce IgM antibodies when activated[55,64]. The IgD+/IgM+ pool of memory B cells is still detectable by around 2 weeks post-vaccination and can remain stable for at least 6 months[72–74]. By contrast, the memory B cells that have undergone CSR and SHM (IgG+. IgA+, IgE+) appear slightly later in the immune response and emerge during the later phase of GC reactions, typically within the first few weeks to a month post-exposure[72–74]. IgG+ B cells tend to dominate the memory B cell pool months after the initial exposure to antigen, while IgD+/IgM+ memory B cells decline over time[75].

1.2 SARS-CoV-2

1.2.1. SARS-CoV-2 Structure

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense, single-stranded RNA virus that belongs to the *Coronaviridae* family and the *Nidovirales* order[76,77]. *Nidovirales* is further divided into the subfamily of *Orthocoronavirinae,* which consists of four genera: *alphacoronavirus, betacoronavirus, gammacoronavirus* and *deltacoronavirus*[76,77]. SARS-CoV-2 is a *betacoronavirus,* a group of coronaviruses that exclusively infect mammalian species and mainly results in respiratory diseases[77]. The RNA genome of SARS-CoV-2 is approximately 29.9 kb in length, 5' capped and 3' polyadenylated[77,78]. At the 5' end of the genome are the open reading frames ORF1a and ORF1b which span two-thirds of the genome[77]. These reading frames encode 16 non-structural proteins involved in processes that include viral RNA synthesis and virus-host interactions[77]. Four structural proteins are encoded at the 3' end of the genome. These proteins include the spike (S), envelope (E) and membrane (M) proteins found in the viral membrane, and the nucleocapsid (N) protein bound to the RNA genome[77].

The N protein is the only structural protein inside the SARS-CoV-2 virion. The primary role is to form a nucleocapsid structure with the viral genome, protecting the RNA and packaging it into a ribonucleoprotein complex during replication[79,80]. In addition, the N protein has been found to act as a viral suppressor of RNA interference within infected cells and an interferon antagonist[81,82]. Structural analysis of the N protein has shown that the genomic RNA of SARS-CoV-2 winds around N protein decamers, allowing for the large genome to be packaged efficiently within the virion[83]. The E protein is found in the viral membrane of SARS-CoV-2 and is responsible for viral lysis and genomic release[84]. The E protein is also responsible or viral assembly and exocytosis by localizing to the endoplasmic reticulum membrane in the infected cells and forming viral protein-containing vesicles[84]. Lastly, the M protein of SARS-CoV-2 is the most abundant structural protein and is also found in the membrane bilayer of SARS-CoV-2. The M protein can bind to all other structural proteins, which aids in stabilizing the nucleocapsid complex within the virions and promoting virion assembly by stabilizing the nucleocapsid upon uptake into the virions[85]. The M protein also heavily interacts with the S protein during viral attachment and entry into host cells[86,87].

Multiple glycosylated S proteins are located on the surface of SARS-CoV-2, forming large protrusions from the viral membrane that gives the appearance of having a crown-like halo around the virion[88,89]. This structure is what gives the coronaviruses their name, *corona* meaning crown in Latin[90]. These proteins are critical for the viral life cycle and for cell entry. For that reason, it is extremely conserved across all coronavirus species and is a highly important target for SARS-CoV-2 therapeutics and treatments[88]. The main function of the S protein is receptor recognition, viral attachment and entry into the host cells to commence infection[88]. The S protein is a roughly 180-200 kDa N-glycosylated type I membrane protein and can be found anchored to the viral membrane of SARS-CoV-2[88,91]. The S protein can also undergo extensive structural rearrangements to facilitate entry during receptor recognition and membrane fusion[88]. The glycosylation of the S protein acts as a camouflage to avoid detection from the innate and adaptive elements of the immune system[92]. The S protein consists of two main subunits: the S1 subunit and the S2 subunit[88]. The S1 subunit contains an N-terminal region and the receptor binding domain (RBD). The RBD portion of the S protein is the region of the S protein that will bind to the host cell receptors, leading to a structural change and membrane fusion[88]. Therefore, this region is designated a key target of therapeutic and vaccine design and is also a critical target for antibody neutralization due to the key role it plays in receptor binding[88]. The S2 region contains the fusion peptide and transmembrane region of the protein, as well as other peptides responsible for viral membrane fusion and cell entry[88]. The fusion peptide is a short segment of hydrophobic residues which anchor to and disrupt the cell membrane after the S protein changes conformation[93]. The S2 region also contains heptapeptide repeat sequences 1 and 2, consisting of a mix of hydrophobic, bulky, polar and hydrophilic residues[88]. Structural changes after receptor binding leads to interactions between the two repeat sequences, which results in closer proximity of the viral envelope and cell membrane[94].

1.2.2. SARS-CoV-2 Life Cycle

The life cycle of a SARS-CoV-2 viral particle begins with binding to angiotensin-converting enzyme 2 (ACE2) on host cells. ACE2 is a type I transmembrane glycoprotein that belongs to the M2 family of zinc metalloproteases and consists of a single active site domain[95,96]. While ACE2 primarily exists as a transmembrane domain protein, it can also exist in a soluble form, which circulates in small amounts in the blood[96]. ACE2 is widely expressed in various cells of the body and was even found in one study to be expressed in 72 different tissues obtained

from three human donors[97]. These include endocrine, gastrointestinal tracts, cardiovascular, kidney, urinary bladder, testes, and muscle tissues[96,97]. High ACE2 expression was also detected in the parenchyma, primary and tertiary bronchi, type I and type II alveolar epithelial cells, as well as oral, nasal and nasopharynx epithelia, which is relevant for the transmission of SARS-CoV-2[96,97]. The main role of ACE2 is to act as a key regulator of the renin-angiotensin system (RAS). The RAS pathway is a hormonal system that regulates blood pressure, fluid balance, and vascular responses[98].

Figure. 1.2-1 outlines the role of ACE2 in the life cycle of SARS-CoV-2 and the replication phases of the virus after it enters the cells. The S1 and S2 domains are responsible for recognizing and binding ACE2 and mediating the fusion of the viral and cellular membranes, respectively. After SARS-CoV-2 enters the respiratory tract, the virus will invade host cells that express ACE2, such as goblet cells, ciliated cells and endothelial cells in the lung[99]. Glycoconjugates on the cell surface are also used by SARS-CoV-2 as attachment factors, which are driven by non-specific electrostatic interactions[99,100]. The RBD portion of the S protein can exist in different conformations. Before recognizing ACE2, the RBD portion is in a “down” state, which shields it from antibody detection[99]. Once ACE2 is present and interacts with SARS-CoV-2, the S protein undergoes a conformational change to expose the RBD portion in an “up” state, which makes it readily available to bind to ACE2[99]. After binding to the cell surface, the S protein undergoes further conformational changes through host cell proteases located on the cell membrane. The first protease cleavage is performed by furin, which recognizes and binds a furin cleavage site at the boundary between the S1 and S2 regions of the S protein[99,101]. The furin separates the two regions, which gives rise to a pre-fusion structure made up of S1 and S2 non-covalently bound to each other[101,102]. The S1 subunit is released, which exposes an S2 cleavage site[99]. The second protease is a transmembrane protein called TMPRSS2, which recognizes and cleaves at the S2 cleavage site[99,101,102]. The resulting conformational change brings the viral and cellular membranes closer together, triggering membrane fusion[99,101,102].

Following membrane fusion, the genomic RNA of SARS-CoV-2 is released into the cytosol of the cell, where replication of the genome occurs[77]. Host ribosomes will utilize the genomic RNA as mRNA and will translate two large open reading frames in the genomic RNA called ORF1a and ORF1b, which results in the synthesis of two large polypeptides called pp1a

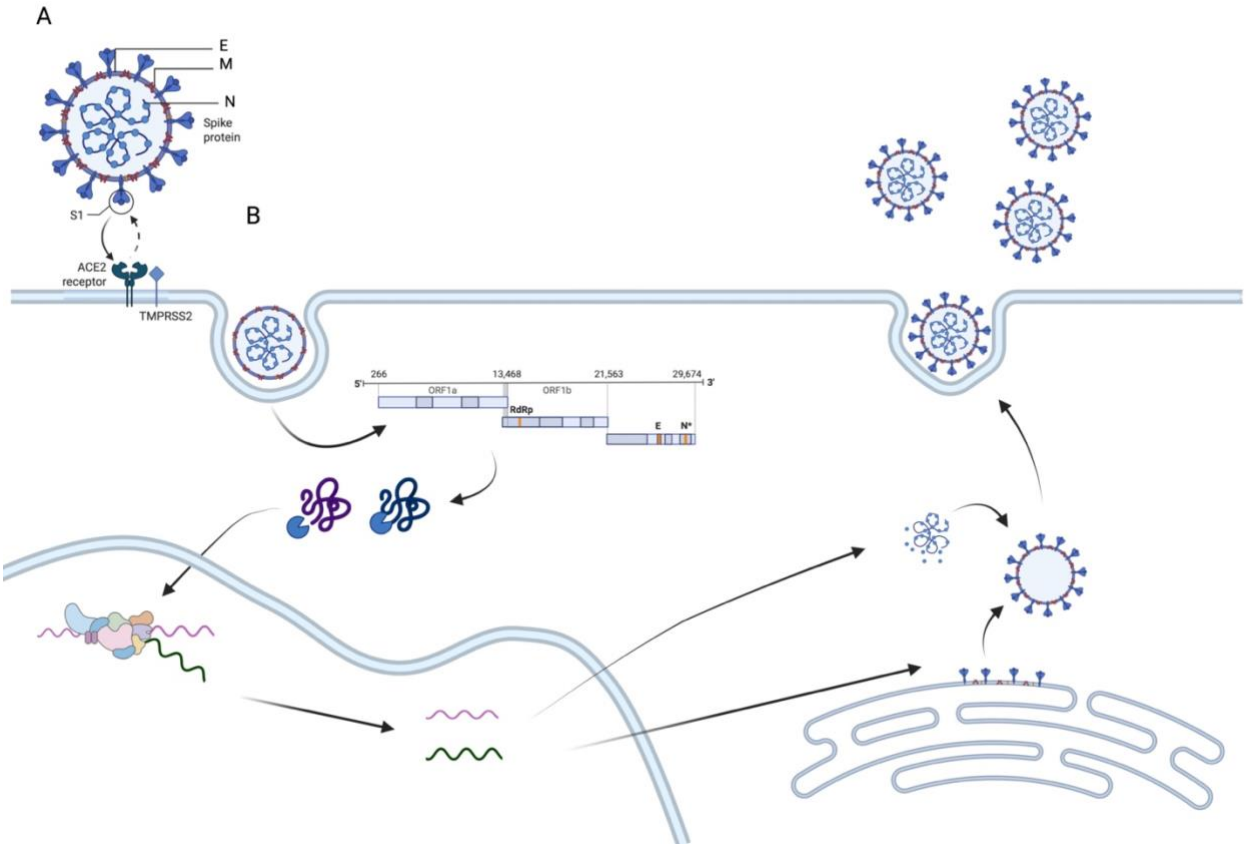


Figure 1.2-1: SARS-CoV-2 Life Cycle: (A) The coronavirus virion consists of the spike, envelope, membrane and nucleocapsid proteins. **(B)** The virion binds to ACE2, which promotes viral uptake and fusion at the membrane. The RNA genome is translated into two large open reading frames, ORF1a and ORF1b. The polyproteins are processed into individual non-structural proteins, which are responsible for RNA replication, packaging and the assembly of the coronavirus virion with the endoplasmic reticulum. The fully formed virion is secreted from the cell by exocytosis. Made in BioRender.

and pp1ab[103,104]. Production of pp1ab is dependent on a ribosomal frameshift upstream of the ORF1a termination codon, thus allowing the ribosome to continue translating the genomic RNA past the stop codon and read the ORF1b codon[104,105]. Sixteen non-structural proteins are released from pp1a and pp1ab following 15 proteolytic cleavages performed by non-structural proteins 3 (Nsp3) and 5 (Nsp5), which are viral encoded proteases[77,104,105]. Some other prominent non-structural proteins important for viral replication include non-structural protein 1 (Nsp1), which is involved in the suppression of the cellular immune responses, the inhibition of cell mRNA translation and host mRNA degradation, and non-structural protein 12 (Nsp12), which forms the RNA-dependent RNA polymerase[106–108]. The non-structural proteins constitute the replication-transcription complex (RTC), which can produce multiple negative sense single strands of the

viral genomic RNA[77,104]. The negative sense strands serve as a template for producing positive sense SARS-CoV-2 genomic RNA for novel viral particles or more RTCs[77]. In addition to generating full-length negative sense strands of the genomic RNA, RTCs also generate negative sense subgenomic RNAs. During transcription, the RTC can be interrupted by transcription regulatory sequences found upstream to most ORFs in the 3' region of the viral genome[77,104,109]. This results in discontinuous synthesis of the negative strand of subgenomic RNAs that are then used as templates to synthesize positive sense subgenomic mRNAs[77,104,109]. These subgenomic mRNAs are translated into the structural proteins that will assemble and form the SARS-CoV-2 virion. The newly synthesized S, E and M protein mRNA molecules are trafficked to the endoplasmic reticulum where they are translated within the membrane[110]. In contrast, the N protein is translated and binds to the genomic RNA to form the nucleocapsid[110]. The nucleocapsid and the S, E and M proteins embedded in the endoplasmic reticulum membrane are assembled at the endoplasmic reticulum-golgi intermediate compartment[110]. The newly formed SARS-CoV-2 virions are transported to the cell surface in vesicles released from the cell via exocytosis[110].

1.2.3. COVID-19 DISEASE

Coronavirus Disease 2019 (COVID-19) is the result of SARS-CoV-2 infection[111]. The primary mode of transmission of SARS-CoV-2 is exposure to respiratory droplets carrying infectious virus particles from close contact or direct transmission from infected individuals[111,112]. This transmission could be from symptomatic individuals, although presymptomatic and asymptomatic individuals have also been shown to transmit SARS-CoV-2 to healthy individuals[111,113,114]. While the main mode of transmission is respiratory droplets, other methods of transmission have been detected, albeit rarely. Fomite transmission from the contamination of inanimate surfaces is a secondary route of transmission of SARS-CoV-2. While the risk is low, SARS-CoV-2 is stable and viable on surfaces such as stainless steel and plastic and has even been detected on hospital floors and equipment. While no strong evidence was found to support human fecal-oral transmission, SARS-CoV-2 can be detected in fecal samples from patients with COVID-19, and animals can be infected with fecal SARS-CoV-2[111,115]. Vertical transmission between mothers and neonates is possible but represents a minority of cases and is rare[111]. Although most infected newborns are asymptomatic or mildly symptomatic,

there are case reports of severe neonatal infection, and maternal infection can increase the risk of adverse pregnancy outcomes such as abortion and prematurity[111,116].

After infection, the median incubation time of SARS-CoV-2 was estimated to be 5.1 days, with most patients developing symptoms within 11.5 days of infection[117]. Although it is estimated that 17.9% to 33.3% of infected patients will remain asymptomatic throughout infection, the spectrum of COVID-19 outcomes varies from asymptomatic patients to clinical illnesses with various consequences[111,118,119]. The most common symptoms associated with COVID-19 are fever, cough and shortness of breath. Research during the initial outbreak of SARS-CoV-2 revealed that 70% of confirmed cases of COVID-19 experienced fever, cough and shortness of breath[120]. Less common symptoms included sore throat, anosmia, dysgeusia, anorexia, nausea, malaise myalgias and diarrhea[120]. These symptoms are what the vast majority of people with COVID-19 would encounter, as roughly 81% of cases are reported to be mild COVID-19 cases[121]. However, 14% of cases are reported to be severe, with 5% of cases being classified as critical[121]. Severe and critical cases usually begin approximately 1 week after the onset of symptoms, with dyspnea and hypoxemia being the most common symptoms of severe disease[122]. Severe cases typically lead to progressive respiratory failure and acute respiratory distress syndrome (ARDS), which is defined as the acute onset of bilateral infiltrates, severe hypoxemia, and lung edema that is not fully explained by cardiac failure or fluid overload[122,123]. Critical cases of COVID-19 can also lead to multiple organ failure in the extrapulmonary organ system, which is generally brought about by direct viral toxicity, vasculitis injury, thrombosis or immune dysregulation[124]. ACE2 receptors can be found in other tissues outside of the epithelial cells of the respiratory tract, resulting in COVID-19-related organ damage[125]. As a result, severe COVID-19 can lead to such conditions as myocarditis, acute kidney injury, gastrointestinal issues and strokes[125]. Across all age groups, the Centers for Disease Control and Prevention (CDC) estimated that COVID-19 had a hospitalization rate of 5.7%, with a rate of <8.3% for all age groups up to 64 years of age[126]. The fatality rate of COVID-19 was estimated at 1.7% for all age groups, with a rate of <1.6% for all age groups up to 64 years of age[126].

The rate of case fatalities and critical cases of SARS-CoV-2 can vary due to various demographic and health factors. Data shows that older adults have higher mortality rates and make up a larger proportion of COVID-19 patients[127,128]. This can be due to several age-related

factors, such as ACE2 expression increasing with advancing age, which would precipitate viral replication and cell entry[129,130]. Aging is also associated with progressive immune system dysregulation, including increased inflammatory reactions and decreased cellular immunity[131,132]. Another factor that causes an increase in COVID-19 severity and mortality is sex, as male cases of COVID-19 tend to be more severe with increased mortality outcomes than female cases[133]. Males have higher expression of ACE2 than females due to the location of the ACE2 gene on the X chromosome and because testosterone has been shown to maintain high levels of ACE2 in the heart and kidney[134–137]. Additionally, females have stronger humoral and cell-mediated immune responses to immunization and infection compared to males, as well as greater baseline immunoglobulin levels, antibody responses and higher expression of antiviral and proinflammatory genes[138–141]. Another risk factor for COVID-19 is ethnicity. The CDC had found that Black people had been hospitalized at 2.0 times the rate and died at 1.6 times the rate of non-Hispanic White people, and Hispanic or Latino people had been hospitalized at 1.8 times the rate and died at 1.7 times the rate of non-Hispanic White people[142]. The outcomes in these populations can be explained by societal factors, including lower socioeconomic statuses and poorer living conditions, limited access to therapeutics and treatments and increased viral exposure among frontline, essential and critical-infrastructure workers[143,144]. While some research has also suggested genetic differences between different human populations may play a role in the disparity of COVID-19 cases and hospitalizations, this is not a foregone conclusion as other research has provided different results[145–147].

Symptoms of COVID-19 that persist beyond 2 weeks are classified as long-term effects of the disease[148,149]. Post COVID-19 condition, or long COVID, is when symptoms of COVID-19 persist for more than 12 weeks after SARS-CoV-2 infection[150]. A survey revealed that approximately 35% of symptomatic individuals had not yet returned to their baseline health after 3 weeks[149]. The Canadian COVID-19 Antibody and Health Survey found that 15% of adults who had COVID-19 experienced longer-term symptoms 3 or more months following infection, and 47% of those adults had symptoms for 1 year or longer[150]. Although severe disease survivors tend to report long-term effects, these symptoms are not solely associated with infection severity, as mild infections that do not require hospitalization may also lead to long-term impacts[149,151]. A major feature of long COVID is the impact on neurological systems and the resulting cognitive impairments, such as memory loss, dizziness, balance issues, loss of smell or taste and

autonomic dysfunction[151,152]. Hearing loss and vertigo have also been noted as long-term effects from COVID-19 infection[153]. Further research has also linked long COVID to gastrointestinal symptoms such as nausea and gut dysbiosis, and reproductive health complications such as irregular menstruations, erectile dysfunction and reduced sperm count[154–158]. The long-term impact of COVID-19 is still not properly understood, and it is still not properly established whether demographic factors, health conditions or infection severity play a role in the risk of developing long-term effects. Still, risk factors potentially include the female sex, type 2 diabetes, attention deficit hyperactivity disorder and autoimmune disorders, although a third of long COVID patients do not have pre-existing conditions[159–161]. Various potential causes of long COVID have been suggested by researchers. SARS-CoV-2 may drive chronic symptoms in patients by persisting in certain tissue reservoirs after acute infection has occurred[162]. Immune dysregulation brought about by SARS-CoV-2 may also cause previously harbored latent pathogens to reactivate, driving new chronic symptoms[162]. The immune dysfunction caused by SARS-CoV-2 may lead to prolonged inflammation of blood vessels, damaged tissue as well as improper activation of immune cells, which could cause blood clotting issues, endothelial cell damage and neurological symptoms typically found in long COVID patients[153,162].

1.2.4. SARS-CoV-2 Pandemic

SARS-CoV-2 was the third highly pathogenic coronavirus to emerge in humans within the last two decades, following SARS-CoV in Foshan, China, on 16 November 2002 and MERS-CoV in Zarqa, Jordan, on 4 April 2012[163–165]. The SARS-CoV epidemic was contained in July 2003, and no more infected human cases have been detected since May 2004[164,166]. Roughly 8000 people fell ill and 774 died from SARS-CoV before the end of the epidemic[167]. While MERS-CoV has led to persistent epidemics in the Middle East, there have been no major outbreaks, with countries such as Saudi Arabia reporting 5 MERS-CoV cases in 2024[164,168]. As of April 2012, there has been a total of 2613 laboratory-confirmed cases of MERS-CoV with 943 associated deaths[169]. While the previous viruses resulted in limited outbreaks, SARS-CoV-2 was declared a pandemic in March 2020 by the World Health Organization (WHO)[170]. By that time, the number of cases had reached 118000 in 114 countries, and 4291 people had died in less than half a year[170]. Analyzing the structures and transmission of SARS-CoV, MERS-CoV, and SARS-CoV-2 provides numerous reasons why the SARS-CoV-2 outbreak led to a global pandemic while the former viruses were endemic. Studies have shown that the S protein of

SARS-CoV-2 has a higher affinity for the ACE2 receptor than SARS-CoV, which enhances transmissibility[171]. MERS-CoV utilizes a different receptor, DPP4, primarily expressed in the alveolar regions of the lower respiratory tract[172]. SARS-CoV-2 also replicates and sheds in the upper respiratory tract, even among presymptomatic patients, which distinguishes the novel virus from SARS-CoV, where replication occurs mainly in the lower respiratory tract[173,174]. The presence of SARS-CoV-2 in the upper respiratory tract, while SARS-CoV and MERS-CoV replicate in the lower respiratory tract, is what allows for rapid human-to-human transmission. Additionally, transmission of SARS-CoV-2 was being facilitated by asymptomatic carriers, which had not been seen in the previous coronavirus outbreaks[175]. The reproduction number (R0), or the average number of cases an infected person will cause during the infectious period, was estimated at around 1 for MERS-CoV and <1-2.75 for SARS-CoV[176,177]. The R0 of SARS-CoV-2, at the start of the pandemic, was estimated to be between 1.5 and 6.68 based on a review of available literature, with an average mean of 3.28[178]. The increased transmission rate of SARS-CoV-2 is what distinguishes it from predecessors and is why there was an explosion of cases in such a short amount of time.

The SARS-CoV-2 pandemic had profound global consequences that continue to affect the population. The virus continues to circulate among the population to this day. As of November 10, 2024, WHO reported over 776.8 million confirmed COVID-19 cases and over 7 million confirmed deaths across 234 countries[179]. However, excess mortality puts the estimated deaths from COVID-19 closer to 27 million[180]. As of September 21, 2024, there were 60871 total confirmed deaths in Canada from COVID-19, although excess mortality could place that number closer to 90000[180,181]. The pandemic also resulted in economic downfalls for many industries and nations, as well as a rise in extreme poverty and malnutrition[182–184]. Erosion in the trust of institutions and scientists declined significantly in the US and signals a worrisome trend if a future pandemic were to arise[185]. Vaccine hesitancy has also seen a rise during the pandemic, with hesitancy increasing around the globe[186,187].

1.2.5. Variants of Concern

SARS-CoV-2 variants are classified into three categories: variant under monitoring (VUM), variant of interest (VOI), and variant of concern (VOC). VUM is a SARS-CoV-2 variant with genetic changes that are suspected to affect virus characteristics with an indication

that it may pose a future risk, but the evidence is unclear and requires further monitoring[188,189]. These viral characteristics can include transmissibility, disease severity, immune escape, as well as diagnostic or therapeutic escape. VOI is a SARS-CoV-2 variant with genetic changes that are suspected to or are known to affect virus characteristics and is identified to cause epidemiological impacts that may suggest an emerging risk to global public health[188,189]. Lastly, VOC is a SARS-CoV-2 variant that meets the definition of VOI but has also been demonstrated to be associated with one or more of the following: an increase in transmissibility, increase in virulence or change in symptoms or a decrease in the effectiveness of public health measure or available therapeutics[188,189]. As of December 9, 2024, the WHO had identified 6 circulating VUMs and 1 circulating VOI[190]. The SARS-CoV-2 pandemic has seen 5 VOCs identified, which are named Alpha, Beta, Gamma, Delta and Omicron[191].

The first VOC was identified in the UK and named B.1.1.7, dubbed the Alpha variant according to the WHO[192,193]. The Alpha variant included 17 mutations in the viral genome, which included 8 mutations in the S protein[189]. Of these mutations, the most notable were N501Y, P681H and an H69/V70 deletion[189]. These mutations enhanced RBD binding affinity to ACE2, altered the furin cleavage site in the S protein, and enhanced immune escape and testing kit failures respectively[194–197]. A study from the UK evaluated the effectiveness of vaccines against the Alpha variant and found that effectiveness against symptomatic disease after one dose was 48.7%, and the effectiveness of two doses was 93.7%[198]. The Alpha variant soon became the dominant strain of SARS-CoV-2 by mid-2021, but few new cases of the Alpha variant have been detected since March 2022, and the strain has now been classified as a previously circulating VOC by the WHO[199].

The B.1.351 (Beta) variant was first detected in South Africa in October 2020[200]. There are 3 notable mutations in the RBD portion of the S protein, allowing the Beta variant to attach more easily to human cells[189]. These mutations included N501Y, K417N and E484K. The E484K mutation conferred resistance to monoclonal antibodies that targeted the RBD portion, while the K417N mutation resulted in a more open S trimer, providing the Beta variant with enhanced ACE2 binding capabilities[201,202]. While the vaccine effectiveness against the Beta variant was slightly lower than the previous variants at 75%, protection against severe forms of the disease such as hospitalization and death were still being reported with greater than 90%

protection[203]. The Beta variant was also classified as a previously circulating variant by the WHO in March 2022 alongside the Alpha variant, due to a lack of detected cases[199].

The Gamma variant, or the B.1.1.28.1 variant, was first detected in four travellers returning to Japan from Brazil in January 2021[204]. This variant harboured 10 mutations in the S protein, with 3 of those mutations being in the RBD region[205]. These mutations (N501Y, K417N and E484K) were similar to the Beta variant and were also associated with enhanced binding affinity to ACE2 and increased transmissibility. The Gamma variant, when compared to previous SARS-CoV-2 variants, was associated with higher disease severity, including increased viral load, elevated risk of death in younger adults, and higher case fatality rates across multiple age groups in Brazil[206–208]. Studies had shown that variants with similar mutations to the Gamma variant were slightly more resistant to neutralization by sera from vaccinated individuals and that mRNA vaccine effectiveness at 7 days after the second dose was 77% against the Gamma variant[209–211]. The Gamma variant did not solely impact Brazil and was detected in nearly 74 countries by July 2021[212]. However, the number of cases of the Gamma variant declined due to the rapid onset of Delta and Omicron cases shortly after July 2021[199,213]. Similarly to the previous variants, the Gamma variant was classified as a previously circulating variant by the WHO in March 2022[199].

India first identified the B.1.617.2 variant in October 2020[214]. On May 31, 2021, the WHO announced a naming system using Greek letters for previous variants and designated the B.1.617.2 variant as a VOC called the Delta variant[215]. Shortly after the change in nomenclature, the Delta variant quickly emerged as the most dominant strain of SARS-CoV-2 globally[216]. The genome of the Delta variant contained 13 mutations, with 4 mutations in the S protein particularly concerning. These mutations include P681R, a change in the furin cleavage site that enabled enhanced cleavage of the S protein into its active configuration, and D164G, which increases the stability of virions[189,217,218]. L452 was another common mutation in the S protein, stabilizing the interaction between the S protein and the ACE2 receptor on host cells, enhancing infectivity[189,219]. Initial studies estimated that the Delta variant was more than 40-60% more transmissible than the wild-type SARS-CoV-2 variant, with an initial R0 of 5.08[220]. Cohort studies from Ontario found that increased risk with the Delta variant was more pronounced at 108% for hospitalization, 235% for ICU admission and 133% for death compared to non-VOC

strains[221]. These results suggest a faster replication rate and enhanced infectiousness and transmission of the Delta variant. Vaccine effectiveness was reduced against the Delta variant compared to the Alpha variant after two doses of the mRNA vaccine, with slightly lower protection against infection and hospitalization also detected[198,222]. During the summer of 2021, the Delta variant became the predominant strain due to the high transmissibility and relative resistance to therapeutics. In the US, Delta rose from 1% of SARS-CoV-2 cases in May to over 50% by June and exceeded 95% of cases by the end of July 2021[223]. However, the emergence of Omicron in November 2021 led to a rapid decline in Delta variant cases, and the WHO reclassified the Delta variant as a previously circulating VOC by June 2022[224].

The initial outbreak of the Omicron variant was first reported to the WHO on November 24, 2021, from specimens collected earlier that month in South Africa and Botswana[225]. On 26 November 2021, WHO designated variant B.1.1.529 a VOC and gave the Omicron designation to the new strain[226]. Since the initial detection of the Omicron variant, various sublineages of the original B.1.1.529 strain have emerged, including BA.1.1, BA.2, BA.3, BA.4 and BA.5[227]. The current circulating strain of SARS-CoV-2 is XEC, followed by KP3.1.1. These two variants are responsible for most cases of COVID-19, with XEC representing 35.9% of sequenced samples in Canada and KP3.1.1 representing 23.1% of sequenced samples as of December 22 2024[228]. These two lineages are subvariants of the B.1.1.529 variant of SARS-CoV-2, also known as the Omicron variant[229,230]. Omicron managed to spread rapidly worldwide in a short period of weeks due to a greater infection rate, higher transmissibility and an ability to evade acquired immunity in vaccinated individuals[231]. This was due to the vast number of mutations in the S protein of the strain, including several that overlap with the variants described above. As of June 2022, Omicron had roughly 50 mutations relative to the original wild-type variant, more than any previous variant of SARS-CoV-2 at the time[232,233]. Twenty-six to 32 mutations were found in the S protein, with 15 mutations in the RBD portion[232,233]. The Delta variant, in comparison, had 9 mutations in the S protein, with 2 mutations in the RBD region[232,234]. Out of the 50 total mutations, 26 mutations are unique to Omicron, including G446S, Q493R, G446S and G496S, which affect antibody binding and neutralization of the strain[191,234,235]. These unique mutations in the S protein and RBD region distinguish the Omicron variant from previous strains and significantly alter factors such as transmission rate and severity. Reviews found that the average initial r0 value for the Omicron variant was 8.2, which was roughly 3 times higher than the Delta

variant[236]. The Omicron variant was also found to have replicated to the highest titres at the earliest time points in the upper respiratory tract compared to the other VOCs[237]. However, despite the increased transmissibility of the Omicron variant, the rate of hospitalizations and deaths among patients infected with Omicron were significantly lower than previous SARS-CoV-2 strains[238–240]. Even though severity was reduced, the enhanced immune evasion due to the amount of S and RBD mutations resulted in limited protection from vaccines against the Omicron variant. Researchers found that no effect against the Omicron variant was noted from 20 weeks after 2 doses of an adenovirus vaccine, while 2 doses of the mRNA vaccine resulted in a vaccine effectiveness of 65.5% at 2-4 weeks and 8.8% at 25 or more weeks[241]. Three doses of mRNA vaccines were found to provide an effectiveness of 42.0% against Omicron infection and 81.7% against hospitalization or death from Omicron infection[242]. Additional research found that sera from individuals vaccinated by two doses of a SARS-CoV-2 vaccine and 20 monoclonal antibody treatments showed weak neutralization activity against the sub-lineages of the Omicron variant[243]. Overall, protection from vaccines against the previous variants was not as effective in stopping Omicron. The lack of protection from therapeutics already provided during the pandemic resulted in a widespread outbreak of the Omicron variant and subsequent sub-lineages.

1.2.6. SARS-CoV-2 Vaccines

1.2.6.1. mRNA Vaccines

mRNA vaccines are comprised of synthetic mRNA molecules that encode for the antigen that will generate the immune response rather than using an inactivated pathogen (Fig. 1.2-2)[244]. The vaccine can be comprised of self-replicating or non-replicating mRNA molecules[245]. The non-replicating mRNA-based vaccines encode the desired antigen for the immune response and are comprised of 5’ and 3’ untranslated regions, open reading frame and a poly(A) tail[245]. The self-amplifying mRNA contains those components and a coding region that codes for viral replication machinery, allowing continuous RNA amplification[245]. Although mRNA had been found to elicit both innate and adaptive immunity in the early 1990s, the development of mRNA faced numerous hurdles due to the known instability of mRNA molecules, the rapid degradation by extracellular RNases, and inefficient delivery systems[245–247]. Additional modifications in these mRNA molecules prevent the traditional limitations of mRNA molecules from arising. For example, the addition of additional nucleotides to the poly(A) tail can result in synthetic mRNA with a longer lifespan[248,249]. Modified nucleotides, such as N(1)-methyl-pseudouridine, can be

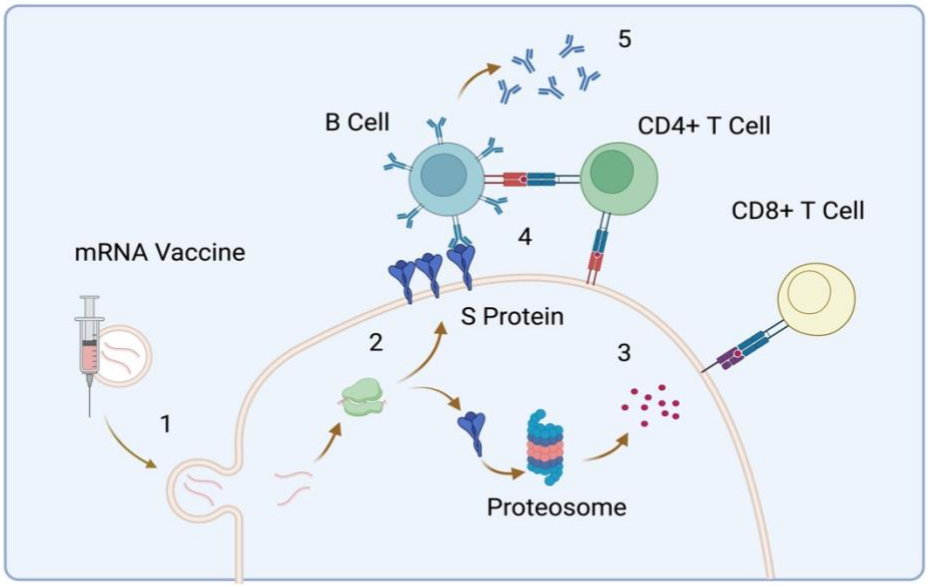


Figure 1.2-2: Overview of mRNA Vaccination and Immune System Response. (1) Injected mRNA molecules are endocytosed by APCs. **(2)** The mRNA molecule is translated into protein by the ribosomes. **(3)** The antigen is broken down into proteosomes, and the fragments can be presented on MHC I proteins to CD8+ T cells. **(4)** Antigen can also be presented on the surface of the APC or on MHC II proteins to recruit B cells and CD4+ T cells, respectively. **(5)** The activated B cells will produce neutralizing antibodies that can recognize and bind to the antigen. Made in BioRender.

added to the molecule to avoid recognition by the immune system and improve translational efficiency[248,250]. Lastly, mRNA vaccine molecules are large and negatively charged, which means they are unable to pass through the lipid bilayer of cell membranes[248]. Lipid nanoparticles (LNPs) are lipid vesicles that encapsulate the synthetic mRNA molecules, protecting them from nuclease degradation and providing an efficient delivery system into the host cells[245,251]. LNPs can also have the proportions of certain lipid components adjusted to provide organ selectivity for the vaccine or treatment[251]. Once they reach the target APCs, the LNPs are internalized through processes such as endocytosis[251]. The lipids are usually trapped in endosomal compartments, resulting in the leak of mRNA molecules into the cytosol of the immune or non-immune cell[251]. The translational processes of the cell will produce the desired antigen, which is then degraded by proteosomes[244,245,248]. The antigenic fragments are then presented on MHC I molecules of the APC, which can activate CD8+ T cells, which can in turn activate other cellular

components of the immune system[244,245,248]. The fragments can also form a complex with MHC II, resulting in the activation of CD4+ T cells, which can lead to the activation of B cells to produce neutralizing antibodies[244,245,248]. The translated antigen may also be presented directly on the surface of the cell, thereby directly activating B cells with BCRs that can recognize the antigen[244,245,248].

While mRNA technology had already begun to materialize for cancer immunotherapy and infectious disease prevention, it was the COVID-19 pandemic that provided the critical impetus for the first major deployment of mRNA vaccines. To date, two prominent mRNA vaccines have gained approval and widespread usage globally. These vaccines are Comirnaty (BNT162b2) and Spikevax (mRNA-1273), produced by Pfizer-BioNTech and Moderna, respectively[252,253]. While both vaccines utilize the same technology, the BNT162b2 vaccine has 30μg per dose in people 12 years of age and older, whereas the mRNA-1273 vaccine has 50 μg per dose in the same age range[254,255]. Both vaccines showed enormous promise in preventing severe COVID-19 disease and reducing infection and transmission. Large-scale studies during the first year of the pandemic found that BNT162b2 was 95% effective in preventing COVID-19 infection[256]. Randomized trials found that the mRNA-1273 vaccine had 94.1% efficacy at preventing COVID-19 illness, including severe disease[257].

mRNA vaccines provide several advantages over traditional vaccine types. Unlike attenuated or viral vector vaccines, mRNA is a non-infectious, non-integrating platform, unlike other vaccine technologies, which may inadvertently contain live viral components[245,258]. This eliminates the potential risk of infection from the pathogen in question. In addition, mRNA is degraded and removed from the host through normal cellular processes, reducing the risk of metabolite toxicity[245]. The molecule itself can be modified to regulate immunogenicity or the half-life of the molecule, which modulates the immune response from vaccination[245]. Additionally, mRNA vaccines have the potential for rapid and inexpensive manufacturing and can be synthesized without the need for cell cultures or live viruses[245,248,258]. Lastly, unlike traditional vaccines, mRNA vaccines do not require an added adjuvant. Generally, an adjuvant is added to vaccines to activate the innate immune system by binding to PRRs of innate immune cells[259]. However, both unmodified and modified exogenous mRNA molecules can act as PAMPs and bind to PRRs such as TLR7 and TLR8[259]. One limitation of using mRNA molecules

as an immunization strategy is that mRNA molecules are extremely unstable and sensitive to temperature. mRNA molecules degrade rapidly due to their unstable nature and require stringent cold-chain logistics to maintain integrity. The BNT162b2 vaccine requires storage between -90°C and -60°C, while the mRNA-1273 vaccine requires storage between -50°C and -15°C continuously, or else vaccine efficacy can be compromised[254,255]. This poses significant challenges for distribution, especially in regions lacking advanced refrigeration infrastructure[260].

1.2.6.2. Other Vaccine Types

Viral vector vaccines consist of viral particles whose genomes have been modified to contain one or more foreign genes that encode the desired target antigen[261,262]. These vectors can also be modified by removing the virulence genes, which enables the viral vectors to deliver target genes to cells without causing disease, known as non-replicating viral vectors[261]. Replicating vector vaccines do not have virulence genes removed and can produce new viral particles in the cells that they enter, which can then go on to enter more cells to make the antigen[261]. The SARS-CoV-2 pandemic has resulted in the production and testing of new viral vector vaccines. Johnson & Johnson, an American biotechnology company, created Ad26.COV2.S, which is comprised of a non-replicating human adenovirus type 26 (Ad26) vector that expresses a SARS-CoV-2 wild-type S protein[263]. The University of Oxford and AstraZeneca developed Vaxzevria, or ChAdOx1 nCoV-19, a non-replicating vaccine based on a simian viral vector originally designed for MERS-CoV[264]. Another example of a viral vector vaccine is Sputnik V, a non-replicating adenoviral vector vaccine developed by the Gamaleya Research Institute of Epidemiology and Microbiology[261]. Ad26.COV2.S was found to have a 66.9% vaccine efficacy rate against moderate COVID-19 and 76.7% vaccine efficacy against severe COVID-19 at least 14 days after administration compared to those who received a placebo[265]. A twenty-article meta-analysis found that Vaxzevria had a vaccine efficacy rate of 78% after the first vaccine dose, while after the second dose, the vaccine efficacy rate was 67%[266].

The subunit vaccines utilize a fragment of a pathogen that can elicit an immune response, rather than a vector or an mRNA molecule that encodes the fragment[267]. The S protein is an ideal target for vaccine development due to its high antigenicity and because developing antibodies against S protein leads to effective neutralization of SARS-CoV-2's ability to recognize

ACE2[268,269]. NVX-CoV2373 vaccine, or Novavax, is a nanoparticle vaccine created by Novavax, Inc. which contains a full-length, wild-type recombinant SARS-CoV-2 S protein and a Matrix-M adjuvant[267]. Novavax was found to have a vaccine efficacy rate of 89.7% at least 7 days after a second dose of the vaccine, and an efficacy rate of 86.3% against the alpha variant[270]. While the vaccine has shown good efficacy, its performance against emerging variants, such as Omicron, has raised concerns about the adaptability of Novavax and other protein-based vaccines. Studies indicate that mRNA vaccines have shown high efficacy in preventing severe disease and hospitalization against Omicron, with three doses providing strong neutralization against Omicron subvariant[271]. Meanwhile, Novavox has moderate efficacy against Omicron, with 31% effectiveness against infection and 50% against symptomatic COVID-19 during Omicron predominance[272]. Additionally, reformulating protein-based vaccines to combat variants takes longer than mRNA counterparts, which may limit the responsiveness of these types of vaccines to new variants or pathogens[273].

1.3. SARS-CoV-2 Vaccine Dose Schedule

1.3.1. Vaccine Rollout

To combat the SARS-CoV-2 pandemic, a large-scale vaccination campaign was implemented in Canada and other nations. This campaign started in March 2020, when Canada announced a $275 million investment in coronavirus research and more than $1 billion in vaccine research[274]. On December 9th, 2020, Health Canada issued an emergency authorization for the Pfizer-BioNTech COVID-19 vaccine under the *Interim Order Respecting the Importation, Sale and Advertising of Drugs for Use in Relation to COVID-19*[275]. During that time, Health Canada provided emergency authorization of the Moderna COVID-19 vaccine on December 23rd, 2020, under the same act as Pfizer-BioNTech[276]. This decision was followed by the approval of the Oxford-AstraZeneca Vaxzevria vaccine on February 26, 2021 and the approval of the Janssen Ad26.COV2.S vaccine on March 5, 2021[277,278].

It is important to note that at the start of the vaccination rollout, each manufacturer recommended a vaccine dose schedule that Canada followed for the first months of the rollout. Pfizer-BioNTech recommended a dose interval of 21 days apart, while Moderna recommended a dose interval of 28 days apart[279]. Shortly after the authorization of the mRNA vaccines, the National Advisory Committee on Immunization (NACI) issued guidelines on how the vaccines

should be administered[280]. Adults 80 years of age and older, health care workers, adults in Indigenous communities, and residents and staff of congregate living settings were all prioritized for vaccination[280]. Eventually, the vaccine was offered to adults 50 years of age or more without a medical condition and 16 years of age or more with a medical condition, as well as first responders, primary caregivers, and adults in racialized and marginalized communities disproportionately affected by COVID-19[281]. However, these were only recommendations, as the NACI acknowledged that it would be up to provincial and territorial immunization programs to implement these guidelines[281]. For example, Manitoba followed NACI guidelines closely according to frequent updates on vaccination progress during the initial months of the vaccine rollout[282–285]. The eligibility criteria in Manitoba were similar to those set up by NACI, and Manitoba also followed its own guidelines when vaccinating the remaining population by age.

While provinces followed and changed their own immunization guidelines, the government of Canada maintained the standard dose interval recommendation until March 1st, 2021, when the NACI voted on revising the recommendations on vaccine dose intervals[286]. On March 3rd, 2021, the NACI issued a statement recommending that in the context of a limited COVID-19 vaccine supply, jurisdictions should maximize the number of individuals receiving a first dose of the vaccine and delay the second dose up to 4 months[286]. This recommendation change was brought about by the fact that COVID-19 vaccine shortages significantly impacted the initial rollout of vaccines in Canada in early 2021. Unfortunately, Canada had placed significant bets on vaccines that arrived later to market, so by the time orders were placed for mRNA vaccines; manufacturers prioritized other nations due to prior agreements, which severely limited Canada's access to these vaccines[287,288]. While Canada eventually made a deal with Pfizer-BioNTech and Moderna in August 2020, the United States had already made those deals in July 2020[289,290]. While other nations invested in domestic vaccine production that was already present, Canada had historically underfunded biomedical production capabilities even before the pandemic[287,288,291,292]. This meant that Canada relied on global vaccine supply chains, which were extremely vulnerable during the pandemic[287,288]. Regardless, Canada had lower vaccine security than other nations and was second to last among the G7 nations in vaccine uptake as of March 11, 2021, with a vaccine uptake of 7%[287,288,293]. Therefore, to combat vaccine shortages, Canada moved to delay the second dose of the SARS-CoV-2 vaccines.

While based on evidence that will be discussed shortly, this decision was criticized by numerous manufacturing and government people. When the decision to delay the dose interval was made in the United Kingdom, Pfizer-BioNTech released a statement that said, "The safety and efficacy of the vaccine has not been evaluated on different dosing schedules as the majority of trials participants received the second dose within the window specified in the study design… There is no data to demonstrate that protection after the first dose is sustained after 21 days."[294]. The European Medicines Agency said that the maximum interval between the first and second doses of the Pfizer vaccine should be respected, while the FDA released a statement disagreeing with the decision, citing a lack of evidence and a potential risk to people who would not be fully protected against SARS-CoV-2[295,296]. When the NACI decided to delay the dosing interval to 4 months, those concerns did not disappear. Moderna Canada came out and stood by their original 4-week dose interval, while Canada's chief science advisor warned that delaying the dose interval was, "a basically population level experiment" and that it is, "really important that we stick with the data and with the great science that gives us these fantastic vaccines, and not tinker with it"[297]. When Quebec opted to extend the dose interval from 3 to 4 months, Pfizer Canada refused to sign off on the plan, claiming that there are no data, "to demonstrate that protection after the first dose is sustained after 21 days"[298]. Additionally, there were concerns from immunologists and virologists that switching to a delayed dose interval could foster vaccine-resistant forms of the virus and fuel viral evolution by providing sub-optimal immunity to the population[299,300].

1.3.2. Initial Evidence for Delayed SARS-CoV-2 Vaccine Dose Interval

The NACI published a statement on April 7th, 2021, that provided a detailed overview of the reasons and studies behind the decision to delay the dose interval of the SARS-CoV-2 vaccine[301]. This statement included published peer-reviewed studies, pre-prints and data from population-based assessments within and outside Canada[301]. While the statement offers a wealth of evidence, the most critical and relevant aspects will be explored in greater detail. The primary evidence used to decide to delay the dosing interval ultimately came from reviews of the data on the efficacy and effectiveness of a single dose of an mRNA vaccine, as well as the duration of protection following the first dose. As previously mentioned, the efficacy rate of the mRNA vaccines 10-14 days after administration of the first dose was quite strong. In addition, it was

determined, based on preliminary data, that a single dose of a SARS-CoV-2 vaccine provided a long duration of protection. Initial research from England measured the effectiveness of the Pfizer-BioNTech vaccine in adults aged 70 and older and found that vaccine effectiveness reached 61% at 28-34 days after vaccination and then plateaued, with protection being maintained for the duration of follow-up (>6 weeks)[302]. Preliminary data from the Comité sur L'Immunisation du Québec and the Institut National de Santé Publique du Québec measured incidences of COVID-19 among healthcare workers and the vaccine effectiveness[303]. Data showed that vaccine effectiveness among healthcare workers in Quebec was 80% at the end of the measured period, which was just over a month, and the effectiveness had plateaued. The data that was available at the time indicated that the first dose of the SARS-CoV-2 vaccine protected against SARS-CoV-2 for a longer period than the 3-4 week schedule implemented by Pfizer-BioNTech and Moderna, which aided the NACI in making their final decision.

Additionally, numerous mathematical models examined the potential outcomes of extending the dose interval of the SARS-CoV-2 vaccine. These models were based on pre-print or published articles and internal models from the Public Health Agency of Canada (PHAC). The internal model from PHAC showed that extending the mRNA vaccine dose intervals was projected to result in 12.1-18.9% fewer symptomatic cases, 9.5-13.5% fewer hospitalizations, and 7.5-9.7% fewer deaths in the population, with the largest reductions in hospitalizations and deaths being observed in the 24-week dose interval[304]. These benefits were driven by accelerating access to the first dose of the vaccine to adults aged 20-74 years. Additional models from outside of PHAC sought to compare the two strategies of vaccination schedules[305]. These models found that a delay of 9 weeks between doses of the Moderna vaccine could avert an additional 17.3 infections, 0.69 hospitalizations and 0.34 deaths per 10,000 people compared to the recommended dose interval. A similar schedule for the Pfizer-BioNTech vaccine also averted an additional 0.60 hospitalizations and 0.32 deaths per 10000 people compared to the initial 3-week schedule. These benefits were determined to be from providing greater vaccine coverage to more people, even when the level of protection of one dose was not as high as that of two doses[301]. This was further confirmed with additional models that directly compared fixed vaccine schedules (schedules that held back doses to ensure enough were available for second doses) and flexible schedules (schedules that provided more first doses to the population) and found the

flexible strategy would result in an additional 23% to 29% of COVID-19 cases averted compared to the fixed strategy[306].

While mathematical models and vaccine effectiveness data are invaluable tools during the initial phases of a pandemic, they can be limited in scope. Models often rely on assumptions and projections that may not reflect the clinical outcomes of vaccination and how those vaccines impact the immune system. Initial data only gave researchers information about how well the vaccine reduced COVID-19 cases and did not delve into the underlying immunological mechanisms or long-term consequences of a delayed dose interval of the SARS-CoV-2 vaccine. These models and preliminary research articles had to work with a sample size that did not fully represent the overall population, such as healthcare workers who were in contact with SARS-CoV-2 and people over 70 years of age.

1.4. COVID Vaccine Immunogenicity Study

1.4.1. Impact of Dose Interval on CD4+ and CD8+ T Cells

The COVID-19 Vaccine Project at the National Microbiology Laboratory and University of Manitoba enrolled participants with either a standard or extended dose interval of the SARS-CoV-2 vaccine and collected blood samples from before the primary dose and after each subsequent dose, providing an opportunity to gain a greater understanding of how the vaccine dose interval impacts the immune system. More specifically, research from the COVID-19 Vaccine Project has shown how an extended dose interval of the SARS-CoV-2 vaccine can impact the proportions of CD4+ and CD8+ T cells in the immune system. mRNA vaccines for SARS-CoV-2 have already shown that they can induce a strong T cell response to SARS-CoV-2 and the variants[307–309]. When the extended dose interval was analyzed, the proportions of CD4+ and CD8+ T cells expressing interferon-γ were comparable between both groups, indicating that an extended dose interval did not impact cell-mediated immunity[310]. The data from the COVID-19 Vaccine Project confirmed those results by assessing CD4+ and CD8+ T cell responses after an extended dose interval (Fig. 1.4-1)[311]. While vaccination led to significant increases in the S-specific T cell population, the proportions did not significantly differ from standard or extended dose intervals. Further subsets of T cells, such as central memory, effector memory and CD45RA-expressing effector memory T cells were assessed and found not to differ between the two vaccination schedules.

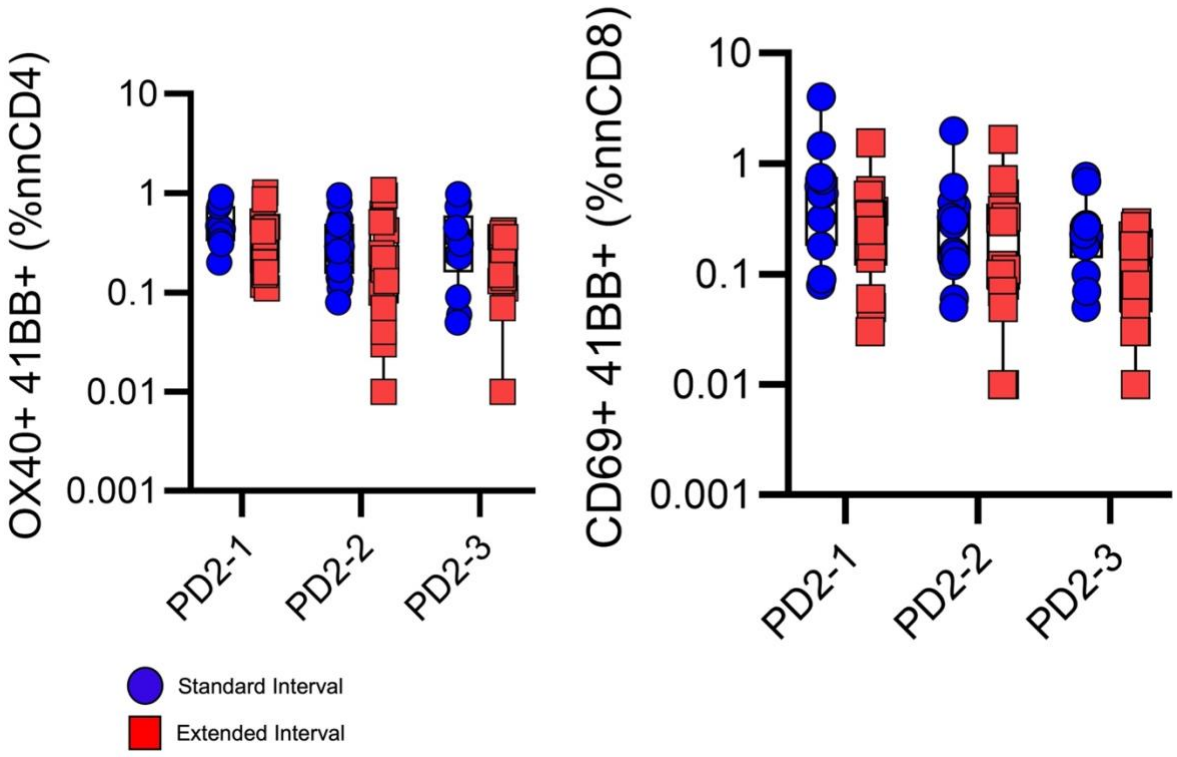


Figure 1.4-1: Impact of a Delayed Dose Interval on CD4+ and CD8+ T Cells. Proportions of non-naïve CD4+ and CD8+ T cells were measured at 10-14 days post-dose 2 (PD2-1), 2-4 months post-dose 2 (PD2-2) and 6 months post-dose 2 (PD2-3).

1.4.2. Impact of Dose Interval on Humoral Immunity

While the impact of an extended dose interval on CD4+ and CD8+ T cells has shown no significant difference with a standard dose interval, research on how dose intervals impact antibody titres have provided different results. RBD-specific antibody titres are significantly greater compared to the standard dose interval group[310]. Additionally, a plaque reduction neutralization tests against the wild-type SARS-CoV-2 and Alpha, Beta and Delta variants showed enhanced neutralization from antibodies harvested from the delayed dose interval group compared to the standard dose interval group[310]. A greater than 3-month dose interval was also analyzed and found to have 31% higher titres of IgG S-specific antibodies and 37% higher titres of IgG RBD-specific antibodies compared to dose intervals that were less than 3 months[312]. Serology data from our COVID-19 Vaccine Project shows that an extended dose interval resulted in a slower decline of S-specific and RBD-specific IgG+ antibodies in participants who

received the extended dose interval compared to participants who received the standard dose interval (Fig. 1.4-2A)[311]. Furthermore, participants who had an extended dose interval had a greater breadth of inhibition of SARS-CoV-2 antigen by plasma antibodies, with greater neutralization of the wild-type, Alpha, Beta and Delta variants after the second dose compared to the standard dose interval (Fig. 1.4-2B)[311].

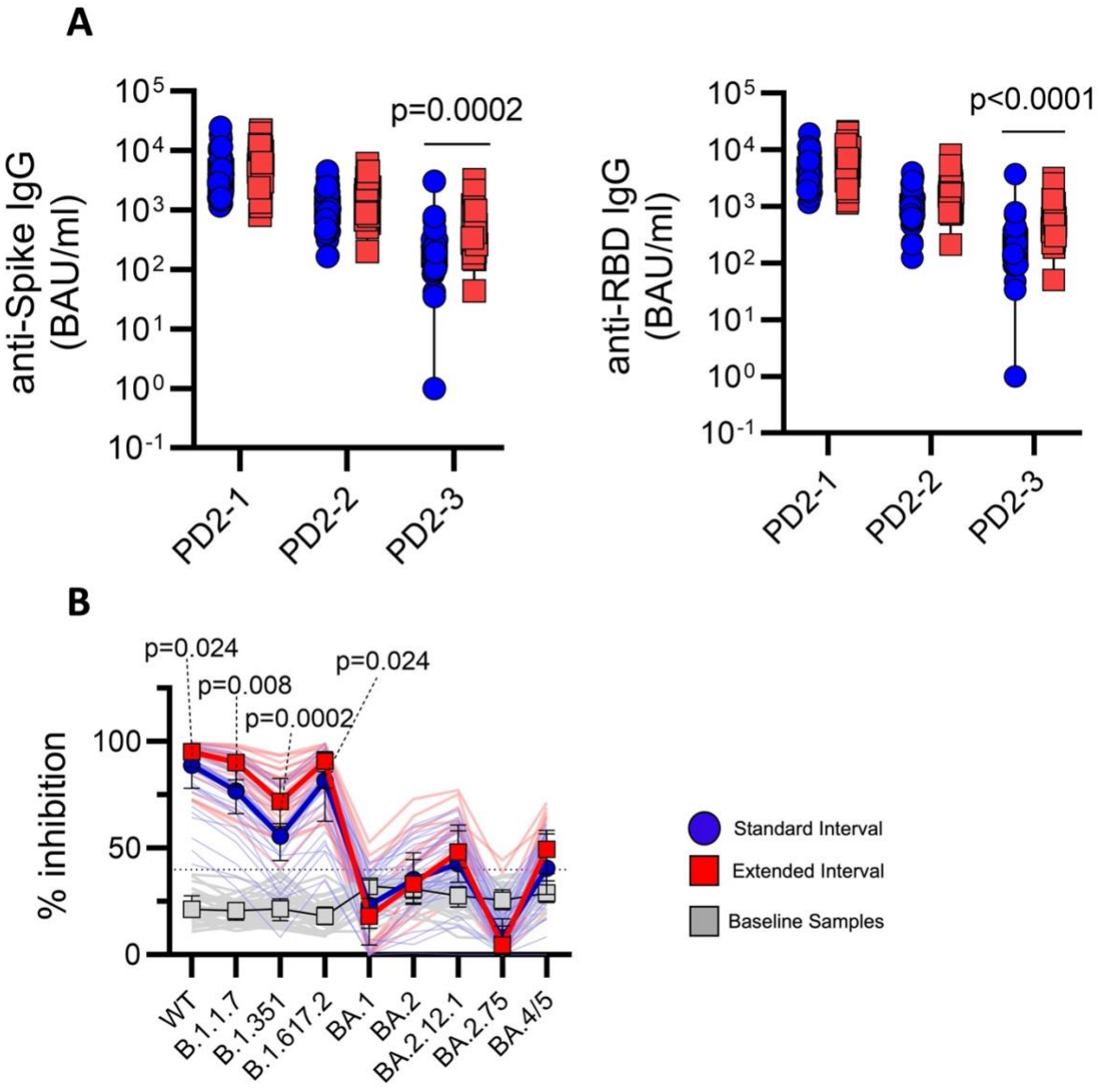


Figure 1.4-2: Proportions of Spike-specific IgG and RBD-specific IgG in the Plasma of Participants with Standard or Extended Dose Interval (A). Neutralization Ability of Antibodies Against SARS-CoV-2 Variants (B). The antibody proportions were measured in binding antibody units (BAU)/mL. Neutralization was measured against wildtype (WT), B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta) BA.1, BA.2, BA.2.12.1, BA.2.75 and BA.4/5 (Omicron subvariants) at 10-14 days post-dose 2.

1.4.3. Current Gaps in Knowledge

1.4.3.1. Impact of Dose Interval on B Cells

While the role of T cells in an extended dose interval has been studied in detail, there is less published information on the impact these schedules have on the B cell population and their subsets. Researchers have already hypothesized that the differences in antibody titres could be due to a difference in memory B cell maturation or proportion[310]. Some research has begun to analyze the proportions of B cells alongside CD4+ and CD8+ T cells. Extended dose intervals have been shown to increase B cell responses and proportions of antigen-specific B cells when compared to standard dose intervals[313,314]. However, a limitation of both of these studies is that the various subsets of B cells (plasma, memory, naïve), as well as their B cell receptor isotype (IgM+/IgD+, IgG+), are not considered and analyzed. This limitation means that while there is research into the subsets of T cells and their response to an extended dose interval, the proportions of B cell subsets are still poorly understood. Although there has been research into the proportions of antigen-specific B cells after vaccination, the relative proportions and scale of the different subsets of mature B cells during different dosing schedules of the SARS-CoV-2 vaccine is not as well known. In addition, most studies are performed on samples collected shortly after immunization, which means that there is less information available about whether any differences detected after immunization with a standard or an extended dose interval are sustained in the long-term following the second dose.

1.4.3.2. Impact of Dose Interval on Antibody Production and Neutralization

While data from our lab and others already shows that an extended dose interval is associated with higher titres of antibody and greater neutralization of SARS-CoV-2 variants after the second dose, this only considers antibodies found in the plasma. The bulk of circulating antibody is derived from plasma cells that emerge soon after the last antigen exposure or long-lived plasma cells, so it does not reflect the antibody that circulating memory B cells are capable of potentially producing at later time points, after maturation in GCs. There is limited evidence on how an extended dose interval impacts antibodies directly produced from plasma cells that differentiate from memory B cells at later time points after vaccination. Thus, plasma antibodies paint an incomplete picture as to how the extended dose interval impacts SMH and the affinity maturation process. The antibodies produced from the activated plasma cells would be produced

from the specific time points the cells were taken from and would reflect the differentiation state of those B cells at the last point of exposure to antigen, providing a glimpse into how the SMH and affinity maturation process is developing antibodies over time, and whether B cell receptor selection for a greater breadth of inhibition for SARS-CoV-2 wildtype and the variants is occurring during this process.

1.4.3.3. Longitudinal Impact of Dose Interval on B Cells, Antibody Production and Neutralization

A final limitation in many of the previous studies is the lack of information on the impact that booster doses have on the immune response following an extended primary dosing interval. Additionally, given the introduction of additional booster doses beyond the primary series, it remains to be seen whether any immunological differences observed after the first two doses persist long term. Many of the studies focus on a single timepoint after the second dose and fail to follow the study participants after subsequent exposures. This means that there is limited evidence of any immunological outcomes of an extended dose interval maintained after a third dose of the SARS-CoV-2 vaccine and if there are any differences in B cell phenotypes in participants with a standard or extended dose interval after a third dose.

1.5. Hypotheses and Objectives

1.5.1. Rationale

The SARS-CoV-2 pandemic posed unprecedented challenges to public health systems, which required urgent decisions to be made about vaccine deployment. At the forefront of these decisions, determining the optimal interval between vaccine doses was paramount. A retrospective analysis of the changes made to the vaccine schedule at an immunological level during the pandemic can provide further evidence as to whether or not a delayed dose interval was the better option for the population. Current studies have demonstrated that while a delayed dose interval of the SARS-CoV-2 vaccine does not appear to have an impact on CD4+ T cell populations, there is an observed impact on SARS-CoV-2-specific antibodies. More specifically, a delayed dose interval resulted in a slower decrease in SARS-CoV-2-specific antibody titre, increased neutralization of the RBD portion of SARS-CoV-2 and increased breadth against SARS-CoV-2 variants. As B cells are responsible for antibody production during infection or vaccination, studies that analyze B cell populations and their neutralization ability after

vaccination are paramount in determining the mechanisms by which an extended dose interval provides an enhanced immune response.

Unfortunately, SARS-CoV-2 may not be the last global pandemic in our lifetime. In addition to providing evidence for past decisions, this research aids in determining vaccine schedules for future outbreaks. On November 13, 2024, the first case of avian influenza was confirmed in Canada[315], and as of December 24, 2024, 65 confirmed cases were detected in the United States[316]. Although no clear human-to-human transmission has occurred, the world may already be on the cusp of a new pandemic, which will require a robust plan of action to combat infection and transmission. Fortunately, the lessons learned from the previous pandemics will inform the decisions for the next pandemic. Vaccine schedules can be based on the knowledge already acquired during the SARS-CoV-2 pandemic, so nations or regions potentially weighing the option of delaying a dosing interval due to vaccine shortages can rely on previous research, rather than having limited data on the effect of mRNA vaccines on the immune system. Therefore, not only is this work beneficial for determining the effectiveness of the delayed dose interval strategy in 2021, but this work will hopefully provide reassurance in the future when similar decisions must be made rapidly.

1.5.2. HYPOTHESES

Prolonging the interval between vaccine doses is anticipated to enhance memory B cell maturation, as evidenced by a higher frequency of antigen-specific memory B cells, as well as more mature B cell phenotypes and an expanded breadth of neutralizing antibody responses following differentiation into activated plasma cells. In addition, the immunological benefits observed with an extended dose interval between the 1st and 2nd vaccine doses will diminish following a 3rd dose of the SARS-CoV-2 mRNA vaccine.

1.5.3. OBJECTIVES

1. Evaluate the proportions and phenotypes of antigen-specific (RBD-specific and S-specific) memory B cells and phenotypic subsets in PBMC samples collected at various time points following the second dose from participants with either a delayed or standard dose interval.

2. Evaluate the neutralization of SARS-CoV-2 variants by the secreted antibodies from antibody-secreting plasma cells differentiated from memory B cells in participants with either a delayed or standard dose interval.
3. Evaluate the effects of a third booster dose of the SARS-CoV-2 mRNA vaccine on antigen-specific memory B cell phenotypes and the antibodies they produce upon antigen exposure and differentiation.

METHODS

2.1. COVID-19 VACCINE PROJECT

2.1.1. ETHICS AND PARTICIPANT SELECTION

Ethical approvals for this thesis project were received by the University of Manitoba Health Research Ethics Board and the Health Canada-Public Health Agency of Canada Research Ethics Board. The study protocol, recruitment, participant information and consent forms, questionnaires and use of data complied with the principles set out in the 2nd edition of the Tri-Council Policy Statement for the ethical conduct of research involving humans.

Participants involved in the study included individuals aged 18-65 in generally good health who received approved mRNA SARS-CoV-2 vaccines. This included laboratory and health care workers from the National Microbiology Laboratory and Cadham Provincial Laboratory. Exclusion criteria included prior SARS-CoV-2 infection. Previous infection was detected through questionnaires or by the presence of SARS-CoV-2 specific N protein antibodies using a BioPlex 2200 SARS-CoV-2 IgG assay (BioRad, Hercules, CA, USA). The presence of SARS-CoV-2 specific antibodies before vaccination or anti-N protein antibodies at any time point were interpreted as prior infection, and the participant was excluded from this analysis.

2.1.2. SAMPLE COLLECTION, BLOOD PROCESSING AND THAWING METHODS

2.1.2.1. SAMPLE COLLECTION

Participants consented to sampling pre- and post-vaccination. Participants at least 18 years of age or older were asked by a study coordinator or research nurse if they were willing to participate in a study on vaccine-induced immunity. Participants filled out a basic questionnaire at the time of enrollment, which collected basic demographic information required for further analysis of potential confounders for vaccine responsiveness, including date of enrollment, age, sex, height, weight, ethnicity, pre-existing conditions, medications, and whether the participant received an annual flu shot. After subsequent visits, participants were asked whether they had experienced any side effects following vaccination, if they had experienced COVID-19-like symptoms, or if they received a positive SARS-CoV-2 test since their last study visit. At each sampling time point, study personnel collected four tubes of EDTA blood taken from a vein in the arm by standard venipuncture phlebotomy. Vaccination and sampling time points are presented in Figure 2.1. Samples were transported to the University of Manitoba or the JC Wilt

Infectious Diseases Research Centre, at which point the samples were processed and stored for further analysis.

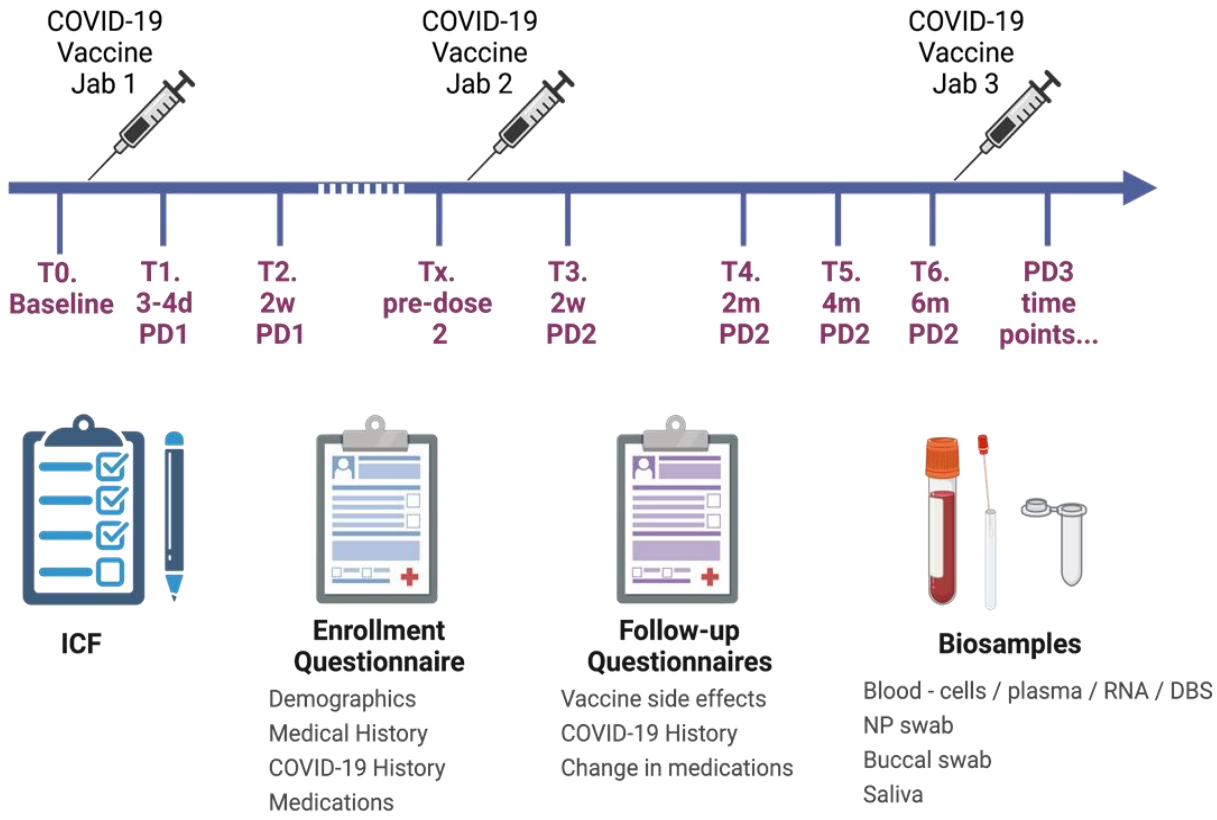


Figure 2.1-1: COVID-19 Vaccine Project Outline and Sampling Timepoints. Samples were collected before initial vaccination and after each subsequent vaccine dose. Participants filled out informed consent forms, questionnaires for data about the demographics of the study and provided blood and saliva samples. Figure prepared using Biorender by Dr. Catherine Card.

2.1.2.2. Blood Processing

Blood tubes were centrifuged for 7 minutes at 450x*g* to separate plasma, which was aspirated and stored at -80°C for downstream serological analyses. Plasma-depleted blood was diluted with sterile phosphate-buffer saline (PBS) supplemented with 2% Premium Plus Fetal Bovine Serum (FBS; ThermoFisher Scientific, Waltham, MA, USA) at a ratio of 1:1 of original blood volume. Peripheral blood mononuclear cells (PBMCs) were isolated from plasma-depleted blood using Lymphoprep in SepMate-50 tubes (STEMCELL Technologies, Vancouver, BC, Canada), which was centrifuged at 1200x*g* for 15 minutes at room temperature. The top layer of

PBMCs were collected in a new 50ml conical tube, enriched with PBS + 2% FBS to 50ml and centrifuged at 300x*g* for 8 minutes. The supernatant was discarded and the pellet was resuspended in 20ml PBS + 2% FBS. PBMCs were counted using a Countess 3 FL automated cell counter (ThermoFisher Scientific, Waltham, MA, USA) and then resuspended in Rosewell Park Memorial Institute 1640 Medium (RPMI, Life Technologies, Carlsbad, CA, USA) containing 40% heat-inactivated FBS and 15% Dimethylsulfoxide (DMSO: MilliporeSigma, Oakville, ON, Canada). The PBMC aliquots were frozen in CoolCell containers overnight at -80°C and then transferred to liquid nitrogen at -180°C for future analyses.

2.1.2.3. Thawing Methods

Cryovials containing frozen PBMCs were retrieved from liquid nitrogen storage and immediately placed in a cooler containing Lab Armour Beads chilled to -80°C. The cryovials were transferred to a 37°C water bath for 30 seconds to 2 minutes, which ensured gradual thawing until a pea-sized pellet of ice remained. During this time, conical tubes were labelled with sample information and filled with 10 ml RPMI (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin (ThermoFisher Scientific, Waltham, MA, USA) herein referred to as R10. The thawed cell suspensions were transferred from cryovials to the conical tubes with warmed R10 media. The suspension was centrifuged at 350x*g* for 8 minutes, at which point the supernatant was discarded and the cell pellet was resuspended in fresh R10 media. Cell counts and viability were assessed using Trypan Blue Solution, 0.4% (ThermoFisher Scientific, Waltham, MA, USA) and a Countess 3 FL automated cell counter. The cell suspension was then centrifuged at 350x*g* for 8 minutes, the supernatant was discarded, and the cell pellet was resuspended in fresh R10 media at $4x10^6$ ml. The R10 cell suspensions were rested in sterile snap-capped FACS cell culture tubes in an incubator at 37°C until either B cell phenotyping or stimulation could begin. Cells set aside for stimulation rested in the incubator for 4hrs, while cells set aside for staining and phenotyping rested overnight.

2.1.3. Serological Data

The Serological analysis of plasma anti-S IgG and anti-RBD IgG was performed using the BioPlex 2200 SARS-CoV-2 IgG assay (BioRad, Hercules, CA, USA). Plasma samples were thawed and vortexed after 30 minutes, then samples were centrifuged at 2000x*g* for 1 minute. The samples were then loaded into the instrument for analysis. Antibodies were quantified using

the WHO International Immunoglobulin Standard for anti-SARS-CoV-2. The presence of any SARS-CoV-2-specific antibodies in baseline samples or anti-nucleocapsid antibodies at any time point were interpreted as evidence of prior infection, and those samples were excluded from the final analysis.

2.2. B Cell Phenotyping

2.2.1. Staining Methods

The cell suspensions, comprised of 8-10x10^6 cells, were rested in the incubator overnight. The next day, the cell suspensions were centrifuged at 400x*g* for 5 minutes. The supernatant was discarded, and the pellet was vortexed. Live/Dead Fixable Near-IR Cell Stain (LD NIR, ThermoFisher Scientific, Waltham, MA, USA) was first diluted 1/100 in PBS, then a master mix was made with the diluted LD NIR, PBS and Human BD FC Block (BD Biosciences, San Jose, CA, USA). The LD master mix is comprised of 7.25 µL/test of diluted LD NIR and 1 µL/test of Human BD FC Block and was added to the cell culture tubes at 100 µL per tube. The tubes were incubated at room temperature for 15 minutes, shielded from light. One mL of Stain Buffer (BD Biosciences, San Jose, CA, USA) was added to each tube, and the cell culture tubes were centrifuged at 400x*g* for 5 minutes. The supernatant was discarded, and the pellet was resuspended by vortexing.

After viability staining, the cells were stained with the probe master mix. The probe master mix comprises the multimerized probes, and Biotin Mouse Anti-Human CD45 (BD Biosciences, San Jose, CA, USA) diluted 1/100 in PEB buffer. It should be noted that an error had been made in the protocol, and that D-Biotin (Thermofisher Scientific, Waltham, MA, USA) should have been used instead of Biotin Mouse Anti-Human CD45. D-Biotin acts as a decoy that reduces non-specific binding during staining. Biotin Mouse Anti-Human CD45 acts as a decoy but also stains CD45+ cells with biotin, which includes all B cells. Therefore, when the decoy streptavidin (SA) probe was added, it would bind free biotin and all B cells that have biotin bound to CD45, which could have resulted in unnecessary B cell loss during the gating step that removes the decoy probe. However, because the Biotin Mouse Anti-Human CD45 was extremely dilute, results indicated that there was no significant difference in B cell proportions between the two protocols, indicating that unnecessary and significant cell loss had not occurred (Supp. Figure 5.2-1). The contents of the probe master mix are listed in Table 2.2-1. Probe

multimerization requires 0.1mg/mL of Recombinant SARS-CoV-2 Spike-Prot (Human Endothelial Kidney)-Biotin (Miltenyi Biotec, Bergisch Gladbach, Germany) and Recombinant SARS-CoV-2 RBD-Prot (Human Endothelial Kidney)-Biotin (Miltenyi Biotec, Bergisch Gladbach, Germany) suspended in sterile ddH_2O. SA-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), PEVio770 (Miltenyi Biotec, Bergisch Gladbach, Germany), BB515 (BD Biosciences, San Jose, CA, USA), PECF594 (BD Biosciences, San Jose, CA, USA) and BV786 (BD Biosciences, San Jose, CA, USA) tags were diluted in PEB buffer (PBS pH 7.2, 0.5% BSA, 2mM EDTA) to 50µg/ml. SARS-CoV-2 wild-type S protein was conjugated at 0.06 mg/mL with SA-conjugated fluorophores SA-PE and SA-PEVio770 at 0.06 µg/mL in separate tubes. RBD protein was conjugated at 0.02 mg/mL with SA-BB515 and SA-BV786 at 0.2 µg/mL in separate tubes. The probes were conjugated for 20 minutes at room temperature in PEB buffer, protected from light, and kept at 4°C until needed. The cell culture tubes were kept at room temperature in the dark for 20 minutes after 100 µL of the probe master mix was added. After 20 minutes, 1 mL of Stain Buffer was added to each tube, followed by centrifugation at 400x*g* for 5 minutes. The supernatant was discarded, and the pellet was vortexed.

Table 2.2-1: Outline of Probe Master Mix for Single Test and for an Example 6 Sample Test

Probe	Volume/test (µL)	6 Sample Master Mix (µL)+10% Volume
S-PE	5	33
S-PEVio770	10	66
RBD-BB515	5	33
RBD-BV786	5	33
decoy SA-PECF594	1.2	7.92
CD45 Biotin (1/100 Dilution) (Should be D-biotin)	1	6.6
PEB buffer	72.8	480.48

After probe staining, the cells were stained with the antibody master mix. Table 2.2-2 outlines the streptavidin-linked probes and fluorescent-conjugated antibodies used in the master mixes for B cell phenotyping, the fluorophore or streptavidin molecule bound to them, the volume required per test and the marker that is recognized. These probes and antibodies recognize the various cell markers and receptors on PBMCs and can isolate and identify the

phenotypes of these cells. The multiple probes are utilized in the staining methods mentioned in a later section in master mixes, which are applied to each of the PBMC samples. These probes are designed to give a comprehensive profile of B cell subsets at various time points after vaccination. The master mix comprises fluorescent antibodies and BD Horizon Brilliant Stain Buffer (BD Biosciences, San Jose, CA, USA). Each tube received 100 µL of the antibody master mix, which was incubated at 4°C in the dark for 30 minutes. Following incubation, 1 ml of Stain Buffer was added, the culture tubes were centrifuged at 400x*g* for 5 minutes, the supernatant was discarded, and the tubes were vortexed for cell resuspension. The cells in each tube were fixed with 100 µL of 0.5% paraformaldehyde (PFA, ThermoFisher Scientific, Waltham, MA, US). The cell culture tubes were incubated at 4°C in the dark for 30 minutes. After incubation, 1 mL of Stain Buffer was added to each tube. The tubes were centrifuged at 400x*g* for 5 minutes, at which point the cells were suspended in 1 mL of stain buffer for flow cytometry acquisition.

Table 2.2-2: Outline of Fluorochromes, Markers and Volumes for the Antibody Master Mix

Lasers	Filters	Fluorochrome	Marker	Clone	Isotype	Source	Vol/test
355	**379/28**	BUV395	CD19	SJ25C1	Mouse	BD Biosciences	0.6 µL
	586/15	BUV563	CD20	2H7	Mouse	BD Biosciences	2.5 µL
	670/30	BUV661	IgM	UCH-B1	Mouse	BD Biosciences	0.3 µL
	740/35	BUV737	IgG	G18-145	Mouse	BD Biosciences	1.25 µL
	820/60	BUV805	CD11c	B-ly6	Mouse	BD Biosciences	2.5 µL
405	**431/28**	BV421	CD27	M-T271	Mouse	BD Biosciences	1.25 µL
	470/15	BV480	CD71	M-A712	Mouse	BD Biosciences	2.5 µL
	610/20	BV605	CD21	B-ly4	Mouse	BD Biosciences	0.6 µL
	780/60	BV786	RBD			BD Biosciences	5 µL
488	**515/20**	BB515	RBD			BD Biosciences	5 µL
	710/50	BB700	IgD	IA6-2	Mouse	BD Biosciences	1.25 µL
561	**586/15**	PE	S			Miltenyi Biotec	5 µL
	610/20	PE-CF594	Decoy			BD Biosciences	1.2 µL
	670/30	PECy5	CD3	UCT1	Mouse	BD Biosciences	1 µL
			CD14	M5E2	Mouse	BioLegend	1 µL
	780/60	PEVio770	S			Miltenyi Biotec	5 µL
	710/50	R718	CD38	HB7	Mouse	BD Biosciences	1.25 µL
	780/60	APCFire750	LD NIR			ThermoFisher Scientific	7.25 µL

A BD FACSymphony A5 Cell Analyzer (BD Biosciences, San Jose, CA, USA) was used to detect the relative fluorescence of the stained cells. Cytometer Setup and Tracking (CST, BD

Biosciences, San Jose, CA, USA) beads were used to verify the performance of the lasers and detectors every run. The positive-control beads used during compensation were BD CompBeads Anti-Mouse Ig (BD Biosciences, San Jose, CA, USA) and BD CompBead Plus Anti-Mouse Ig (BD Biosciences, San Jose, CA, USA). The negative-control beads used during compensation were BD CompBeads Negative Control (BD Biosciences, San Jose, CA, USA) and BD CompBead Plus Negative Control (BD Biosciences, San Jose, CA, USA). Lastly, Sphero Rainbow 8-Peak Calibration Beads (BD Biosciences, San Jose, CA, USA). were run on the flow cytometer to validate instrument performance. Each independent experiment included $5x10^5$ cells as a no-probe control. Samples were vortexed before loading, and 5000000-10000000 events per sample were acquired to provide robust statistical analysis. To ensure that enough cells were collected, given the rarity of antigen-specific B cells, the entire sample was acquired by the flow cytometer.

2.2.2. Gating Strategy

Flow cytometry results were analyzed using FlowJo software (BD Biosciences, San Jose, CA, USA). The gating strategy used for phenotyping S-specific and RBD-specific B cells is outlined in Figure 2.2-1. To summarize, a forward scatter area vs forward scatter height was used to identify single cells. Additionally, cells that were bound to the LD NIR probe will be gated out, as will cells that were bound to the anti-CD3 and anti-CD14 antibodies to remove T cells and monocytes. Once those cells had been identified and excluded, B cells were identified by CD19 expression. The D-Biotin/SA-PECF594 probe was present to act as a decoy for cells that non-specifically bound biotin, although the use of CD45-Biotin precluded this. Fortunately, the concentration of CD45-Biotin was dilute enough that it did not interfere with results (Supp. Figure. 5.2-1). S- and RBD-specific B cells were identified by binding to the PEVio770-S and PE-S probes or BB515-RBD and BV786-RBD probes, respectively. The CD19+ B cell population was also further analyzed and separated into mature B cells (CD27+) and immature B cells (CD27-). Within the mature B cell population, the combined expression of CD20 and CD38 was used to distinguish memory B cells (CD20+, CD38-) from antibody-secreting plasma cells (CD20-, CD38+). The memory B cells were further analyzed for the relative presence of IgM, IgD or IgG to determine the type of B cell receptor present, as well as whether CD71 expression was present on the cell, which indicates whether a cell is rapidly proliferating[317,318]. The

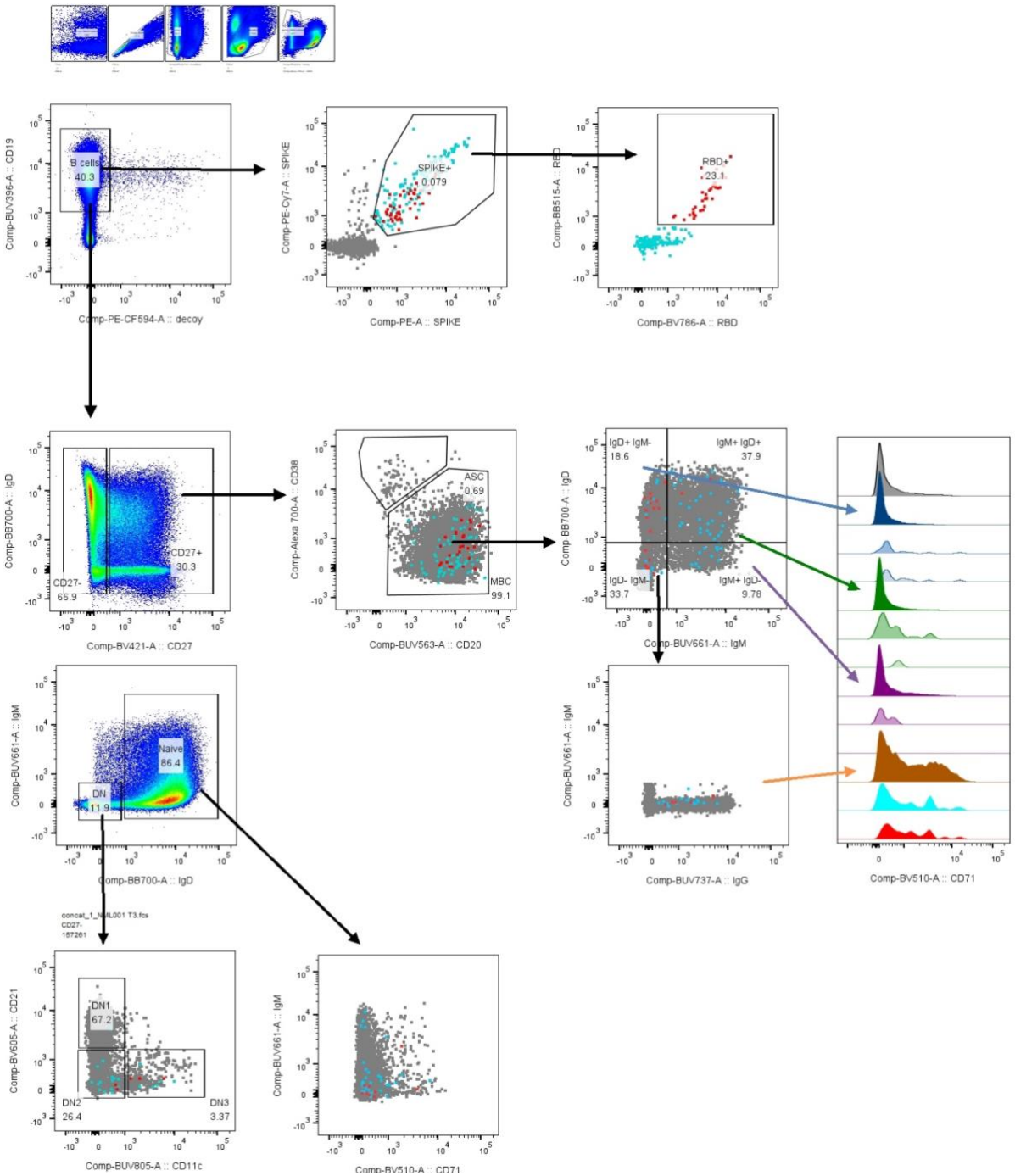


Figure 2.2-1 Gating Strategy of a B Cell Assay. The gating protocol starts by removing macrophages and T cells, and keeps single cells, leukocytes and living cells. The protocol isolates B cells from the rest of the PBMCs and can then detect Spike-specific and RBD-specific probes, as well as fluorescent antibodies that isolate memory B cells, plasma cells, naïve B cells, double negative B cells and resting B cells. Outline from FlowJo.

immature B cells were separated into a naïve B cell population, with IgM and/or IgD present, or double-negative B cells, with no IgM and IgD present on the cell surface. Anti-CD11c and anti-

CD21 distinguished between the various double-negative B cells[319]. The B cell phenotypes were analyzed as a percentage of S-specific or RBD-specific B cells.

2.3. B Cell Differentiation into Plasma Cells and Antibody Neutralization

2.3.1. IL-2 and R848 Stimulation Protocol

Roughly 1x10^6 PBMCs per sample were incubated at 37°C for 3-4 hours in a 24 well plate with 250 µL of R10 media. A stimulation cocktail comprised of 2.5 µg/mL of Human IL-2 Recombinant Protein (ThermoFisher Scientific, Waltham, MA, USA) and 30 U/mL of R848 (STEMCELL Technologies, Vancouver, BC, Canada) was made before stimulation. 250 µL of the cocktail was added to each of the wells containing cell suspension, for a total of 500 µL per well. Unstimulated controls from each participant were included with each assay. Empty wells were filled with PBS to humidify the plate. The 24-well plate was incubated at 37°C for 6 days. After 6 days, the plates were centrifuged at 400x*g* for 5 minutes, 250 µL of supernatant was removed from each of the wells and 250 µL of new stimulation cocktail was added to each of the wells. The non-stimulated control had 250 µL of old R10 media removed and 250 µL of new R10 media was added. The plates were placed in a 37°C incubator for an additional 4 days. After 10 days had passed in total, the plates were removed from the incubator and centrifuged at 400x*g* for 5 minutes. The supernatant from each of the wells was harvested in 100 µL aliquots and frozen at -80°C for future use.

2.3.2. Mesoscale Discovery Neutralization Assay

Frozen supernatant samples were thawed on ice. The V-PLEX SARS-CoV-2 Key Variant RBD Panel 1 (ACE2) Kit) (Meso Scale Discovery, Rockville, MD, USA) was used to quantify neutralizing antibodies that block the binding of ACE2 to SARS-CoV-2 RBD antigens from WT (Wuhan), B.1.1.7 (Alpha), BA.1.351 (Beta), B.1.617.2 (Delta) and Omicron subvariants BA.1, BA.2, BA.2.12.1, BA.2.75 and BA.4/BA.5. The assay was run according to manufacturer's instructions with additional changes made by members of the laboratory to accommodate supernatant antibodies instead of plasma antibodies, and plates were read on a 1300 Meso QuickPlex SQ 120MM (Meso Scale Discovery, Rockville, MD, USA). 96-well plates are provided pre-coated with SARS-CoV-2 antigens on 10 spots in each well, with each spot

corresponding to a different SARS-CoV-2 RBD variant, while the final spot is comprised of SARS-CoV-2 N protein. MSD Blocker A (Meso Scale Discovery, Rockville, MD, USA) was dispensed into 96-well plates to prevent non-specific binding. Plates were shaken at room temperature (RT) for 30 minutes at 700 RPM before being washed and rinsed with 150 µL of MSD 1X Wash Buffer (Meso Scale Discovery, Rockville, MD, USA) 3 times using a BioTek 405 Plate Washer (BioTek, Winooski, VT, USA). The negative control was Diluent 100 (Meso Scale Discovery, Rockville, MD, USA) with no supernatant. The positive control was a plasma sample identified in-house as having high neutralizing capability against all variants included in the panel. The plasma sample was diluted 1:50 with Diluent 100. All standards, samples, and controls were added at 25 µL onto the plate with 2 technical replicates per timepoint and incubated at RT on a plate shaker for 1 hour at 700 RPM. After incubation, 25 µL of a 1:200 dilution of the human ACE2 protein conjugated with MSD SULFO-TAG (Meso Scale Discovery, Rockville, MD, USA) in Diluent 100 was mixed with the supernatant samples and incubated at RT, shaking for 1 hour at 700 RPM. After 1-hour incubation, plates were washed and soaked 3 times with 150 µL of the Wash Buffer and 150 µL of MSD GOLD Read Buffer B (Meso Scale Discovery, Rockville, MD, USA) was added after soaking. Plates were immediately read on 1300 Meso QuickPlex SQ 120MM (Meso Scale Discovery, Rockville, MD, USA). Light emitted from the MSD SULFO-TAG was measured as an ECL signal. A strong signal from any of the spots in a well indicated that ACE2 protein with a SULFO-TAG was able to bind to the RBD antigen at that spot, and that very little of the antibody present in that well managed to neutralize the variant. A weak signal from any of the spots in a well indicated that ACE2 protein with a SULFO-TAG was prevented from binding to the RBD antigen at that spot, indicating that the antibody present in that well was a strong neutralizer of the variant.

2.3.3. MESOSCALE DISCOVERY PROGRAM

Results were analyzed using the Discover Bench 4.0 software (Meso Scale Discovery, Rockville, MD, USA). Results were reported as percent (%) inhibition of ACE2 binding by the sample. The negative control served as a 0% inhibition reference.

$$\%\ neutralization = 1 - \frac{Average\ Sample\ ECL\ Signal}{Average\ ECL\ Signal\ of\ Negative\ Control} \times 100$$

A threshold of 40% inhibition was used for a positive response, based on the mean neutralization output from the baseline supernatant controls plus 2 standard deviations. Samples below 40% inhibition were assigned 0% inhibition for statistical analyses.

2.4. Statistical Analyses

2.4.1. GraphPad Analysis

The statistical analysis of the B cell proportion and antibody neutralization data was conducted using GraphPad Prism Software, version 10.1.2. The analysis of B cell phenotypes over time regardless of dose interval was performed using Kruskal-Wallis tests with multiple comparisons. Paired comparisons of two time points were done using the Wilcoxon matched-pairs signed rank test. Comparisons between the extended dose interval and standard dose interval groups after the second and third doses of the vaccine were performed using Mann-Whitney U tests, with corrections for multiple comparisons using the Holm-Šídák method. Correlation analyses were performed using the Spearman test. Comparisons between male and female outcomes of extended dose intervals were done using two-way ANOVA tests with multiple comparisons.

RESULTS

3.1. Total Participants Involved in Project

3.1.1. Age Range, Sex, and Previous SARS-CoV-2 Infection Status

Participants were selected from the National Microbiology Laboratory and Cadham Laboratory cohorts. In total, 50 participants had received a short-dose interval of the vaccine, and 36 participants had received a long-dose interval of the vaccine. From that total, 14 individuals from both standard and delayed dose interval groups were selected for B cell phenotyping. Additionally, PBMCs from 11 participants who received a short-dose interval and 11 participants who received a delayed-dose interval from the initial 14 individuals were stimulated to measure antibody neutralization had PBMCs stimulated to measure antibody neutralization. The age range of the standard dose interval group (n=14) was from 22-52 years, with an average age of 39 years. There were eight females and six males in this group. The dose intervals ranged from 20-27 days between doses 1 and 2, with an average of 21 days between doses. The age range of the extended dose interval (n=14) group was from 23-57 years, with an average age of 41 years. There were eleven females and three males in this group. The dose intervals ranged from 41-83 days between doses 1 and 2, with an average of 61 days between doses. Serology tests and forms indicated that no SARS-CoV-2 infections occurred in any of the participants in the standard or extended dose interval groups at any time point presented in this study. A summary of the participant demographics can be found on Table 3.1-1.

	Dose Interval Group		
Characteristic	**standard n=14**	**extended n=14**	**p-value**
Age at enrollment	41 (22-52)	43.5 (23-57)	0.54
Female sex	8 (57.14%)	11 (78.57%)	0.42
BMI (kg/m^2)	25.18 (16.54-35.14)	27.64 (21.74-57.6)	0.14
Asthma/Allergy	3 (21.43%)	2 (14.29%)	1.0
Length of Dose Interval (days)	21 (20-27)	66.5 (41-83)	<0.0001
[+]Continuous variables are presented as median (range). Categorical variables are presented as n (%).			

Table 3.1-1: Outline of Demographics of Participants[*].

3.2. Impact of Dose Interval on Magnitude and Phenotype of SARS-CoV-2-specific Memory B cell responses

3.2.1. Assessment of SARS-CoV-2-Specific B cells at Longitudinal Time Points After dose 2

B cells that had BCRs that could recognize the S protein and RBD region of SARS-CoV-2 were analyzed between the two dose interval groups after the second dose of the vaccine. The analysis of participants, regardless of dose interval, found a significant increase in S-specific B cells from 10-14 days post-dose 1 to 10-14 days post-dose 2 and 4-6 months post-dose 2 (Figure 3.2-1A). This trend was also seen when measuring RBD-specific B cells in all participants, both as a percentage of all B cells and of S-specific B cells. Participants who had received the extended dose interval had similar proportions of S-specific B cells compared to participants who had received the standard dose interval at 10-14 days post-dose 1 (Figure 3.2-1B). However, after 10-14 days post-dose 2, participants who had received the extended dose interval were found to have a significantly greater proportion of S-specific B cells than participants who had received the standard dose interval ($p=0.002$), with a median proportion of 0.090% and 0.056% of all B cells, respectively. After 4-6 months post-dose 2, the difference in proportions of S-specific B cells between the extended dose interval and the standard dose interval are no longer statistically significant ($p=0.794$), with median proportions of 0.092% and 0.082% of all B cells, respectively. A similar outcome is seen with the proportions of RBD-specific B cells (Figure 3.2-1B). Participants who received the extended dose interval had a significantly greater proportion of RBD-specific B cells than those who received the standard dose interval at 10-14 days post-dose 2 ($p=0.003$), with a median proportion of 0.032% and 0.017% of all B cells, respectively. After 4-6 months post-dose 2, the proportions of RBD-specific B cells are not statistically significant between the extended and standard dose interval groups ($p=0.434$), with median proportions of 0.02% and 0.027% of all B cells, respectively. Additionally, when analyzing the proportion of RBD-specific B cells as a subset of S-specific B cells, the proportion is similar between the two participant groups at 10-14 days post-dose two as well as between 10-14 days post-dose 1 and 10-14 days post-dose 2 (Figure 3.2-1B). This would indicate that the proportion of S-specific B cells that target the RBD portion of the S protein is unaffected by the dose

interval. However, the overall proportion of B cells that are RBD-specific and/or S-specific are impacted by an extended dose interval. Analyzing results by sex showed no significant differences in the proportions of S-specific B cells as a percentage of total B cells, RBD-specific B cells as a percentage of total B cells and as a percentage of S-specific B cells between male and female participants who had a standard or extended dose interval following the 2nd dose of the vaccine (Supp. Figure 5.2-2).

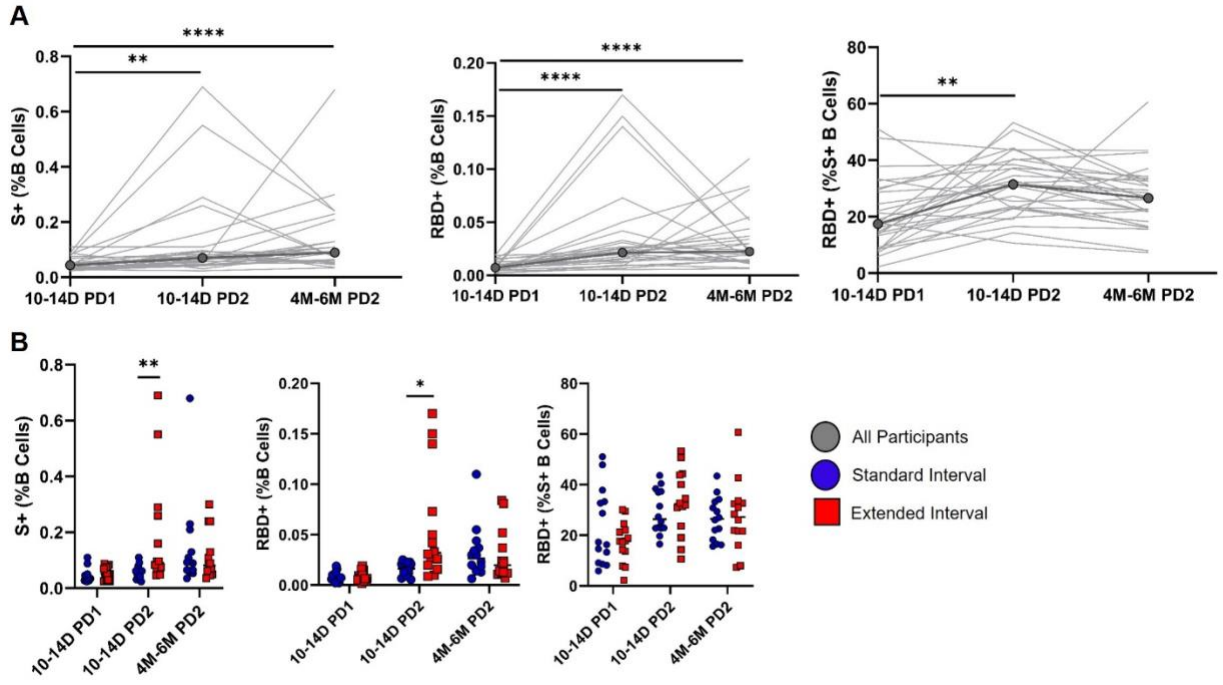


Figure 3.2-1: Assessment of S-Specific and RBD-Specific B Cells as a Percentage of B cells and S-specific B cells. (A) Trajectory of S-specific and RBD-specific B cells as a percentage of B cells from 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Time points were compared using the Kruskal-Wallis test with Dunn's post-test. Bold line show median values. **(B)** Comparison of S-specific B cells and RBD-specific B cells as a percentage of B cells, and RBD-specific B cells as a percentage of S-specific B cells, between the standard and extended dose interval groups. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

3.2.2. Assessment of memory B cell phenotypes among SARS-CoV-2-specific memory B cells After Dose 2

3.2.2.1. Assessment of SARS-CoV-2-Specific Memory B Cells at Longitudinal Time Points After Dose 2

Further assessment of B cell subsets can provide a greater understanding as to how the booster interval of a vaccine can impact memory B cell maturation. To determine how B cell maturation is impacted, the proportion of S-specific and RBD-specific B cells that are memory B

cells was also assessed. At 10-14 days post-dose 2, there are significant increases in the proportion of S-specific and RBD-specific memory B cells in both extended and standard dose interval groups (Figure 3.2-2A). However, when the results are compared between the dose interval groups, there is no significant difference (Figure 3.2-2B). The proportions of S-specific B cells that are memory B cells are not statistically different either 10-14 days (p=0.98) or 4-6 months (p=0.23) after the second dose. Similar results are seen when analyzing RBD-specific B cells that are memory B cells. After 10-14 days (p=0.18) and 4-6 months (0.76) post-dose 2, there are no significant differences between extended and standard dose interval groups. This would indicate that the interval between doses has minimal impact on the overall proportion of

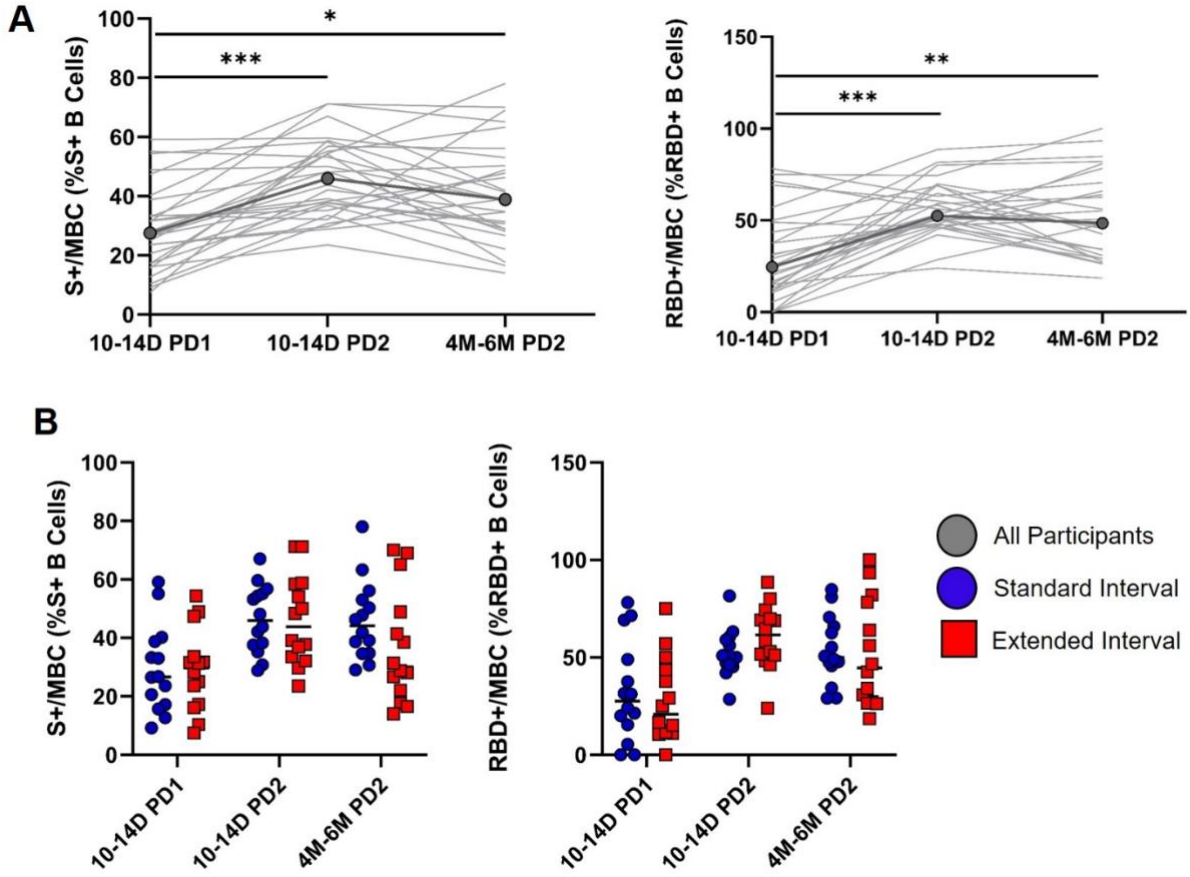


Figure 3.2-1: Assessment of Memory B Cells as a Percentage of S-specific and RBD-specific B cells. (A) Trajectory of memory B cells as a percentage of S-specific and RBD-specific B cells from 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Time points were compared using the Kruskal-Wallis test with Dunn's post-test. Bold lines show median values. **(B)** Comparison of memory B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

S-specific or RBD-specific B cells that have been activated and matured into memory B cells. Further analysis showed no significant differences in the proportions of S-specific and RBD-specific B cells that were memory B cells after the 2nd dose between male and female participants who had received a standard or extended dose interval of the vaccine (Supp. Figure 5.2-3).

3.2.2.2. Assessment of SARS-CoV-2-Specific IgG+ and IgD+/IgM+ Memory B Cells at Longitudinal Time Points After Dose 2

While the proportion of S-specific and RBD-specific cells that are memory B cells showed very little difference between the dose interval groups after vaccination, further assessment of the different BCR isotypes of memory B cells can be done to assess the impact dose interval has on B cell maturation while considering CSR. At 10-14 days post-dose 1, the proportions of S-specific and RBD-specific B cells that were IgG+ memory B cells was similar in both extended and standard dose interval groups (Figure 3.2-3A). However, by 10-14 days post-dose 2, there is a significant increase in the proportions of S-specific and RBD-specific B cells that are IgG+ memory B cells in both groups. Participants with an extended dose interval have a greater proportion of S-specific IgG+ memory B cells at 10-14 days post-dose 2 compared to participants with a standard dose interval ($p=0.004$), with median proportions of 34.45% and 13.85% of S-specific B cells, respectively (Fig 3.2-3B). However, by 4-6 months post-dose 2, the proportions of S-specific B cells that are IgG+ memory B cells are no longer significantly different between the two interval groups ($p=0.33$). A similar outcome is seen in RBD-specific B cells that are IgG+ memory B cells, as after 10-14 days post-dose 2, extended dose interval participants have a greater proportion of RBD-specific B cells that are IgG+ memory B cells than standard dose interval participants ($p=0.0002$), with median proportions of 44.1% and 13.2% of RBD-specific B cells, respectively. While the proportions of memory B cells are unchanged with an extended dose interval, an increase in IgG+ memory B cells in the extended dose interval group would indicate that dose interval impacts the class-switching process of memory B cells. RBD-specific B cells that are IgG+ memory B cells are not significantly different following 4-6 months after the second dose ($p=0.84$). However, there no significant differences in the proportions of both S-specific and RBD-specific B cells that were

IgG+ memory B cells between males and females who had received an extended dose interval after the 2nd dose (Supp. Figure 5.2-4).

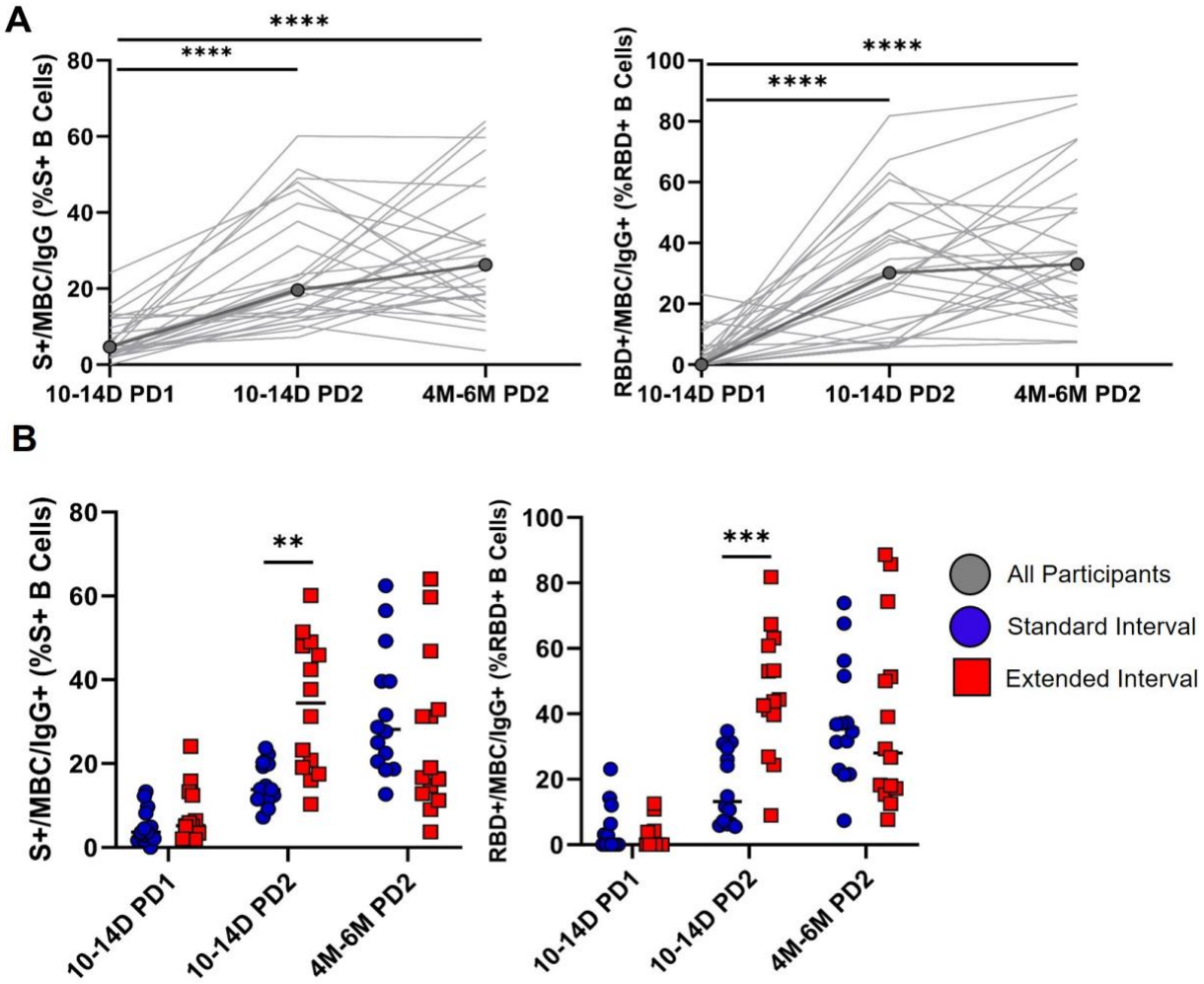


Figure. 3.2-2: Assessment of IgG+ Memory B Cells as a Percentage of S-specific and RBD-specific B cells. (A) Trajectory of IgG+ memory B cells as a percentage of S-specific and RBD-specific B cells from 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Time points were compared using the Kruskal-Wallis test with Dunn's post-test. Bold lines show median values **(B)** Comparison of IgG+ memory B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

The proportions of SARS-CoV-2 specific IgD and IgM memory B cells, cells that have undergone memory B cell maturation but are not yet IgG+ or IgA+ B cells, were also assessed to detect any differences between the standard and extended dose interval groups. SARS-CoV-2 vaccination results in a significant decrease from 10-14 days after the first dose to 1-month

following the third dose in both RBD-specific and/or S-specific B cells that are either IgD+/IgM+, and IgD+ only memory B cells (Figure 3.2-4A). S-specific memory B cells that are IgM+ see a similar significant decrease after vaccination, but those cells that also recognize RBD do not see a significant decrease. At 10-14 days post-dose 2, standard dose interval participants have a significantly greater proportion of S-specific memory B cells that are IgD+/IgM+ than extended dose interval participants ($p=0.004$), with an median proportions of 8.67% and 3.35% of S-specific B cells, respectively (Figure 3.2-4B). After 4-6 months post-dose 2, the difference in proportions of S-specific B cells that are IgD+/IgM+ memory B cells between the two groups are no longer significant ($p=0.97$). The proportions of S-specific B cells that are IgD+/IgM- memory B cells follow a similar trend, with a greater proportion in the standard dose interval participants compared to the extended dose interval participants at 10-14 days post-dose 2 ($p=<0.0001$), with median proportions of 9.17% and 3.2% of S-specific B cells, respectively. Similarly, the difference in proportions of S-specific B cells that are IgD+/IgM- memory B cells are not significantly significant following 4-6 months post-dose 2 ($p= 0.84$). However, this outcome is not detected in S-specific B cells that are IgD-/IgM+ memory B cells, as both participant groups maintain similar proportions of S-specific B cells that areIgD-/IgM+ memory B cells 10-14 days ($p=0.18$) and 4-6 months ($p=0.70$) after the second dose.

The subset of S-specific B cells that recognize RBD and are also IgD+ and/or IgM+ memory B cells follow a similar trend, with some differences (Figure 3.2-4A). While there are significant differences between dose interval groups in S-specific IgD+/IgM+ memory B cells following the second dose, the proportions of RBD-specific IgD+/IgM+ are similar following the second dose in the standard and extended dose interval participants (Figure 3.2-4B). The proportions of RBD-specific IgD+/IgM- memory B cells also follow the same trend, with a greater proportion of those cells in standard dose interval participants when compared to extended dose interval participants at 10-14 days post-dose 2 ($p=0.0003$), with an average proportion of 14.98 and 3.99, respectively. The proportions of RBD-specific IgD-/IgM+ memory B cells also follow the trend of S-specific IgD-/IgM+ memory B cells, as there is no

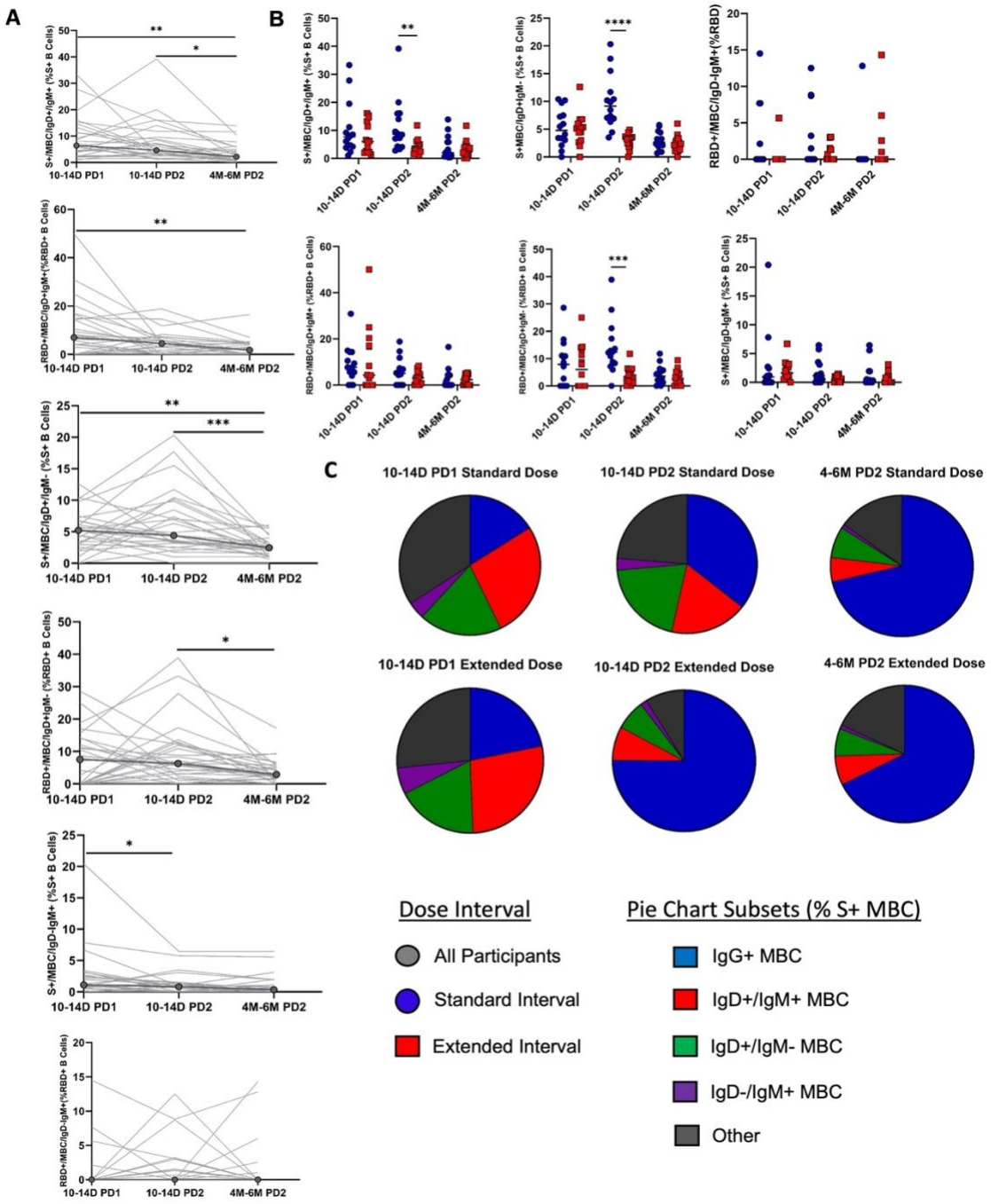


Figure. 3.2-3: Assessment of IgD/IgM Memory B Cells as a Percentage of S-specific and RBD-specific B cells. (A) Trajectory of IgD/IgM memory B cells as a percentage of S-specific and RBD-specific B cells from 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Time points were compared using the Kruskal-Wallis test with Dunn's post-test. Bold lines show median values. **(B)** Comparison of IgD/IgM memory B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values. **(C)** Proportions of the different memory B cell subsets (IgG+, IgD+IgM+, IgD+IgM-, IgD-IgM+) before and after the second dose in standard and extended dose interval participants. The other category includes memory B cell subsets not analyzed, such as IgA+ memory B cells.

significant difference between the two groups. Taken all together, the proportion of SARS-CoV-2 specific IgD+ and/or IgM+ memory B cells is greater in participants who received a standard dose interval of the SARS-CoV-2 vaccine than in participants who received an extended dose interval of the vaccine at 10-14 days post-dose 2, unlike the proportions of SARS-CoV-2 specific IgG+ memory B cells, which were greater in the extended dose interval participants after the second dose. Therefore, the extended dose interval leads to an increase in SARS-CoV-2 specific IgG+ memory B cells 10-14 days after the second dose, while a standard dose interval results in a greater proportion of mature memory B cells that have not yet class-switched to IgG+ and are still presenting IgD+ and/or IgM+ B cell receptors 10-14 days after the second dose. By 4-6 months post-dose 2, there are no differences between the groups. Figure 3.2-4C outlines the changes in proportions of different subsets of memory B cells after the second dose of the vaccine in standard or extended dose interval participants. However, there no significant differences in the proportions of both S-specific and RBD-specific B cells that were IgD+/IgM+ memory B cells between males and females who had received an extended dose interval after the second dose (Supp. Figure 5.2-5,6).

3.2.2.3. Assessment of Proliferating B Cell Phenotypes Among SARS-CoV-2-Specific Memory B Cells

SARS-CoV-2 specific memory B cells that were CD71+, which indicates cells undergoing proliferation, were also assessed in the two groups. The proportions of both S-specific and RBD-specific memory B cells that were CD71+ increased significantly from 10-14 days after the 1st dose to 10-14 days after the 2nd dose (Figure 3.2-5A). The proportions of S-specific memory B cells that were CD71+ were found to be similar between the standard and extended dose interval groups at 10-14 days post-dose 1. At 10-14 days post-dose 2, there was a significant increase in the proportion of S-specific memory B cells that are CD71+ in participants who had received the extended dose interval compared to the standard dose interval participants ($p=<0.0001$), with median proportions of 83.65% and 38.7% of S-specific memory B cells, respectively (Figure 3.2-5B). After 4-6 months post-dose 2, the proportions between the extended and standard dose groups are not significantly different ($p=>0.9999$), with median proportions of 76.7% and 76.2% of S-specific memory B cells, respectively. At 10-14 days post-dose 1, there were similar proportions of RBD-specific memory B cells that are CD71+ in both

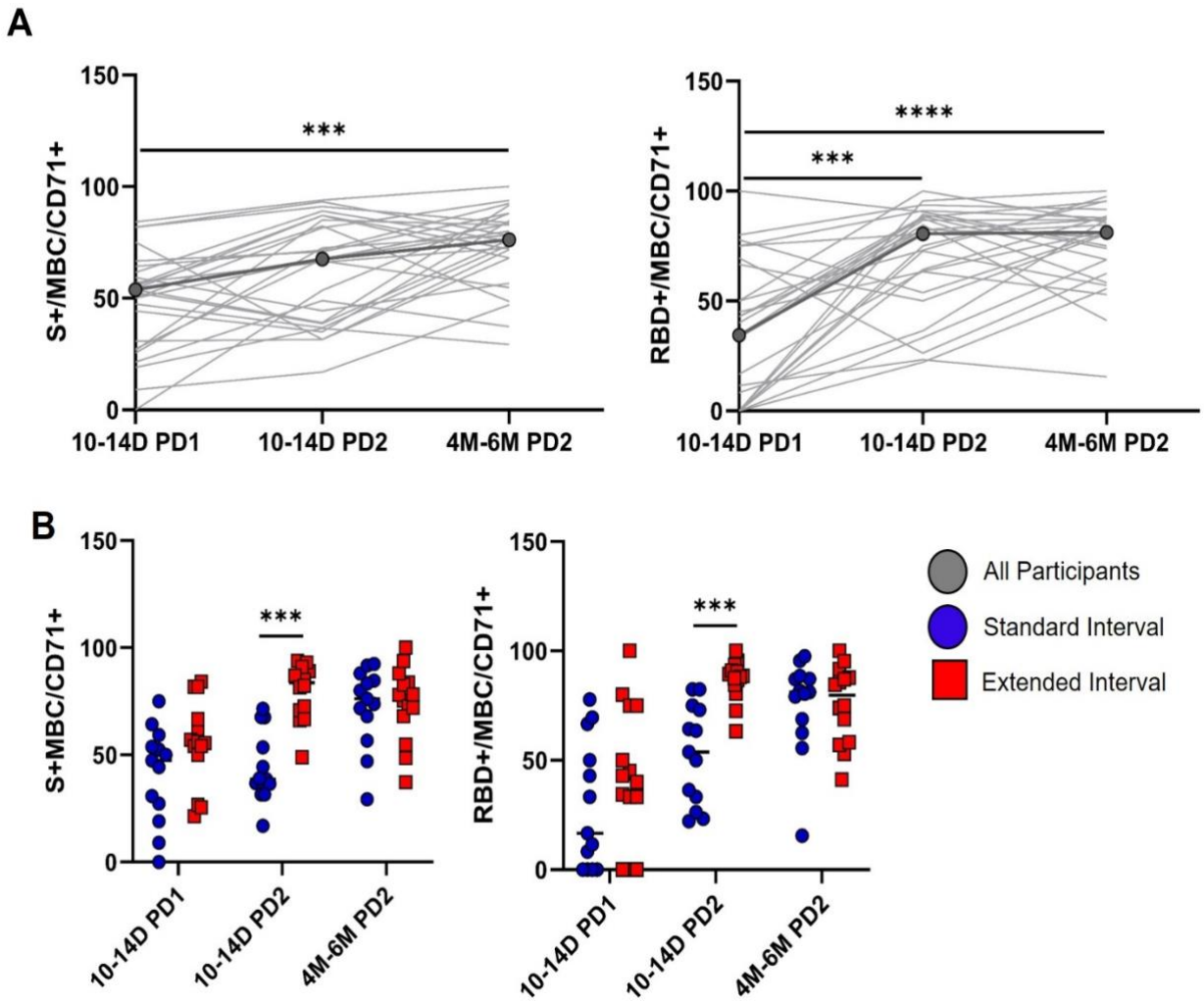


Figure 3.2-4: Assessment of CD71+ B Cells as a Percentage of S-specific and RBD-specific memory B cells. (A) Trajectory of CD71+ B cells as a percentage of S-specific and RBD-specific memory B cells from 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Time points were compared using the Kruskal-Wallis test with Dunn's post-test. Bold lines show median values. **(B)** Comparison of CD71+ B cells as a percentage of S-specific and RBD-specific memory B cells between the standard and extended dose interval groups. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

standard and extended dose interval groups. After 10-14 days post-dose 2, a significant increase and difference is seen in the proportion of RBD-specific memory B cells CD71+ in the extended dose interval group compared to the standard dose interval group (p=<0.0001), with median proportions of 88.35% and 53.8% of RBD-specific memory B cells, respectively. After 4-6 months post-dose 2, the proportions between the extended and standard dose groups are not significantly different (p=0.858), with median proportions of 79.8% and 81.2% of RBD-specific

memory B cells, respectively. However, there no significant differences in the proportions of both S-specific and RBD-specific memory B cells that were CD71+ between males and females who had received an extended dose interval after the second dose (Supp. Figure 5.2-7)

3.2.3. Assessment of Naïve B Cell Phenotypes Among SARS-CoV-2-Specific Memory B Cells

The S-specific and RBD-specific B cells that express a naïve B cell phenotype, defined by an absence of CD27, were also analyzed between the dose interval groups. SARS-CoV-2 vaccination results in a significant decrease in S-specific and RBD-specific B cells that are naïve B cells from 10-14 days after the 1st dose of the vaccine to 10-14 days after the 2nd dose (Figure 3.2-6A). At 10-14 days post-dose 1, the proportions of S-specific B cells that are naïve are similar between the standard and extended dose interval groups (Figure 3.2-6B). There is no significant difference between the standard and extended dose interval groups at 10-14 days post-dose 2 ($p=0.511$) or after 4-6 months post-dose 2 ($p=0.489$). The proportions of RBD-specific B cells that are naïve also significantly decreased in both groups between 10-14 days post-dose 1 and 10-14 days post-dose 2, but there is no significant difference between the standard and extended dose interval groups after 10-14 days post-dose 2 ($p=0.511$) and 4-6 months post-dose 2 ($p=0.489$). Therefore, these results indicate that the dose interval has no significant impact on the proportions of naïve B cells after the 2nd dose. Furthermore, there was no significant differences in the proportions of both S-specific and RBD-specific B cells that were naïve B cells between males and females who had received an extended dose interval after the second dose (Supp. Figure 5.2-8).

3.2.5. Correlation of SARS-CoV-2-Specific B Cells and Length of Dose Interval

A correlation analysis was performed to compare the proportions of various phenotypes of B cells to the length of the dosing interval of the SARS-CoV-2 vaccine in days. Dose interval length was calculated from the vaccine data provided by the participants. These associations were seen at 10-14 days post-dose 2. Spearman correlation analyses showed that an increase in the length of the vaccine dose interval had a significant positive association with an increase in proportions of S-specific B cells ($p=0.0035$, $r=0.53$), and RBD-specific B cells ($p=0.0097$, $r=0.48$) as a percentage of total B cells (Figure 3.2-7). However, there was no correlation between the length of the dose interval and the proportion of RBD-specific B cells as a

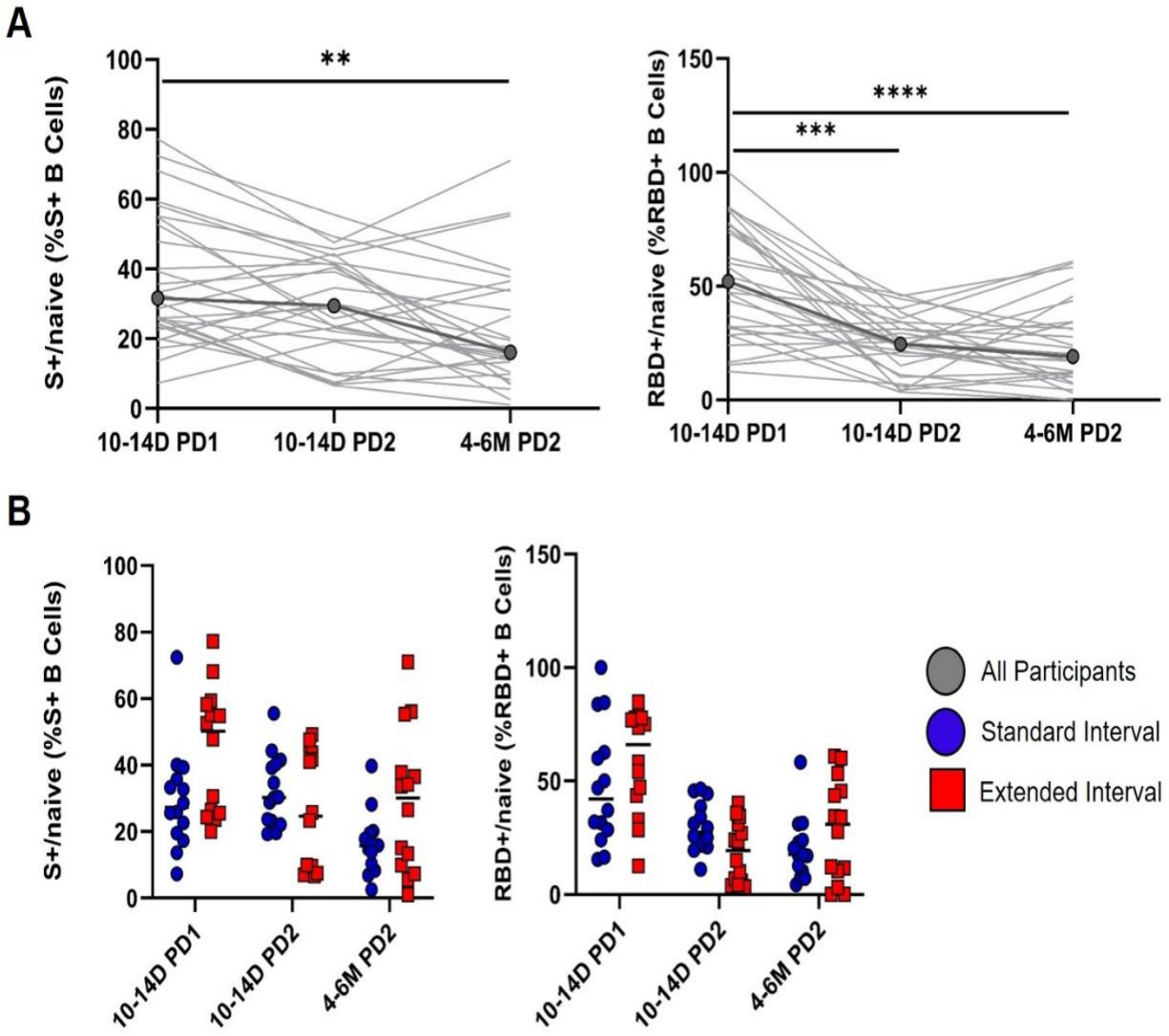


Figure 3.2-5: Assessment of Naïve B Cells as a Percentage of S-specific and RBD-specific B cells. (A) Trajectory of naïve B cells as a percentage of S-specific and RBD-specific B cells from 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Time points were compared using the Kruskal-Wallis test with Dunn's post-test. Bold lines show median values. **(B)** Comparison of naïve B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

percentage of S-specific B cells (p=0.42, r=0.16). Dose interval also correlated with the proportions of S-specific B cells that are IgG+ memory B cells (p=0.0016, r=0.57), RBD-specific B cells that are IgG+ memory B cells (p=0.0001, r=0.66), S-specific memory B cells that are CD71+ (p=<0.0001, 0.72) and RBD-specific memory B cells that are CD71+ (p=<0.0001, r=0.73) (Figure 3.2-7). The length of the vaccine dose interval had a significant inverse

association with proportions of S-specific B cells that are IgD+/IgM+ memory B cells (p=0.0001, r=-0.67). A similar trend was observed with proportions of RBD-specific B cells that are IgD+/IgM+ memory B cells, although not significant (p=0.10, r=-0.31) (Figure 3.2-7). At 4-6

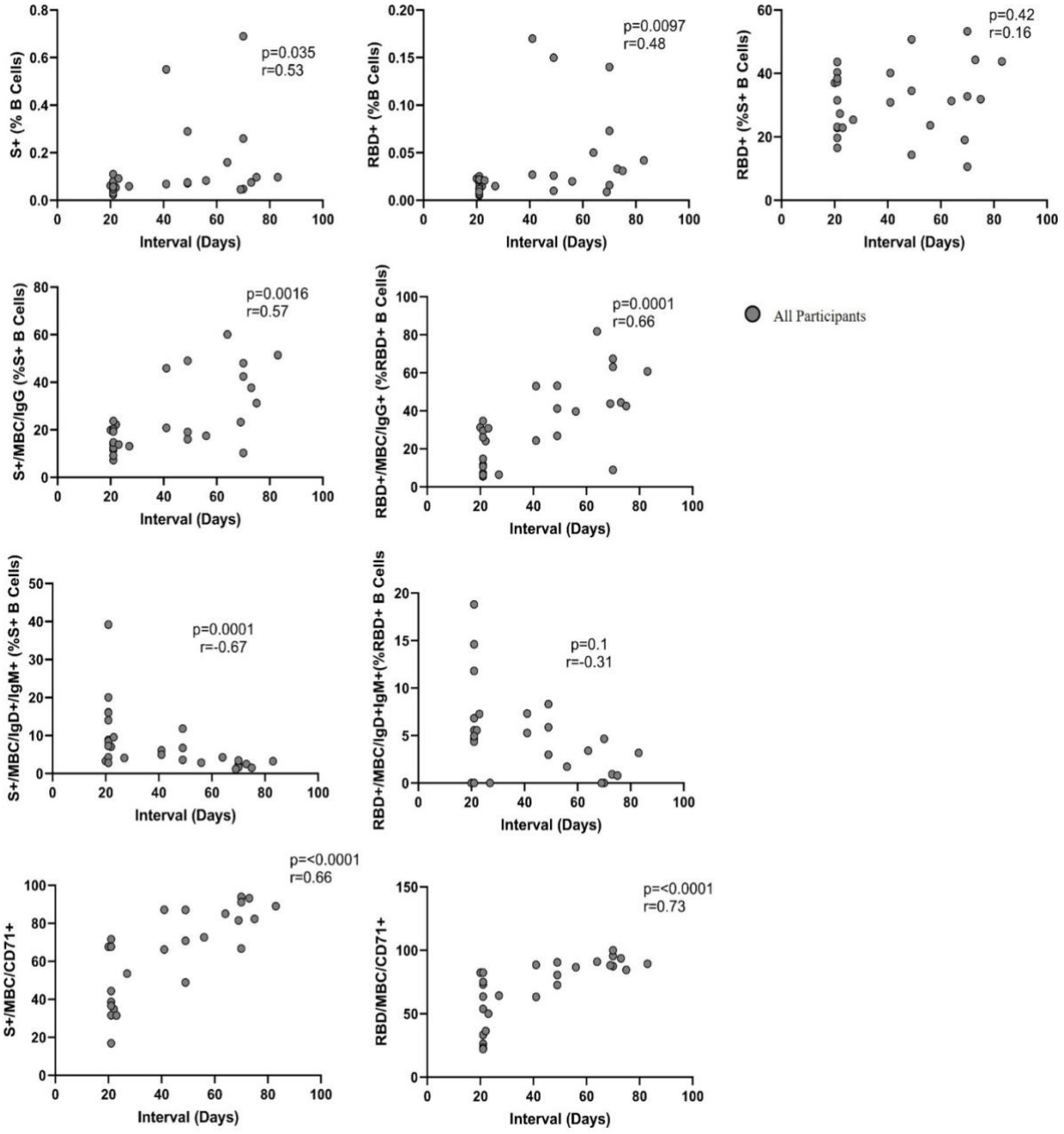


Figure 3.2-6: Correlations between the length of the first and second dose interval (days) and the proportions of S-specific and RBD-specific B cells and subpopulations. Correlations were determined using Spearman correlations.

months post-dose 2, no associations between the length of the dose interval and the proportions of the various B cell phenotypes were observed.

3.3. Impact of Dose Interval on Neutralizing Antibodies Produced by Memory B Cells Upon Secondary Exposure

3.3.1. Longitudinal Assessment of Neutralizing Antibody Responses Against Wild-Type SARS-CoV-2

PBMCs were stimulated using IL-2 and R848. After 10 days, the supernatants containing antibodies produced by the activated plasma cells were collected. The MSD Key Variant Panel was used to assess the inhibition of these antibodies against wild-type SARS-CoV-2 RBD as well as variant SARS-CoV-2 RBD. Participants who had received the extended dose interval were found to have a similar rate of inhibition of the wild-type when compared to participants who had received the standard dose interval at 10-14 days post-dose 1, with a median % RBD inhibition of 30.8% and 27.3%, respectively (Figure 3.3-1A). However, participants who had received the extended dose interval were found to have a better inhibition of the wild-type RBD than participants who had received the standard dose interval at 10-14 days post-dose 2 ($p=<0.0001$), with median % RBD inhibitions of 93.1% and 56.6%, respectively. After 4-6 months post-dose 2, participants with an extended and standard dose intervals showed similar inhibition of wild-type RBD with median % RBD inhibitions of 64.3% and 66.6%, respectively. These results indicate that an extended dose interval results in better neutralizing antibodies against wild-type SARS-CoV-2 RBD at earlier timepoints following the second dose.

3.3.2. Assessment of Breadth of Neutralizing Antibody Responses Against SARS-CoV-2 Variants

The % inhibitions of antibodies from a standard and extended dose interval against SARS-CoV-2 RBD variants were also measured. The second dose of the SARS-CoV-2 vaccine saw a significant increase in antibody neutralization against Wild-Type, Alpha, Beta and Delta variants, regardless of dose interval (Figure 3.3-1B). At 10-14 days post-dose 1, both groups exhibited inhibition similar to the stimulated baseline controls of the alpha, beta, delta and omicron lineages (Figure 3.3-1C). At 10-14 days post-dose 2, the extended dose interval group were found to have significantly greater inhibitions of alpha ($p=0.0001$), beta ($p=0.0003$) and delta ($p=0.0005$) lineages compared to the standard dose interval group, with a nearly two-fold

increase in neutralization between the two groups. Both groups were found to have inhibition similar to the unstimulated baseline controls of the omicron lineages at 10-14 days post-dose 2. These results provide further evidence that an extended dose interval allows for the generation of antibodies with stronger neutralization abilities of both wild-type and variants closely related to the wild-type strain of SARS-CoV-2 at earlier timepoints following the second dose. By 4-6

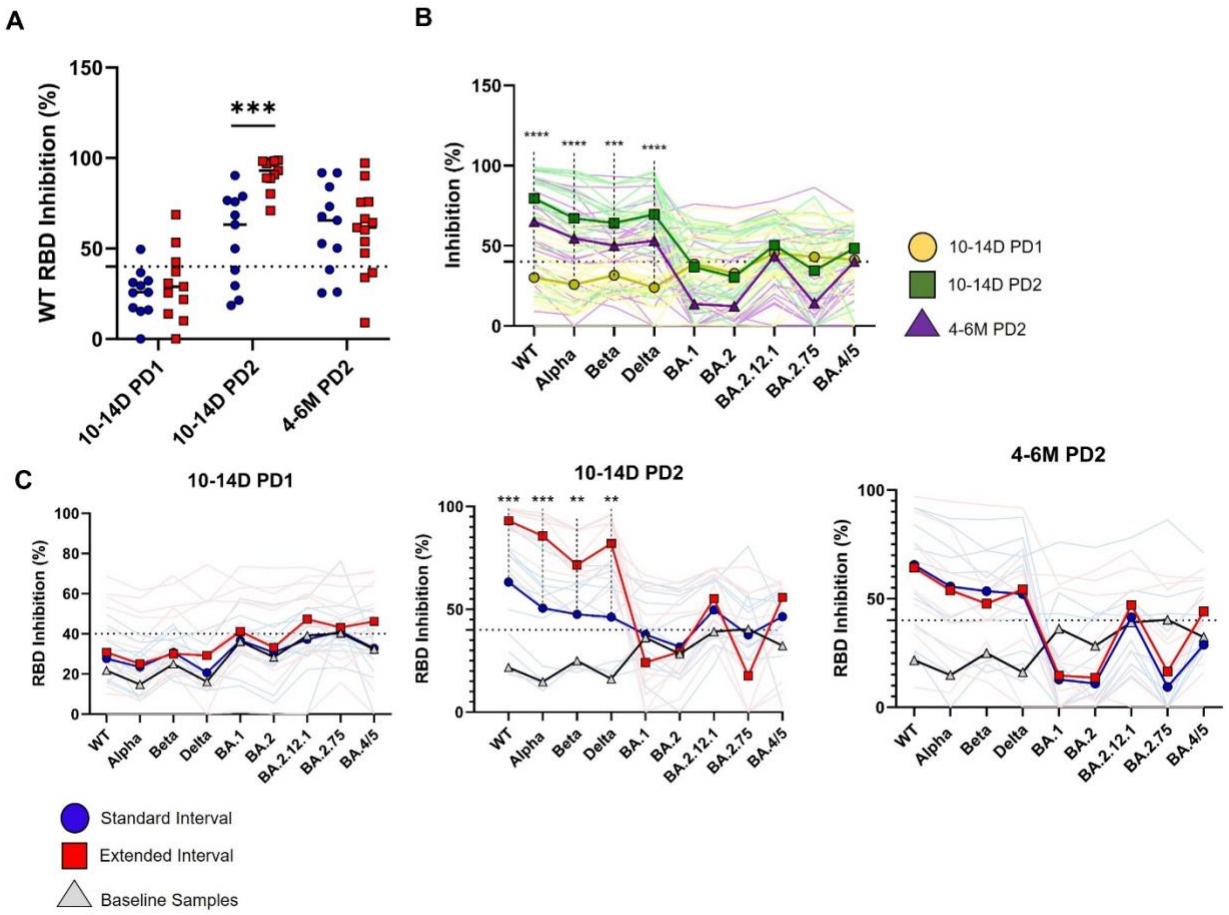


Figure 3.3-1: Comparisons of Stimulated B Cell Antibody Neutralization against SARS-CoV-2 RBD and Variants. (A) Comparisons of plasma cell-derived antibody neutralization against wild-type RBD between standard and extended dose interval participants at 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values. Dotted line indicates cut-off. **(B)** Comparisons of plasma cell-derived antibody neutralization against SARS-CoV-2 RBD variants between 10-14 days post-dose 1, 10-14 days post-dose 2 and 4-6 months post-dose 2. Groups were compared using two-way ANOVA tests with multiple comparisons. Dotted line indicates cut-off. **(C)** Comparisons of plasma cell-derived antibody neutralization against SARS-CoV-2 RBD variants between standard and extended dose interval participants at 10-14 days post-dose 1 and 10-14 days post-dose 2. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values. Dotted line indicates cut-off.

months post-dose 2, there are no significant differences in the inhibitions of SARS-CoV-2 variants.

3.3.3 Correlation of Neutralizing Antibody Responses with Circulating Binding Antibody Titres

The correlation between the neutralization ability of plasma cell antibodies and the RBD-specific IgG+ antibody titres in plasma at matched time points was assessed. Almost no correlation between RBD-specific IgG+ antibody titres and plasma cell antibody neutralization ability was detected for any of the time points except for 10-14 days post-dose 2, where a significant positive association ($p=0.02$, $r=0.53$) was detected between RBD-specific IgG+ plasma antibody titre and wild-type neutralization of plasma cell RBD-specific antibodies (Figure 3.3-2). There were no significant associations between RBD-specific IgG+ plasma antibody titre and inhibition of Alpha ($p=0.06$, $r=0.45$), Beta ($p=0.17$, $r=0.34$) and Delta ($p=0.15$, $r=0.35$) variants of SARS-CoV-2 at 10-14 days post-dose 2. Those positive associations were no longer detected by 4-6 months post-dose 2 or 1-month post-dose 3.

3.3.4. Correlation of Neutralizing Antibody Responses with Proportions of SARS-CoV-2-Specific B Cells

The correlation between the neutralization ability of antibodies produced by the differentiated cells and the proportions of SARS-CoV-2 specific B cells was assessed to determine associations between the proportion of different B cell subpopulations and the neutralization ability of the antibodies produced by stimulated B cells. The proportion of S-specific B cells at 10-14 days post-dose 2 was found to have a significant positive association with cell-derived antibody neutralization against wild-type ($p=0.002$, $r=0.68$), Alpha ($p=0.003$, $r=0.66$), Beta ($p=0.019$, $r=0.55$) and Delta ($p=0.028$, $r=0.52$) variants at the same time point (Figure 3.3-3). The proportion of RBD-specific B cells at 10-14 days post-dose 2 was also found to have a significant positive association with antibody neutralization against wild-type ($p=0.0002$, $r=0.77$), Alpha ($p=0.0002$, $r=0.77$), Beta ($p=0.006$, $r=0.62$), and Delta ($p=0.0074$, $r=0.61$) variants. There was no significant association between the proportion of S-specific or RBD-specific B cells and antibody neutralization of any SARS-CoV-2 variants after 4-6 months post-dose 2 or 1-month post-dose 3.

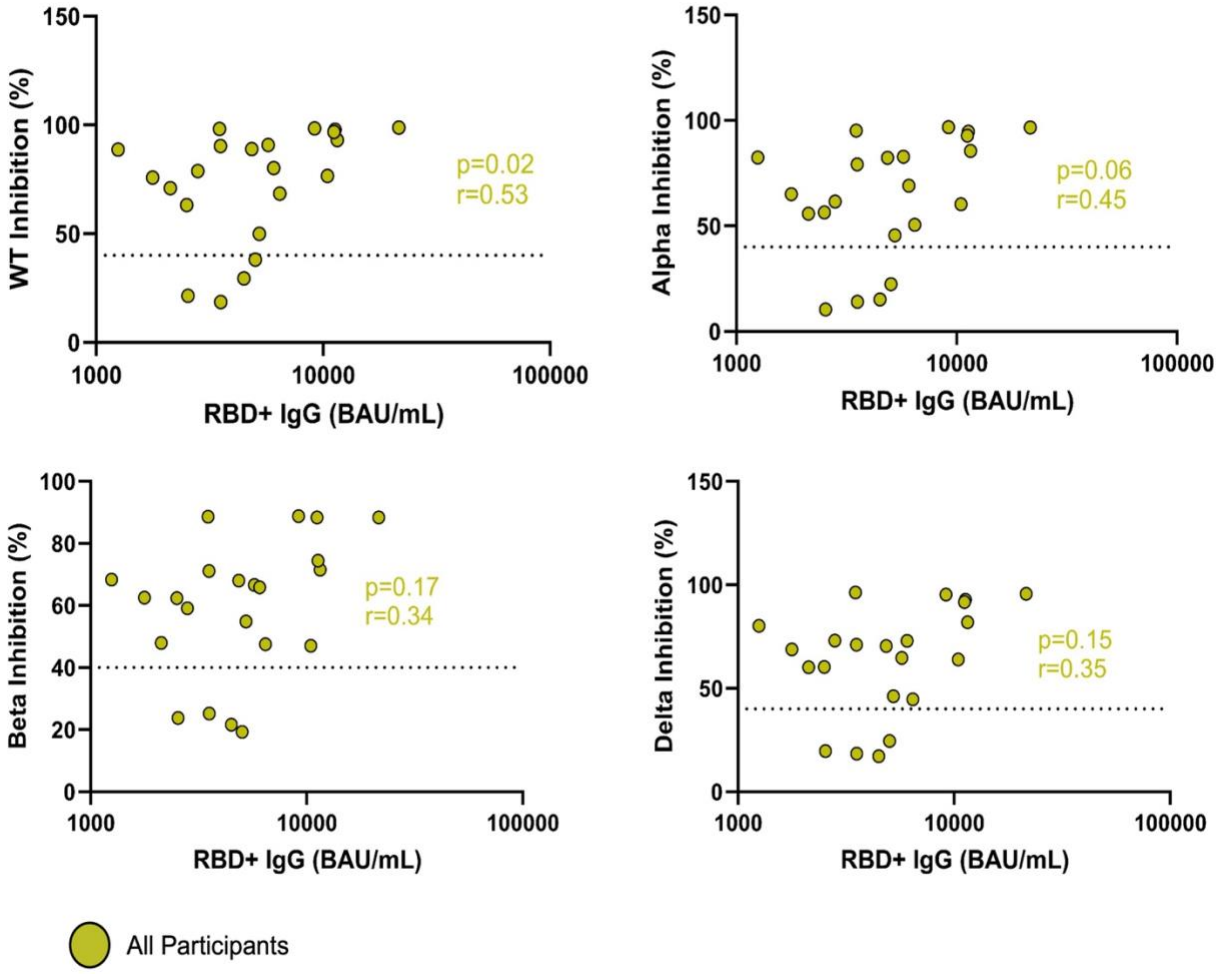


Figure 3.3-2: Correlation between Plasma Cell Antibody RBD+ Neutralization and Plasma RBD-Specific IgG+ Antibody Titres. The titre of RBD-specific IgG+ antibodies from plasma (BAU/mL) from 10-14 days post-dose 2 was compared to the neutralization of RBD variants by the RBD-specific antibodies produced directly from stimulated B cells (% inhibition) at 10-14 days post-dose 2. Spearman Correlation tests were performed to assess correlations. Dotted line indicates cutoff.

Furthermore, at 10-14 days post-dose 2, there was a significant positive association between the proportion of S-specific B cells that are IgG+ memory B cells and antibody neutralization against wild-type (p=<0.0001, r=0.82), Alpha (p=<0.0001, r=0.80), Beta (p=0.002, r=0.68) and Delta (p=0.008, r=0.61) variants. After 4-6 months post-dose 2 and 1-month post-dose 3, there was no positive association between S-specific IgG+ memory B cells and antibody neutralization. There was a similar significant positive association at 10-14 days post-dose 2 between the proportion of RBD-specific IgG+ memory B cells and antibody neutralization against wild-type (p=0.0007, r=0.72), Alpha (p=0.002, r=0.69), Beta (p=0.025, r=0.53) and Delta

(p=0.023, r=0.53) variants. There were no significant associations between the proportions of RBD-specific IgG+ memory B cells and antibody neutralization of SARS-CoV-2 variants at 4-6 months post-dose 2 and 1-month post-dose 3.

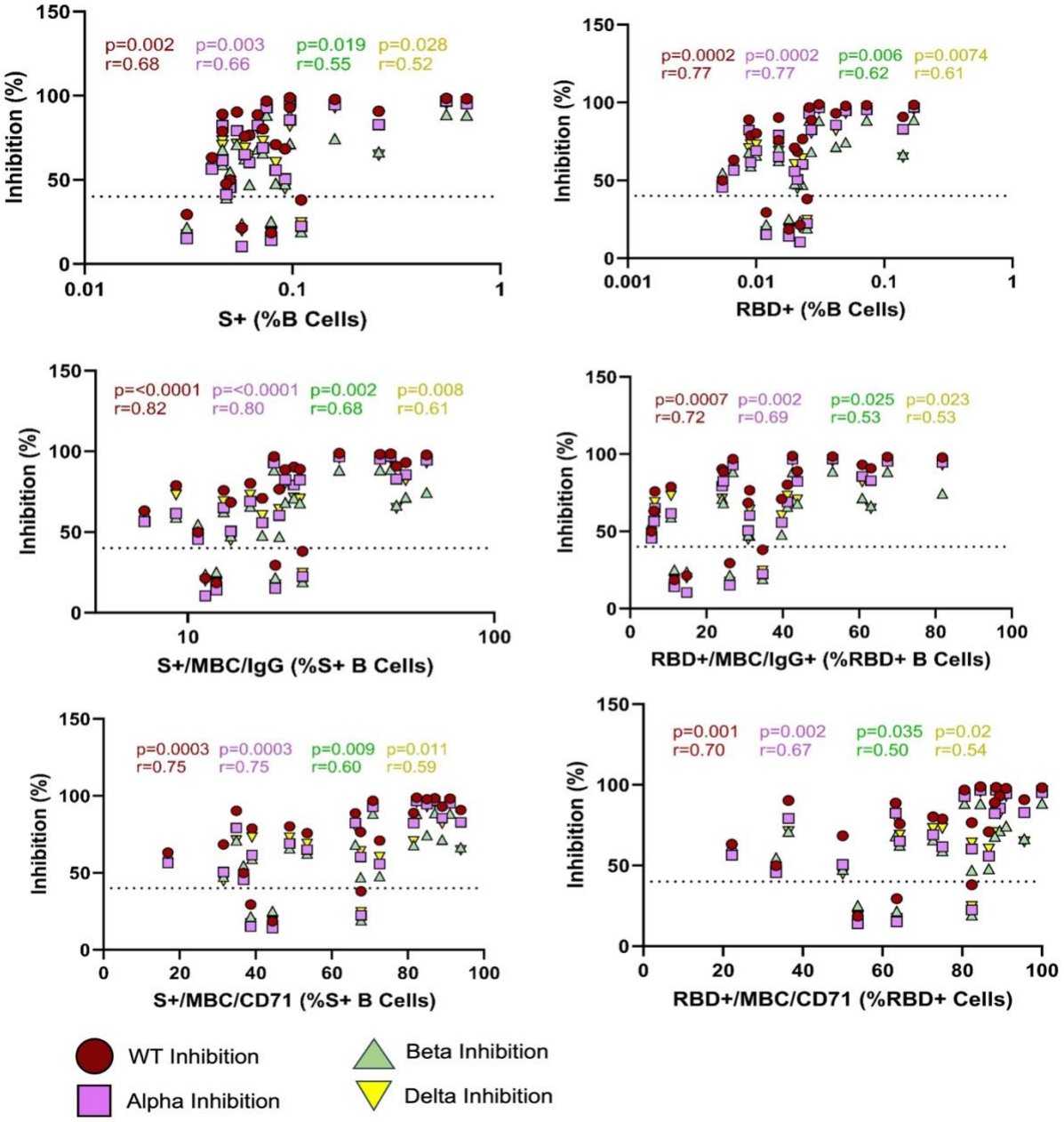


Figure 3.3-3: Correlation between Plasma Cell Antibody RBD+ Neutralization and SARS-CoV-2 Specific B Cell Proportions. The plasma cell antibody RBD+ neutralization ability against different SARS-CoV-2 variants was compared to the proportion of different S-specific and RBD-specific B cell populations at 10-14 days post-dose 2. Spearman Correlations tests were performed to assess correlations. Dotted line indicates cutoff.

At 10-14 days post-dose 2, there was a significant positive association between the proportion of S-specific CD71 memory B cells and antibody neutralization against wild-type (p=0.0003, r=0.75), Alpha (p=0.0003, r=0.75), Beta (p=0.009, r=0.60) and Delta (p=0.011, r=0.59) variants. A similar trend was observed in the proportion of RBD-specific CD71 memory B cells and antibody neutralization against wild-type (p=0.001, r=0.70), Alpha (p=0.002, r=0.67), Beta (p=0.035, r=0.50) and Delta (p=0.02, r=0.54) variants.

3.3.5. Correlation of Proportions of SARS-CoV-2 Specific B Cells with Circulating Binding Antibody Titres

The correlation between the proportions of SARS-CoV-2 specific B cells and plasma neutralizing antibody titres was assessed to determine if antigen-specific B cell populations after vaccination predict the titre of SARS-CoV-2 plasma antibodies at matched or subsequent timepoints. At 10-14 days post-dose 2, the proportions of S-specific B cells as a percentage of total B cells did not have a significant positive association with S-specific IgG antibody titres (p=0.20, r=0.25) (Figure 3.3-4). The same outcome was detected when comparing the proportions of RBD-specific B cells as a percentage of total B cells and RBD-specific IgG antibody titres (p=0.15, r=0.28). Additionally, there was not a significant positive association between S-specific (p=0.38, r=0.18) or RBD-specific (p=0.35, r=0.19) B cells that are CD71+ memory B cells and S-specific or RBD-specific plasma IgG antibody titres, respectively. However, there was a significant positive association between the proportions of S-specific (p=0.03, r=0.41) and RBD-specific (p=0.03, r=0.41) B cells that are IgG+ memory B cells with S-specific and RBD-specific IgG plasma antibody titres, respectively.

When the S-specific and RBD-specific IgG plasma neutralizing antibody titres from 4-6 months post-dose 2 were compared to the B cell proportions from 10-14 days post-dose 2, there were even greater positive associations detected. There was a significant positive association between S-specific (p=0.0002, r=0.66) and RBD-specific (p=<0.0001, r=0.74) B cells as a percentage of total B cells from 10-14 days post-dose 2 when compared to S-specific or RBD-specific IgG plasma neutralizing antibody titres from 4-6 months post-dose 2, respectively (Figure 3.3-4). Similarly, there was a significant positive association between S-specific (p=0.0005, r=0.64) and RBD-specific (p=<0.0001, r=0.71) B cells that were IgG+ memory B cells from 10-14 days post-dose 2 when compared to S-specific or RBD-specific IgG plasma neutralizing antibody titres from 4-6 months post-dose 2, respectively. Lastly, there was a

significant positive association between S-specific (p=0.0009, r=0.64) and RBD-specific (p=0.001, r=0.60) memory B cells with CD71+ from 10-14 days post-dose 2 when compared to S-specific or RBD-specific IgG plasma neutralizing antibody titres from 4-6 months post-dose 2, respectively.

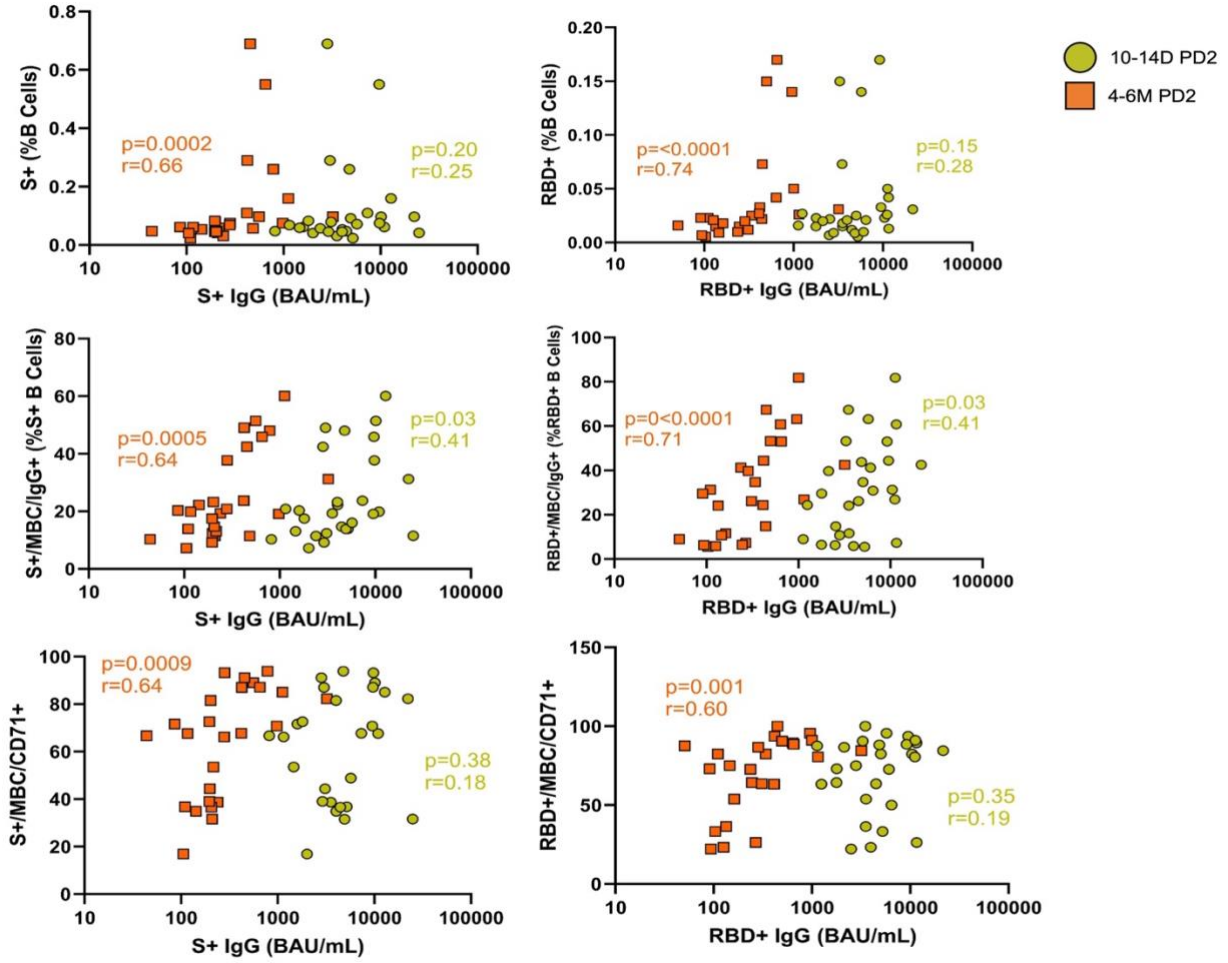


Figure 3.3-4: Correlation between Plasma S- and RBD-specific IgG Antibody Titres and SARS-CoV-2 Specific B cell Proportions at Different Timepoints. The plasma S-specific and RBD-specific IgG antibody titres at 10-14 days post-dose 2 and 4-6 months post-dose 2 were compared to various SARS-CoV-2-specific B cell populations from 10-14 days post-dose 2. Spearman Correlation tests were performed to assess correlations.

3.4. Impact of a Third Dose on Memory B Cell Responses and Relationship to Primary Dose Interval

3.4.1. Assessment of SARS-CoV-2-specific B cells after dose 3

Phenotyping the PBMCs also included samples from 4-6 months post-dose 2 and 1-month post-dose 3. This provides an analysis of whether the extended dose interval provides a

long-term benefit after a 3rd dose of the vaccine or if the enhanced immune response is only temporary. Paired comparisons between 4-6 months post-dose 2 and 1 month post-dose 3 show significant increases in S-specific B cells and RBD-specific B cells as a subset of B cells or S-specific B cells after the third dose (Figure 3.4-1A). At 1-month, post-dose 3, extended dose interval participants have similar proportions of S-specific B cells and RBD-specific B cells as a subset of B cells or S-specific B cells as standard dose interval participants, unlike at 10-14 days post-dose 2 (Figure 3.4-1B). While immunization leads to a steady and significant increase in the proportion of S-specific B cells throughout 3 doses, it is only after the second dose that a significant difference in the two groups is detected. Similarly, there are no differences in the

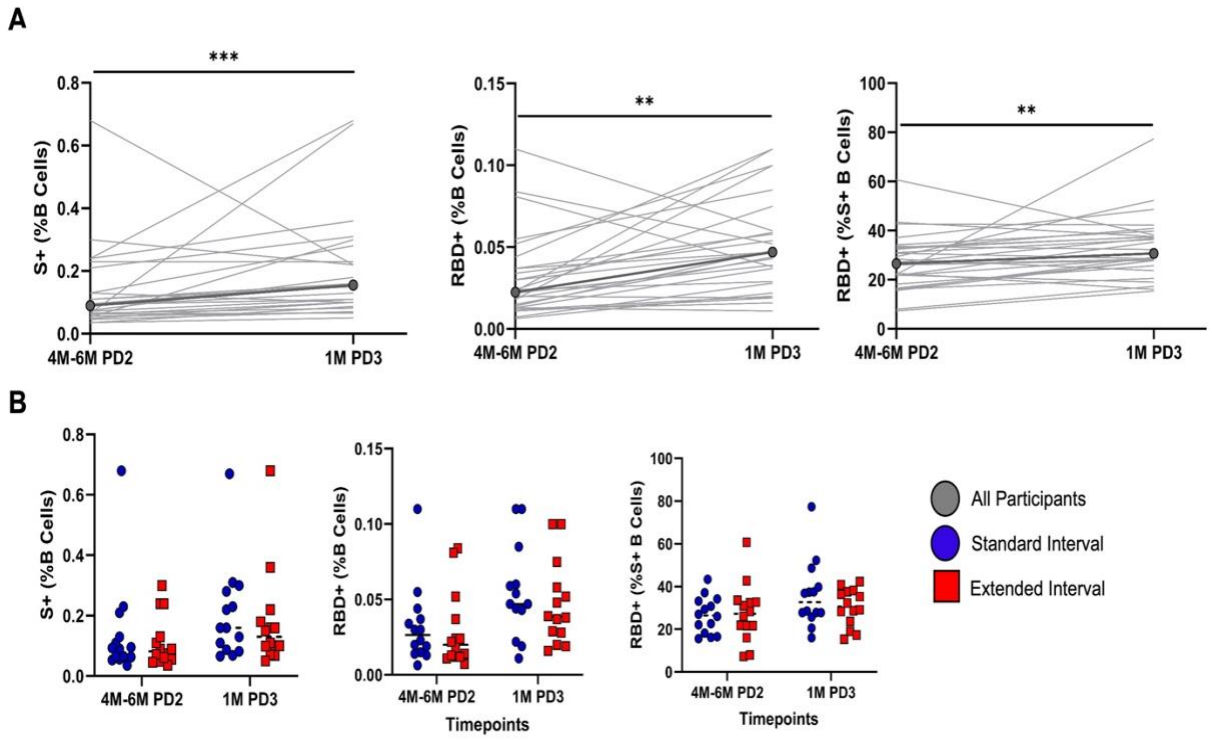


Figure 3.4-1: Assessment of Spike-Specific and RBD-Specific B Cells as a Percentage of B cells and Spike-specific B cells After a Third Dose of the SARS-CoV-2 Vaccine. (A) Trajectory of Spike-specific and RBD-specific B cells as a percentage of B cells from 4-6 months post-dose 2 and 1-month post-dose 3. Time points were compared using the Wilcoxon matched-pairs signed rank test. Bold line show median values. **(B)** Comparison of Spike-specific B cells and RBD-specific B cells as a percentage of B cells, and RBD-specific B cells as a percentage of Spike-specific B cells, between the standard and extended dose interval groups after a third dose. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

proportions of B cells that are S-specific or RBD-specific between males and females who have had either the standard or extended dose intervals after the third dose of the SARS-CoV-2 vaccine (Supp. Figure 5.2-2)

3.4.2. Assessment of memory B cell phenotypes among SARS-CoV-2-specific memory B cells After Dose 3

3.4.2.1. Assessment of SARS-CoV-2-Specific Memory B Cells After Dose 3

A longitudinal assessment of SARS-CoV-2 specific memory B cells was done to determine the impact of a delayed dose interval on memory B cell populations after a 3rd dose. There was a significant increase in the proportions of S-specific and RBD-specific B cells that are memory B cells after the 3rd dose of the vaccine (Figure 3.4-2A) Participants with an extended dose interval saw no significant difference in the proportions of S-specific and RBD-specific B cells that are memory B cells after the 3rd dose of the vaccine compared to standard dose interval participants, unlike after the 2nd dose (Figure 3.4-2B). While the data shows that both immunization strategies results in a steady increase in SARS-CoV-2 specific memory B cells after 3 doses, the dose interval does not impact any long-term difference in the population of memory B cells that are S-specific and RBD-specific. Similarly, there are no differences in the proportions of S-specific or RBD-specific B cells that are memory B cells between males and females who have had either the standard or extended dose intervals after the third dose of the SARS-CoV-2 vaccine (Supp. Figure 5.2-3).

3.4.2.2. Assessment of SARS-CoV-2-Specific IgG+ and IgD+/IgM+ Memory B Cells After Dose 3

IgG+ memory B cells were assessed at 4-6 months post-dose 2 and 1 month post-dose 3 to determine the length of time that extended dose interval participants have a greater proportion of SARS-CoV-2 specific IgG+ memory B cells. A 3rd dose of the SARS-CoV-2 results in a greater proportion of S-specific and RBD-specific B cells that are IgG+ memory B cells, regardless of dose interval (Figure 3.4-3A). While there was a significant increase in the overall proportion, extended dose interval participants did not have a significant difference in the proportions of S-specific and RBD-specific B cells that are IgG+ memory B cells compared to standard dose interval participants after 1-month post-dose-3 (Figure 3.4-3B). This is in stark contrast to the results from post-dose 2, where a significant difference between dose interval

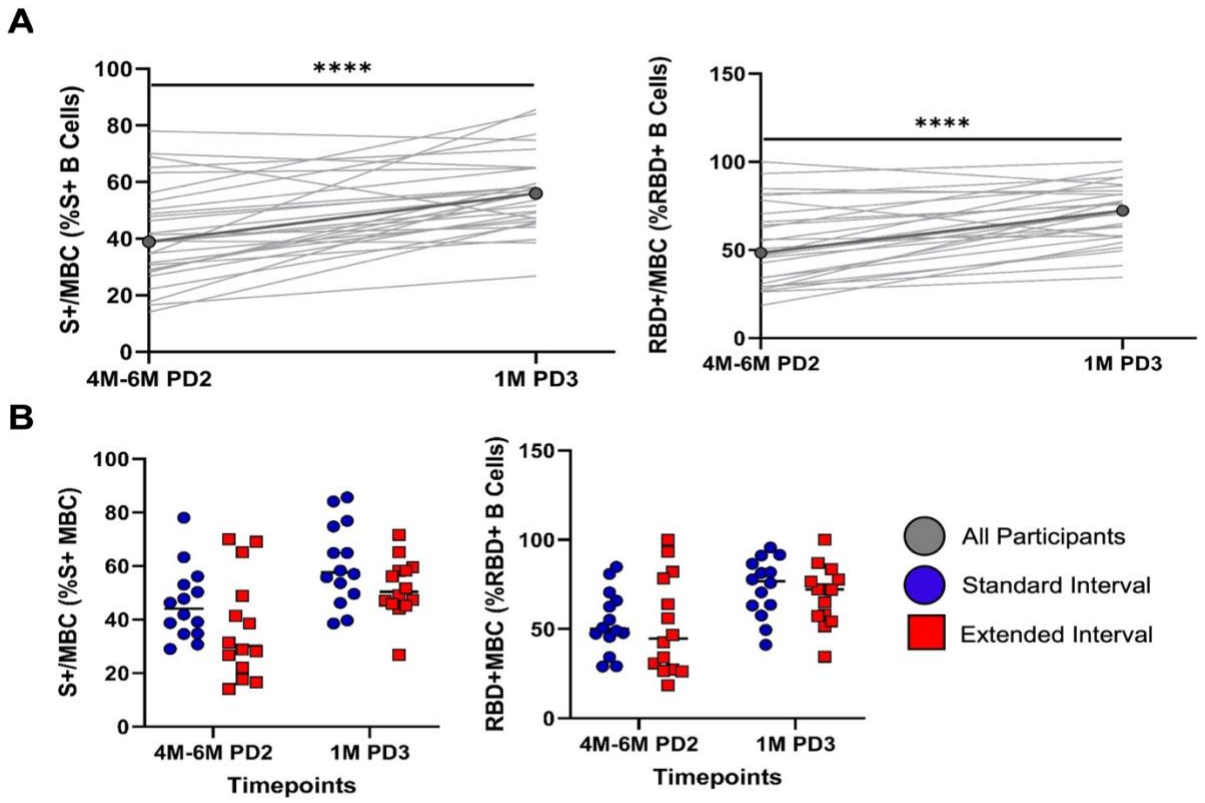


Figure 3.4-2: Assessment of Memory B Cells as a Percentage of S-specific and RBD-Specific B cells After a Third Dose of the SARS-CoV-2 Vaccine. (A) Trajectory of memory B cells as a percentage of S-specific and RBD-specific B cells from 4-6 months post-dose 2 and 1-month post-dose 3. Time points were compared using the Wilcoxon matched-pairs signed rank test. Bold line show median values. **(B)** Comparison of memory B cells as a percentage of S-sepcific and RBD-specific B cells between the standard and extended dose interval groups after a third dose. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

groups was detected. There are no differences in the proportions of S-specific or RBD-specific B cells that are IgG+ memory B cells between males and females who have had either the standard or extended dose intervals after the third dose of the SARS-CoV-2 vaccine (Supp. Figure 5.2-4).

The proportions of S-specific and RBD-specific B cells that expressed IgD or IgM present was also assessed after the 3rd dose to determine whether the differences in proportions between the extended and standard dose interval groups carried on beyond the 2nd dose. After the 3rd dose, there is no significant decrease in the proportions of both S-specific and RBD-specific B cells that are IgD+/IgM+, IgD+/IgM- or IgD-/IgM+ memory B cells, regardless of dose interval (Figure 3.4-4A). This is in contrast to after the 2nd dose, where significant decreases were seen after vaccination. Additionally, there were no significant differences in the proportions

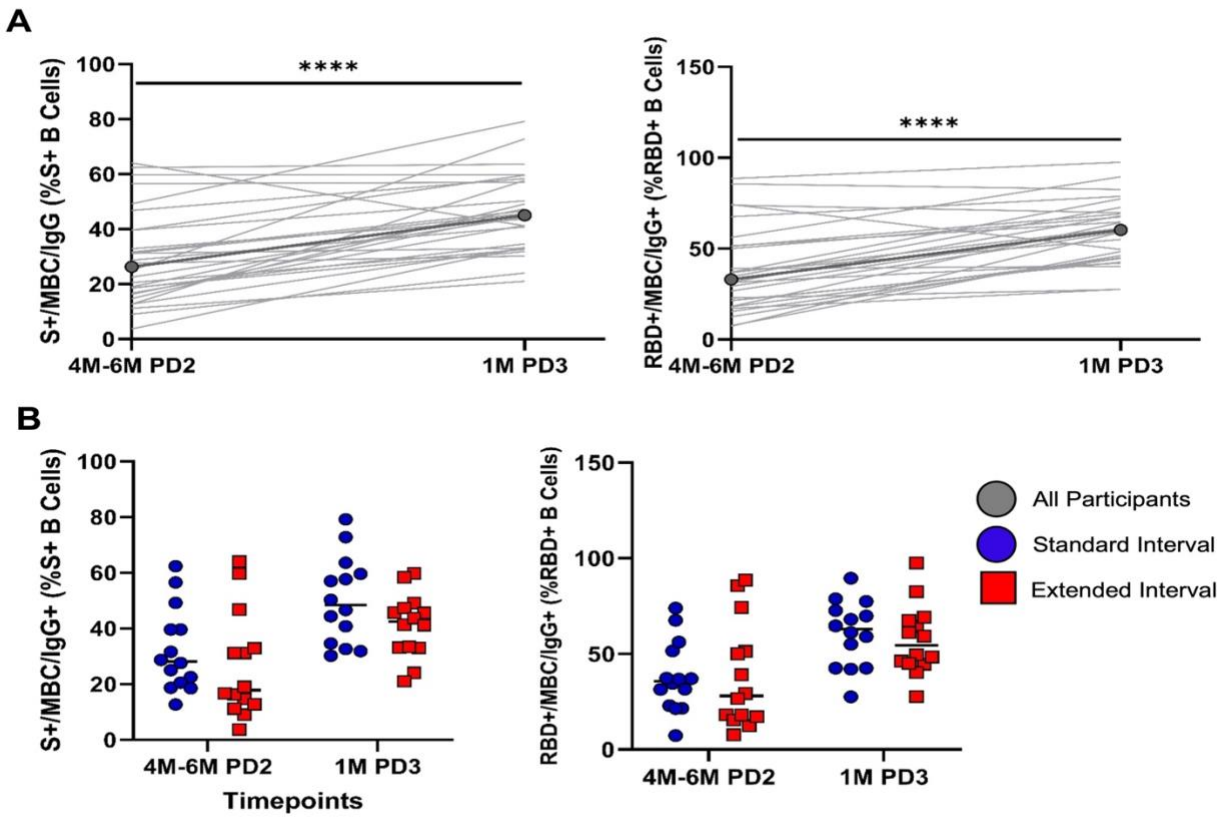


Figure 3.4-3: Assessment of IgG+ Memory B Cells as a Percentage of S-specific and RBD-Specific B cells After a Third Dose of the SARS-CoV-2 Vaccine. (A) Trajectory of IgG+ memory B cells as a percentage of S-specific and RBD-specific B cells from 4-6 months post-dose 2 and 1-month post-dose 3. Time points were compared using the Wilcoxon matched-pairs signed rank test. Bold line show median values. **(B)** Comparison of IgG+ memory B cells as a percentage of S-sepcific and RBD-specific B cells between the standard and extended dose interval groups after a third dose. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

of both S-specific and RBD-specific B cells that are IgD+/IgM+, IgD+/IgM- or IgD-/IgM+ memory B cells between the extended and standard dose interval participants after the 3rd dose (Figure 3.4-4B). This is also in contrast to after the 2nd dose, where significant differences in the proportions of mature resting B cells between the extended and standard dose interval participants were detected after the 2nd dose. Figure 3.4-4C outlines the changes in proportions of different subsets of memory B cells after the second dose of the vaccine in standard or extended dose interval participants. There are no differences in the proportions of S-specific or RBD-specific B cells that are IgD+/IgM+ memory B cells between males and females who have had either the standard or extended dose intervals after the third dose of the SARS-CoV-2 vaccine

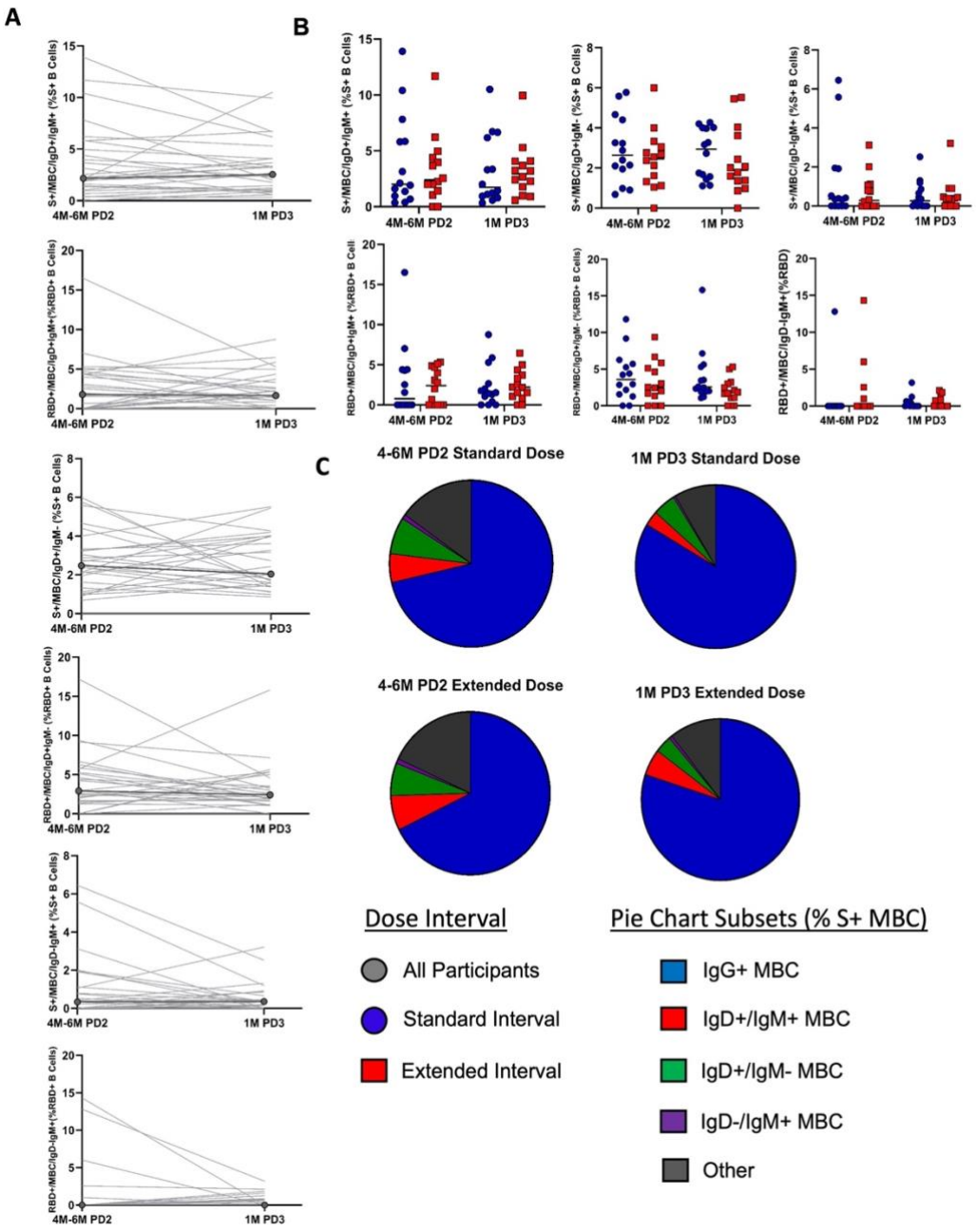


Figure 3.4-4: Assessment of IgD/IgM Memory B Cells as a Percentage of S-specific and RBD-specific B cells After a Third Dose of the SARS-CoV-2 Vaccine. (A) Trajectory of IgD/IgM memory B cells as a percentage of S-specific and RBD-specific B cells from 4-6 months post-dose 2 and 1-month post-dose 3. Time points were compared using the Wilcoxon matched-pairs signed rank test. Bold lines show median values. **(B)** Comparison of IgD/IgM memory B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups after a third dose. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values. **(C)** Proportions of the different memory B cell subsets (IgG+, IgD+IgM+, IgD+IgM-, IgD-IgM+) before and after the third dose in standard and extended dose interval participants. The other category includes memory B cell subsets not analyzed, such as IgA+ memory B cells.

(Supp. Figure 5.2-5,6). However, there was a significant positive association between the ages of the participants and the proportions of S-specific B cells that are IgD+/IgM+ memory B cells at 1-month post-dose 3 (Supp. Figure 5.2-7).

3.4.2.3. Assessment of Proliferating B Cell Phenotypes Among SARS-CoV-2-Specific Memory B Cells After Dose 3

The proportions of S-specific and RBD-specific memory B cells that expressed CD71+ were also assessed up until 1-month post-dose 3. The proportion of S-specific and RBD-specific memory Bcells that expressed CD71+ saw a significant increase between 4-6 months post-dose 2 and 1-month post-dose 3 (Figure 3.4-5A). While there was an overall significant change in the proportions of S-specific and RBD-specific memory B cells that express CD71 after the 3rd dose, there was no significant difference seen in those cell types when comparing the extended and

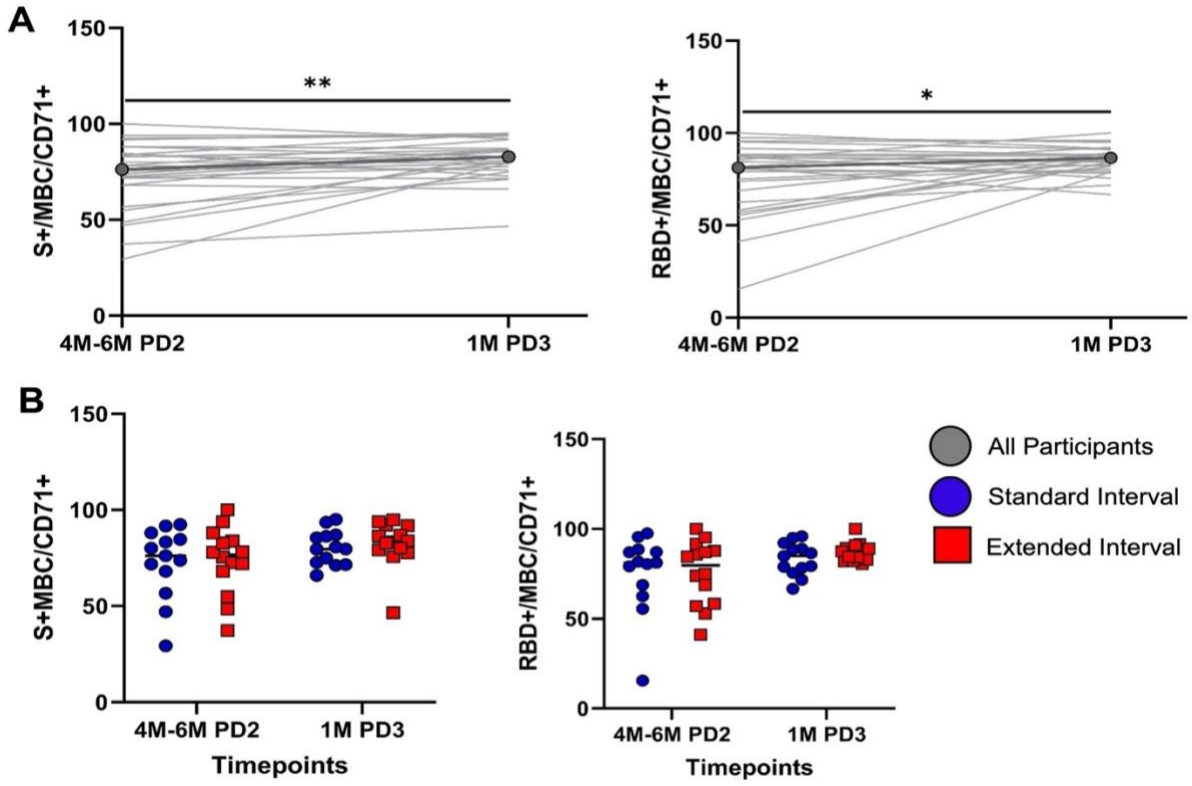


Figure 3.4-5: Assessment of CD71+ B Cells as a Percentage of S-specific and RBD-Specific Memory B cells After a Third Dose of the SARS-CoV-2 Vaccine. (A) Trajectory of CD71+ B cells as a percentage of S-specific and RBD-specific memory B cells from 4-6 months post-dose 2 and 1-month post-dose 3. Time points were compared using the Wilcoxon matched-pairs signed rank test. Bold line show median values. **(B)** Comparison of CD71+ B cells as a percentage of S-sepcific and RBD-specific memory B cells between the standard and extended dose interval groups after a third dose. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

standard dose interval groups after the 3rd dose (Figure 3.4-5B). There are no differences in the proportions of S-specific or RBD-specific memory B cells that are CD71+ between males and females who have had either the standard or extended dose intervals after the third dose of the SARS-CoV-2 vaccine (Supp. Figure 5.2-8).

3.4.3. Assessment of Naïve B Cell Phenotypes Among SARS-CoV-2-Specific Memory B Cells After Dose 3

The proportions of S-specific and RBD-specific naïve B cells were measured from baseline to 1-month post-dose 3 as well. There was a significant decrease in the proportions of S-specific and RBD-specific B cells that are naïve after the 3rd dose of the vaccine (Figure 3.4-6A.). Similar to post-dose 2, there was no significant difference in the proportions of S-specific and RBD-specific B cells that are naïve between the standard and extended dose interval post-dose 3 (Figure 3.4-6B). Similarly, there are no differences in the proportions of S-specific or

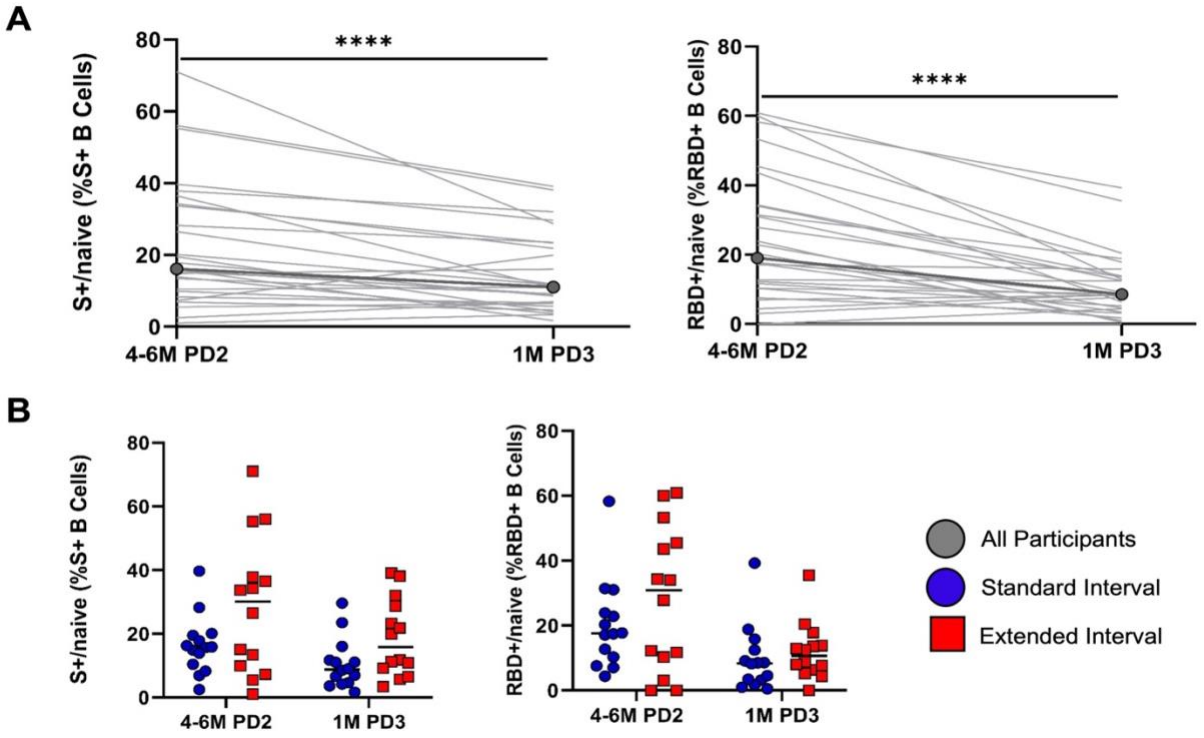


Figure 3.4 6: Assessment of Naïve B Cells as a Percentage of S-specific and RBD-Specific B cells After a Third Dose of the SARS-CoV-2 Vaccine. (A) Trajectory of naïve B cells as a percentage of S-specific and RBD-specific B cells from 4-6 months post-dose 2 and 1-month post-dose 3. Time points were compared using the Wilcoxon matched-pairs signed rank test. Bold line show median values. **(B)** Comparison of naïve B cells as a percentage of S-sepcific and RBD-specific B cells between the standard and extended dose interval groups after a third dose. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values.

RBD-specific B cells that are naïve B cells between males and females who have had either the standard or extended dose intervals after the third dose of the SARS-CoV-2 vaccine (Supp. Figure 5.2-9).

3.4.4. Impact of Dose Interval on Neutralizing Antibodies Produced by Memory B Cells After Dose 3

Longitudinal analysis was performed on supernatant antibodies from the standard and extended dose interval groups. A significant increase in the neutralization of Wild-Type, Alpha, Beta and Delta variants was detected between 4-6 months post-dose 2 and 1 month post-dose 3, regardless of dose interval (Figure 3.4-7A) Participants who had received an extended dose interval were found to have comparable levels of inhibition of the wild-type SARS-CoV-2 RBD and the variants to participants who had received a standard dose interval at 4-6 months post-

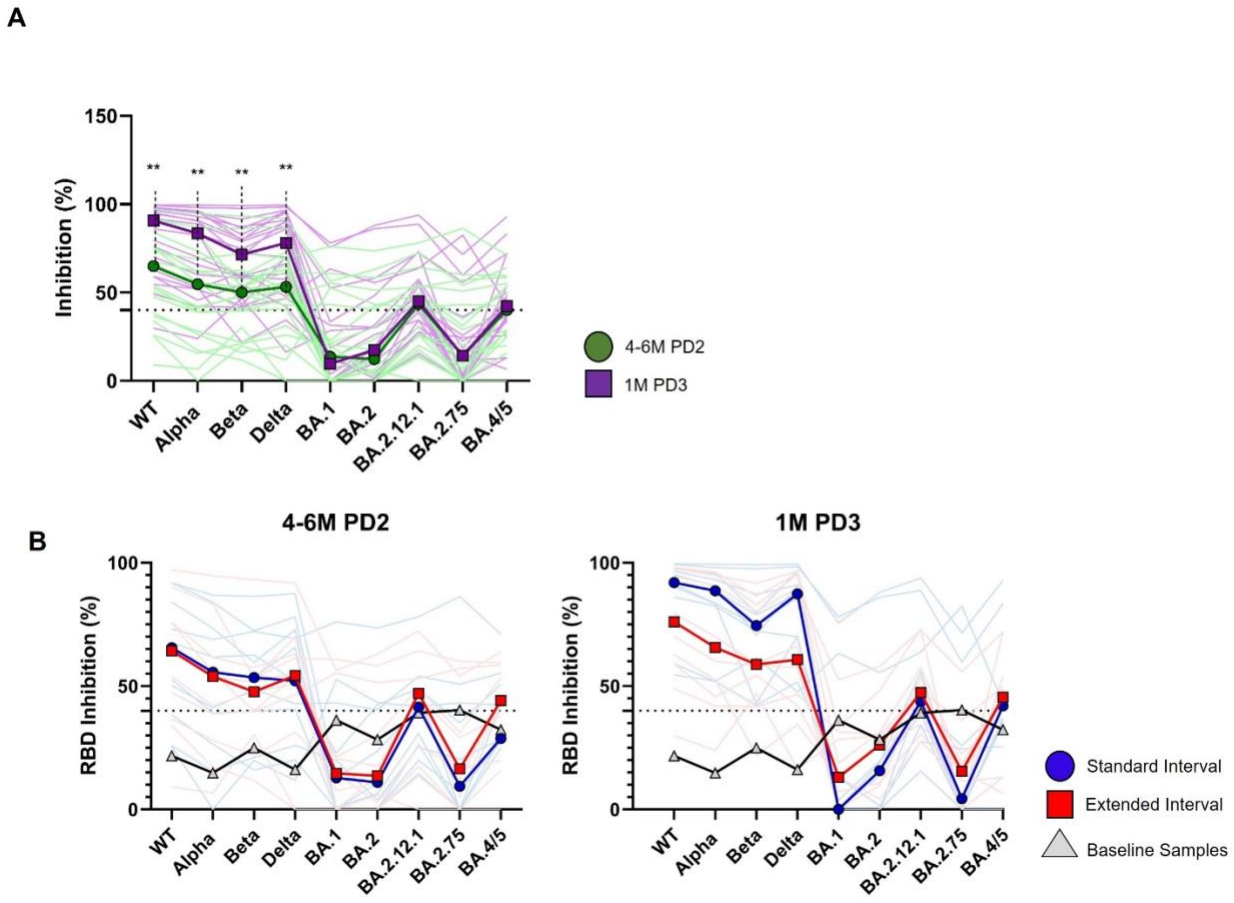


Fig. 3.4-7: Comparisons of Stimulated B Cell Antibody Neutralization against SARS-CoV-2 RBD and Variants After a Third Dose of the SARS-CoV-2 Vaccine. (A) Comparisons of plasma cell-derived antibody neutralization against SARS-CoV-2 RBD variants between 4-6 months post-dose 2 and 1 month post-dose 3. Groups were compared using the Wilcoxin matched-pairs signed rank tests with multiple comparisons. Dotted line indicates cut-off. **(B)** Comparisons of plasma cell-derived antibody neutralization against SARS-CoV-2 RBD variants between standard and extended dose interval participants at 4-6 months post-dose 2 and 1-month post-dose 3. Groups were compared using the Mann-Whitney tests with Holm-Šídák corrections. Lines show median values. Dotted line indicates cut-off.

RBD and the variants to participants who had received a standard dose interval at 1-month post-dose 3 (Figure 3.4-7B).

DISCUSSION

4.1. SUMMARY

The second dose of the mRNA COVID-19 vaccine resulted in an increase in the proportion of S-specific and RBD-specific B cells, as well as S-specific and RBD-specific B cells that were IgG+ or CD71+ memory B cells. This increase in IgG+ memory B cells coincided with a significant decrease in the proportion of S-specific and RBD-specific naïve B cells and resting mature B cells (IgD/IgM), irrespective of dose interval. Furthermore, a delayed dose interval had an impact on SARS-CoV-2 specific memory B cell populations, but only at 10-14 days post-dose 2. A positive association between the length of the first and second dose interval and the proportions S-specific and RBD-specific B cells, S-specific and RBD-specific B cells that are IgG+ memory B cells and S-specific and RBD-specific memory B cells that are CD71+ was detected. The length of the first and second dose interval also had a negative association with S-specific and RBD-specific B cells that are IgM+/IgD+ memory B cells. There were no significant differences detected between male and female extended dose participants after the 2nd dose of the vaccine.

Memory B cells were stimulated with IL-2 and R848 to differentiate into plasma cells, and the resulting antibodies were harvested and analyzed for inhibition of ACE2 binding to RBD variants. Participants who received a delayed dose interval produced antibodies with significantly greater magnitude and breadth of SARS-CoV-2 RBD inhibition at 10–14 days post-dose 2, compared to those with a standard interval. These antibodies more effectively blocked ACE2 binding to Wild-type, Alpha, Beta, and Delta variants. Neutralization capacity was significantly correlated with RBD-specific IgG titres in plasma at 10–14 days post-dose 2. Additionally, higher proportions of S- and RBD-specific B cells, IgG+ and CD71+ memory B cells, and IgD+/IgM+ memory B cells were all positively associated with antibody titres and neutralization potential at 10-14 days and 4-6 months post-dose 2. Notably, neither dosing regimen elicited neutralization of Omicron sublineages after the second dose

A 3rd dose of the SARS-CoV-2 vaccine results in significant increases of S-specific and RBD-specific B cells, regardless of dose interval. At 4-6 months post-dose 2, the proportions of mature S-specific and RBD-specific B cells in extended and standard-dose interval participants are similar. Additionally, the proportions of S-specific and RBD-specific B cells that are IgG+, IgM+/IgD+ and CD71+ memory B cells in both extended and standard dose interval participants

are similar. The administration of a 3rd dose significantly increased the proportion of S-specific and RBD-specific B cells that are IgG+ and CD71+ memory B cells, which was accompanied by a decline in S-specific and RBD-specific B cells that are IgD+/IgM+ memory B cells in both standard and extended dose interval participants, with no significant differences between the two vaccine dose interval groups. After the 3rd dose, both standard and extended dose interval participants have activated B cells that produce antibodies with similar breadths of inhibition for SARS-CoV-2 RBD. An extended dose interval of the SARS-CoV-2 vaccine did not result in significant differences in the proportions of S-specific and RBD-specific B cells and their subtypes in males or females after the 3rd dose.

4.2. Mechanisms Underlying the Impact of Delayed Dose Intervals on Immunogencity

4.2.1. Antibody Class Switching and B Cell Maturation Processes Benefit from a Delayed Dose Interval

Participants who received an extended dose interval of the SARS-CoV-2 vaccine had greater proportions of S-specific and RBD-specific B cells, as well as IgG+ and CD71+ memory B cells after the second dose than participants who received a standard dose interval of the vaccine. Conversely, those who received a standard dose interval of the SARS-CoV-2 vaccine had more S-specific and RBD-specific IgD+/IgM+ and IgD+ only memory B cells after the second dose than participants who received an extended dose interval of the vaccine. IgG+ memory B cells are class-switched B cells that have undergone SHM[64]. IgD+/IgM+ antibodies are most commonly found on naïve B cells[64]. Memory B cells that present IgD+/IgM+ on their cell surface are mature B cells that have yet to finish CSR, will not produce high-affinity IgG antibodies and once activated, can re-enter the GC to continue SHM[64]. These data are consistent with prior research articles showed that a 16-week dose interval of a SARS-CoV-2 vaccine resulted in a higher magnitude and a more mature phenotype of RBD-specific B cells (IgG+ memory B cells) than the 3-week dose interval[314]. Additional research has shown the progression of the B cell response following vaccination, by which antigen-specific B cells at early time points following vaccination are primarily IgM memory B cells, IgD/IgM memory B cells or plasmablasts and S-specific IgG+ memory B cells are predominant months after vaccination[320]. CSR has been shown to occur 1.5 days after vaccination and can last up to a week before the B

cells enter the GC to undergo SHM[44]. A standard dose interval may cause the second dose to act as a prolonged primary response rather than a separate secondary immune response, resulting in a CSR process that has not had time to fully take place in many B cells, leaving short-lived plasmablasts and IgD+/IgM+ memory B cells as the dominate circulating B cell phenotypes after the second dose. By contrast, an extended dose interval may allow for a longer period of CSR and allow for a greater number of B cells to enter the GC, thus resulting in a greater proportion of IgG+ memory B cells rather than IgD+/IgM+ memory B cells once the second dose is administered. The proportions of plasmablasts and plasma cells in standard and extended dose interval participants were also measured following a second dose. However, no significant differences were detected at 10-14 days post-dose 2, and the proportions of plasma cells were extremely low following the third dose.

The extended dose interval additionally resulted in a greater proportion of S-specific and RBD-specific memory B cells that are CD71+ compared to the standard dose interval at 10-14 days post-dose 2. CD71 is a well-established marker of metabolic activity and cellular proliferation, and the expression of CD71 on memory B cells has been associated with recent activation[317,318]. A distinct population of B cells, identified as pro-germinal center B cells, express CD71 and also exhibit early expression of AID, the enzyme essential for initiating SMH and CSR[321]. The presence of CD71 on memory B cells may reflect recent engagement with GCs, as well as recent proliferation and amplification within the GC. In the context of an extended dosing interval, the increased proportion of SARS-CoV-2 specific memory B cells with CD71 present may indicate additional rounds of selection and mutation are occurring, or that there is increased GC activity after an extended dose compared to a standard dose. This would lead to a greater number of immunoglobulin gene mutations, leading to enhanced antibody affinity as well as a greater proportion of mature memory B cells after vaccination. To our knowledge, this is the first study that analyzed CD71+ memory B cells after an extended or standard dose interval of the SARS-CoV-2 vaccine. In addition, double-negative B cells were included in the analysis due to the association between severe SARS-CoV-2 infection and inflammatory immune activation[322]. No significant differences in the proportions of double-negative B cells were detected between extended and standard dose interval participants at any timepoint following vaccination.

4.2.2. Prolonged Dose Interval Results in Greater Neutralization of SARS-CoV-2 RBD

To measure the neutralization capacity of antibodies encoded by memory B cells at various time points, we stimulated B cells with IL-2 and R848 and collected the antibodies produced by the activated plasma cells. An extended dose interval of the SARS-CoV-2 vaccine resulted in antibodies produced by B cells that had a greater breadth of inhibition against SARS-CoV-2 WT, Alpha, Beta and Delta variants compared to a standard dose interval. Previous research from the COVID-19 Vaccine Project showed that an extended dose interval resulted in a greater neutralization of SARS-CoV-2 variants from circulating plasma antibodies[311]. As previously mentioned, the interruption of CSR and SHM due to the administration of a second dose in those with a standard dose interval may result in an increase in the proportion of IgD+/IgM+ memory B cells or short-lived plasmablasts that matured through GC-independent means. This is consistent with the present data suggesting that the antibodies produced by these B cells have a limited breadth of inhibition of the antigen compared to B cells that could enter the GC and undergo SHM. GCs have been shown to form nearly 4-8 days after the initial antigen encounter, while GC B cells have been detected in humans as long as 6 months after SARS-CoV-2 vaccination[55,323]. While some B cells may have enough time to undergo CSR and enter the GC, receiving a booster 3-4 weeks after the primary dose will result in the early exit of GC B cells due to B cell activation and antigen encounter.

Higher titres of plasma-derived RBD-specific IgG antibodies positively correlated with increased neutralization of the original strain of SARS-CoV-2 by antibodies from memory B cells at 10-14 days post-dose 2, although it should be noted that this improved functionality had been detected even when absolute plasma antibody titres are not different between groups, which may suggest differences in affinity and avidity beyond differences in antibody titres[311,324]. In the stimulation assays from this study, it was observed that B cells from participants with an extended dose interval produced antibodies with a greater breadth of inhibition for SARS-CoV-2 variants, despite the previous work from the COVID-19 Vaccine Project finding comparable circulating antibody titres shortly after the second dose[311]. In addition, the dose interval did not impact the neutralization of the Omicron subvariants by B cell derived antibodies, and Omicron neutralization was consistently lower than the other variants. Thus, the dose interval does not provide additional protection against variants with a substantially different S protein structure

compared to the wild-type SARS-CoV-2. At the time of sample collection, Omicron had not yet emerged as a VOC, and the vaccines used at the onset of the pandemic were not designed to elicit an immune response against Omicron, which could explain the lack of neutralization. However, other studies have found that an extended dose interval was associated with improved antibody neutralization of Omicron[325–327]. This outcome may be explained by a reduced sensitivity of the assay using cell supernatants.

Recent models have attempted to elucidate the mechanisms by which antibodies produced during longer vaccine dose intervals have improved neutralization of antigens[328]. Antigen availability within GCs is a key factor that influences affinity maturation. The B cells in the LZ of the GC compete for antigens presented on FDCs. If the antigen is scarce, there will be increased selection stringency on B cells with high affinity for the antigen. A longer dose interval would allow for affinity maturation to proceed further, as well as allow for more antigen decay between doses. In contrast, a short dose interval would certainly interrupt affinity maturation but would also provide more antigen for the GC response just 3-4 weeks after the initial dose, relaxing the selection stringency against the B cells and allowing for the production of B cells with lower binding ability of the target antigen. The prolonged GC response to SARS-CoV-2 vaccination suggests that antigens presented by the vaccines may be long-lasting, and as a result, a longer dose interval allows for both prolonged affinity maturation processes and antigen decay in the GCs[328–330]. This outcome may also occur in vaccines with lower doses of antigen and may provide an even greater benefit to antibody neutralization when combined with a longer dose interval between boosters. A dose interval that is too long or too low of a dosage may result in GC collapse, so an optimum dose interval should be determined before implementing new vaccine schedules[328]. Antibodies have also been shown to bind to the antigen presented in the GC, which would further enhance the selection stringency against maturing B cells and the affinity of antibodies against the antigen[328]. This explains how an extended dose interval results in greater neutralization of the vaccine version of the antigen but does not explain the improved breadth of neutralization of SARS-CoV-2 variants. This outcome may reflect conserved epitopes that shape the memory response after Wild-type S protein vaccination are recognizable on the original strain of SARS-CoV-2 and closely related variants but are not as conserved on genetically distinct variants such as Omicron. Additional research found that extending the SARS-CoV-2 vaccine dose interval after natural infection also resulted in increased breadth of

inhibition against variants[326]. Extended GC responses have been shown to improve cross-reactivity of antibodies, and antibodies derived from memory B cells generated by infection from the original strain of SARS-CoV-2 have shown increased neutralization of variants when compared to plasma antibodies produced by long-lived plasma cells[329,331].

4.2.3. Extended Dose Interval Benefits are not Maintained and are Limited to the Second Dose

An extended dose interval of the SARS-CoV-2 vaccine results in a greater humoral immune response against SARS-CoV-2 compared to a standard dose interval. However, it is important to note that the magnitude of inhibition of antibodies produced by activated B cells against SARS-CoV-2 and variants seen in extended dose interval participants decreases slightly after 4-6 months and is not consistently maintained. By 4-6 months post-dose 2, the differences between groups in B cell phenotypes seen after the 2nd dose are no longer present, as the frequency of SARS-CoV-2-specific B cells in standard dose interval participants increased to levels comparable to those in the extended interval group. This convergence does not imply a loss of memory B cells or immune protection, as SARS-CoV-2-specific B cell populations are still readily maintained in both groups months after the second dose. While B cell populations are readily maintained between the second and third dose of the vaccine, the slight decline in the ability of plasma cell neutralizing antibodies in the extended interval group to bind RBD after 4-6 months is unexpected. While GC reactions have been shown to continue months after immunization, early GCs have a high output of memory B cells and plasma cells within the first month of vaccination, while late GCs are thought to be responsible for the maintenance of a flexible, hypermutated B cell repertoire in case of infection rather than increasing antibody levels[332]. Neutralizing antibody responses to seasonal human coronaviruses, as well as to SARS-CoV-1 and MERS-CoV, have also been shown to be short-lived[333]. Recent research has hypothesized that vaccination against SARS-CoV-2 does not result in long-lived plasma cells being present in the bone marrow, which are generally required for the secretion of neutralizing antibodies for decades after activation[334]. Additional research has also found that prolonged GC reactions after the 2nd and 3rd dose result in a substantial increase in the proportion of IgG_4+ memory B cells and IgG_4 antibodies, which are largely non-neutralizing when compared to IgG_1 and IgG_3[335–337]. It should also be noted that there are differences between immunization brought

about by vaccination or by natural infection, which could explain any potential differences in immune responses detected[338]. Another possibility is that participants in the extended interval group exhibited an early burst of antibody production due to the extended dose, characterized by a greater proportion of CD71+ and IgG+ SARS-CoV-2-specific B cells. It is possible that the extended interval group had a sudden and transient increase in neutralization that later stabilized months after the second dose.

4.2.4. A Third Dose Increases B Cell Proportions and Neutralization Independent of Dose Interval

A third dose of the vaccine resulted in a significant increase in both SARS-CoV-2-specific B cell phenotypes and an increase in neutralization by plasma cell antibodies, irrespective of the dose interval, highlighting the impact and importance of boosters. This indicates that while the extended dose interval was associated with differences in the humoral immune response at time points early after the second dose, these group-specific differences were no longer apparent following the third dose. Unlike the first and second dose of the SARS-CoV-2 vaccine, sufficient time had passed between the second and third dose to produce mature SARS-CoV-2 specific B cells in both groups of participants. These results are consistent with previous research showing the impact of a third dose on increased antibody titres and a wider breadth of neutralization of SARS-CoV-2 variants, regardless of the dose interval[311,339]. Other research articles show enhanced B cell responses after a third dose of the vaccine, although dose interval is not considered[340,341]. While there are no significant differences between interval groups at 1-month post-dose 3, the standard interval participants often showed higher median responses across several measures when compared to the extended interval participants, including antibody binding and IgG+ memory B cell proportions. This may reflect the longer effective interval between the second and third doses in the standard group, since those participants received their primary series closer together and consequently had a longer resting period before boosting. Additional research has shown that a fourth dose results in a comparable neutralizing antibody response to the Omicron variant, which may indicate that additional boosting will restore the humoral immune response but not lead to any differences from the third dose[342]. To our knowledge, this is the first study that analyzes B cell responses and proportions to a third dose of the SARS-CoV-2 mRNA vaccine while comparing a standard or extended dose interval schedule.

4.3. Strengths and Limitations

4.3.1. Participant Recruitment

One of the strengths of this study is that samples had been collected before vaccination had occurred, after each subsequent dose, and during a time when most participants had not yet encountered the virus. While previous research has examined the effects of a different dose interval on immune responses, plenty of research contends with the issue of previous infection, which may inadvertently misconstrue the actual outcomes of vaccination in the overall results. Additionally, long-term studies evaluating the impact of a different dosing schedule of a vaccine on the immune system are also in short supply, as most studies look cross-sectionally after the second dose. The benefits of this study come from the longitudinal collection of samples, which enabled a broad assessment of the immune response over time. The novelty of SARS-CoV-2 at the time also means that the impact on the immune system is not from any previous SARS-CoV-2 infection. A positive SARS-CoV-2 test from a participant would result in exclusion from the analysis further ensuring that the effects of the extended dose interval of a SARS-CoV-2 were analyzed independently from SARS-CoV-2 infection. Additionally, longitudinal monitoring of nucleocapsid-specific antibody responses was performed on the participants to exclude people who had experienced previous infection throughout the study.

4.3.2. B Cell Phenotypes

Another benefit of this study is the wide range of phenotypes that are being analyzed during flow cytometry. The B cell population in the immune system is comprised of numerous subpopulations, all with diverse roles in ensuring the immune response is rapid and effective. Previous research into the impact of a delayed dose interval on the B cell population was limited. The current research available that analyzed the impact of a delayed dose interval of a SARS-CoV-2 vaccine on B cells focused primarily on RBD-specific B cells only and avoided further investigation into memory B cells and their subpopulations. Thus, the overall impact on B cells is potentially overlooked. This research analyzes SARS-CoV-2 specific B cells, both S-specific and RBD-specific. In addition, B cells that have undergone maturation or that are still naïve, plasma cells and memory B cells, as well as IgG/IgD/IgM and CD71+ expressing B cells.

4.3.3. Sample Size and Post-Dose 4 Samples

While the participant recruitment and timeline were touted as strengths of the study, it should be noted that the participants also had limitations. Fifty participants had a short-dose interval, and 36 participants had a long-dose interval, but only 14 from each group were selected for B cell phenotyping and antibody neutralization assays. This was because while there were plenty of participants to choose from, not all of the participants gave samples at the time points required for this study. Additionally, 2 of the participants were excluded due to an autoimmune disorder. The participants skewed female over male. While care was taken to avoid oversampling female participants for this project, it could not be avoided for the extended dose interval cohort and resulted in small sample sizes when analyzing the data by sex.

Lastly, it should be noted that while samples were present post-dose 4 and beyond, analysis of repeated boosting was outside of the scope of this project. Furthermore, issues with choosing samples from those time points are similar to the previous issue of lack of coverage. Not all participants gave samples for time points post-dose 4 and beyond. It was also at this time that infections from SARS-CoV-2 became more prominent in the participants. Therefore, even if a sample for a time point exists, a previous infection would make it impossible to analyze vaccine-only responses. In addition, before dose 3, strict schedules were set up as to when people could get vaccinated. After dose 3, those restrictions began to change, and participants could get vaccinated at different times, which means the dose intervals began to vary widely between participants. Therefore, analyzing samples from after a 4^{th} dose, while beneficial for understanding how repeated boosting impact B cells, was not something considered for this project.

An argument could be made that the differences between the standard and extended dose interval groups at post-dose 2 are partly due to the total elapsed time from post-dose 1 rather than due to the interval. Due to the lack of samples from certain timepoints, analysis between groups based on samples that are taken at the same timepoint post-dose 1 is impossible. While studies looking at samples rooted after the first dose are limited, certain studies analyzed samples based on time following the first dose and found that an extended dose interval resulted in a greater proportion of RBD-specific B cells and enhanced antibody responses[314,343]. Therefore, the results from this study could still be explained by the difference in interval rather than the time the sample was collected.

4.3.4. Issues with Neutralization Assay

The Meso Scale Discovery SARS-CoV-2 RBD inhibition assay was beneficial for numerous reasons. A plate with SARS-CoV-2 RBD variants already in place negates the need to culture SARS-CoV-2 for traditional neutralization assays. The neutralization assays may then avoid the time and cost of culturing viral particles while allowing work to occur outside Biosafety Level 3 labs. The plates provided ten possible SARS-CoV-2 variants in each well, whereas ELISPOT assays would have only one SARS-CoV-2 variant per plate. Plaque reduction neutralization tests (PRNT) are the gold standard for the detection of neutralizing antibodies but require live pathogens and access to biosafety level 3 laboratories, while also being time-consuming and expensive[344]. The Meso Scale Discovery SARS-CoV-2 RBD inhibition assay allows the researcher to rapidly analyze the neutralization of multiple variants simultaneously without the need for multiple plates or live pathogens. For these reasons, the Meso Scale Discovery neutralization assays were an obvious choice for this project. However, a drawback of this assay is that it is designed for analysis of plasma samples and is not validated for cell culture supernatants. In contrast to the good performance observed with plasma samples, we observed high Coefficiant of Variation (CV) values between replicates and high background inhibition from negative control baseline samples, indicating that changes would have to be made to the assay to optimize the detection of supernatant antibodies.

Specifically, the amount of IL-2 and R848 did not impact the stimulation process, as different levels of IL-2 in the cytokine solution still resulted in antibodies being detected in ELISA assays. The Blocker A solution, according to our laboratory protocol, can be left on plates for at least 30 minutes. However, it was determined that leaving Blocker A on the plates for too long would impact the final results of supernatant antibodies by causing extremely high CVs and neutralization. Plasma samples on the same plate would not have this issue. Leaving Blocker A on plates for exactly 30 minutes rectified this issue, and the CVs would be much lower after assay completion (1-30%). This difference between plasma antibodies and supernatant antibodies might be because the titre of antibodies in plasma samples was significantly higher than that in supernatant samples. While plasma samples were diluted 1:50 to compensate for that fact, it may still play a factor in why Blocker A caused an issue with supernatant samples but not with plasma samples. Additionally, the dilution of plasma may reduce total protein content, whereas supernatant samples contain undiluted culture media with

cytokines, serum and other components, potentially contributing to interference during the assay. Secondly, the original method for stimulating B cells was to thaw them and leave them in an incubator overnight, and then, once the B cell phenotyping was complete, the B cells set aside for stimulation would be treated with the cytokine solution. This meant that the B cells would be in an incubator at 37°C for approximately for nearly 24 hours. This was not seen as an issue by our previous lab student, as she had been working with a partner when testing the new protocol, who would do the B cell phenotyping assay while she stimulated, which meant that the cells were not in an incubator for 24 hours. Changing the protocol to complete the first day of the stimulation protocol on the same day as thawing the cells improved results dramatically. It should be noted that despite optimization, the supernatant samples still have a lower magnitude of inhibition when compared to plasma antibody samples. Post-dose 3 plasma samples show inhibition of Omicron and near 100% inhibition of early variants while antibodies stimulated from cell cultures do not. Further optimization to improve sensitivity will be necessary.

4.4. Implications for Future Vaccine Schedules

4.4.1. Clinical Outcomes of Extended Dose Intervals

The results from this study and previous work provide a greater understanding of how a delayed dose interval of the SARS-CoV-2 vaccine impacts the immune system, most notably memory B cell maturation. The research discussed in previous sections has also shown that delaying the dose interval is thought to help enhance the humoral immune response to SARS-CoV-2. However, this data is primarily from the first few months after the initial outbreak of SARS-CoV-2. Additionally, *in vitro* studies that solely analyze B cells fail to consider the impact of an extended dose interval of the SARS-CoV-2 vaccination on the entirety of the immune system and the overall outcomes from infection. More research has looked at the clinical outcomes of a delayed dose-interval on a population level, to see if the enhanced immune response translated to a reduction in infections, hospitalizations, and mortality like the initial models suggested.

During the last 5 years of the SARS-CoV-2 pandemic, researchers have analyzed cohorts of people who received a standard or extended dose interval to determine whether the delayed dose interval was associated with any potential benefits. A study in Hong Kong analyzed the outcomes of 417497 BNT162b2 and 354283 CoranaVac second dose recipients[345]. Among those

participants, 3.8% of the BNT162b2 and 28.5% of the CoronaVac recipients received a delayed dose interval. Delaying the dose interval of the second dose was not significantly associated with all-cause mortality, a risk of emergency department visits or a risk of an unscheduled hospitalization. Researchers from the UK used data on vaccine doses, COVID-19 hospital admissions, deaths, and positive and negative PCR test results provided by the National Health Services (NHS) to compare the extended dose interval chosen by the UK to a mathematical model which simulated the outcome if the UK had decided to continue with the recommended dose interval of 3-4 weeks[346]. Rapidly providing first doses to a larger proportion of the population and delaying the dose interval successfully reduced COVID-19 hospitalizations and deaths. The extended dose interval was estimated to have averted 58000 COVID-19 hospital admissions and 10100 deaths from COVID-19. At the same time, the 3-4 week strategy would have resulted in more infections compared to the extended dose interval strategy. In British Columbia and Quebec, the vaccine effectiveness of two doses was assessed using PCR-confirmed SARS-CoV-2 infection data from provincial databases[347]. Vaccine effectiveness was significantly higher against both infection and hospitalization with the extended dose interval compared to the standard dose interval. In British Columbia, the standard dose interval had a vaccine effectiveness of 85% against infection while the delayed dose interval had a vaccine effectiveness of 91% against infection. In Quebec, the standard dose interval had a vaccine effectiveness of 79% against infection, while the delayed dose interval had a vaccine effectiveness of 89% against infection. A similar outcome was seen in COVID-19 hospitalizations in both provinces. In the US, the effectiveness of the standard dose interval, a slightly delayed dose interval and an extended dose interval were compared among >6 million mRNA vaccine recipients in Georgia[348]. The results found that the slightly delayed dose interval (26-49 days) provided the lowest risk compared to the other schedules, and that delaying the second dose by approximately 1-2 weeks from the initial recommended dose interval would help to reduce the risk of SARS-CoV-2 infection in the longer term.

There has been relatively little data that has looked at the clinical outcomes of delaying a dose interval. Published data on *in vivo*, population-wide outcomes appear to match what *in vitro* studies have shown, that a delayed dose interval provides a stronger humoral immune response. The immunological results from this study help explain these early benefits by demonstrating that an extended dose interval leads to greater SARS-CoV-2-specific IgG+ memory B cell

presence in the blood shortly after the second dose, which in turn results in the development of B cells that produce antibodies with greater breadth of inhibition against the original strain of SARS-CoV-2 and variants. This outcome positively correlates with greater titres of SARS-CoV-2-specific Ig plasma antibodies at 10-14 days post-dose 2 and 4-6 months post-dose 2, which further explains the clinical outcomes detected. Although the available research indicates that delaying the dose interval may lead to positive outcomes against SARS-CoV-2 infection, hospitalizations and mortality, more work is always needed to properly measure the *in vivo* outcomes in an extended dose interval vaccine program for future outbreaks.

4.4.2. Impact of Delayed Dose Strategy for Non-SARS-CoV-2 Vaccines

With the knowledge that extended dose intervals impact the neutralization capacities of antibodies and the cells that produce them and that extending the dose interval reduces mortality and infections, the next question would be whether these results apply to only mRNA vaccines or if this outcome is seen in other types of vaccines that target infections other than SARS-CoV-2. Fortunately, research already exists that assesses optimal vaccine dose schedules for many vaccines, although more work is needed.

Research into human papillomavirus (HPV) vaccines and dose schedules has shown that antibody responses were stronger with an interval of 6-12 months between the first two doses compared to an interval of 2-6 months in 9- to 14-year-old females and males[349]. A 6-month dose interval of an HPV Cervarix vaccine demonstrated higher titres of antibodies in participants compared to a 2-month dose interval after a 24-month follow-up[350,351]. Serological assays performed 1-month post-dose 2 have also shown that a 6-month interval resulted in a 40-91-fold increase in antibody titre and a 3–8-year interval resulted in a 60-82-fold increase in antibody titre[352,353]. There is similar evidence for hepatitis A virus vaccines, as in adolescents and adults who received a delayed dose interval by 20-77 months, antibody responses were non-inferior to the standard dose interval of 6-12 months[354,355]. These results provided evidence for a persistent and often enhanced immune response beyond the recommended vaccine schedule.

The evidence that currently exists for varicella zoster virus (VZV) vaccines only examines antibody responses and shows that longer dose intervals provide a non-inferior response to a certain point. A 6-month dose interval using a subunit VZV vaccine provides a

non-inferior antibody response compared to a 2-month dose interval, but the 12-month dose interval was inferior after 4 weeks post-vaccination[356]. Inactivated polio vaccines have also been investigated for the optimal dose interval. Non-randomized trials with six-week-old infants in Panama and in the Dominican Republic found that antibody titres against poliovirus virus types 1, 2 and 3 were higher 4 weeks after the second dose when the dose interval was 22 weeks instead of 4 weeks[357].

Current research into extended dose intervals for different vaccines has shown either a non-inferior or a beneficial immune response to an extended dose interval. While the research only analyzes antibody titres at singular time points after the second dose, it nonetheless provides similar results to the outcomes seen in extended the SARS-CoV-2 vaccines. This could potentially signify that the different vaccine types are impacting the CSR and SHM processes of B cell maturation in a similar manner to the SARS-CoV-2 vaccines, although more work would be needed to confirm this hypothesis. Additionally, the research on delayed dose intervals has assessed vastly different intervals, ranging from months to even years, and have all seen similar results. An optimal dose interval might be different for certain vaccine types, pathogens or even demographics.

4.4.3. Revisiting Global Scheduling Policies

As more information about the effects of an extended SARS-CoV-2 dose interval emerges, along with known results from other vaccines and their delayed dose intervals, it will also be necessary to evaluate the current strategies implemented by different vaccine programs. As previously mentioned, extended dose intervals for vaccine schedules can provide benefits, not just to a single person, but to an entire population struggling with vaccine shortages. Both current SARS-CoV-2 and non-SARS-CoV-2 vaccine programs should be examined to determine if they are utilizing an extended dose interval. Previous sections outlined the initial vaccine schedule of the SARS-CoV-2 vaccine implemented in Canada, which was 3-4 weeks between dose intervals. Currently, the NACI recommends a one-dose schedule of the SARS-CoV-2 vaccine per year for most individuals who are previously vaccinated, although a 3- or 6-month interval may be used in certain situations[358]. Vaccine programs in various provinces, such as Ontario and Quebec are currently implementing these recommendations[359,360]. The CDC has also implemented changes to their recommendations as of October 31, 2024, where people aged 65 years and older or

immunocompromised individuals are recommended to receive 2 doses of a SARS-CoV-2 vaccine 2-6 months apart[361]. The SARS-CoV-2 vaccines are not the only vaccines that have received updates to their recommended vaccine schedules. For example, the Advisory Committee on Immunization Practices (ACIP) recommended on October 24, 2024 to extend the dose interval of a meningococcal serogroup B vaccine to 6 months between doses for adolescents and young adults based on improved immunogenicity[362]. While changes to vaccination schedules to enhance immunity are beneficial, further research could be done to determine whether current global vaccination strategies could also be improved. The updated WHO and CDC guidelines outline the immunization schedules required for specific vaccines and provide information on the recommended time intervals between doses for effective immunization[363]. For example, both the WHO and the CDC recommend a 4-week dose interval between the 1st and 2nd dose and a 4–8-week dose interval between the 2nd and 3rd dose of the Hepatitis-B vaccine. While research has found that extending the 1st and 2nd dose interval to greater than 8 weeks resulted in no difference in risk of infection from Hepatitis B, they acknowledge that research into extended dose intervals is scarce, as older research had found that delaying the 2nd and 3rd dose interval to 10 months provided a greater titre of antibodies against Hepatitis B[364,365]. There is concern that longer intervals between the last two doses, while resulting in higher antibody titres against Hepatitis B, could result in an increase in the acquisition of Hepatitis B infection[364]. While a re-examination of global vaccination policies is always beneficial, it is important to note that weighing the benefits of a longer dose interval with the urgency of vaccination is also essential. Emergency situations, such as a global pandemic or the outbreak of a virulent pathogen, may result in the prioritization of a shorter dose interval to provide essential initial protection against infection rather than delaying the dose interval of the vaccine. Results from this analysis showed that participants with a standard dose interval (3-4 weeks) eventually ended up with a humoral immune response similar to participants with an extended dose interval. The balance between providing the optimal humoral immune response and ensuring people receive immediate protection during an emergency must be considered.

4.5. Future Research Directions

4.5.1. RNA Sequencing

This study managed to detect the ability of antibodies to recognize and neutralize the RBD portion of SARS-CoV-2, which can inadvertently measure the SHM process and maturation of B cells after vaccination. However, it is not a direct measurement of SHM, as SHM acts on the immunoglobulin genes that encode the B cell receptor and secreted antibodies. This research also fails to capture which pathways and proteins are expressed and amplified at the various time points following vaccination, which would also provide greater information on how a delayed dose interval impacts these processes and influences the expression of certain genes.

Future research could answer these questions using bulk B cell or single-cell RNA sequencing (scRNA-seq). RNA sequencing is a genomic technique that can detect and quantify the transcriptome, or mRNA molecules, of a biological sample[366]. This method, which would include bulk B cell RNA sequencing, generally relies on the analysis of millions of cells, which does not allow for a detailed assessment of the transcripts of individual B cells or B cell subsets[366]. Flow cytometry permits the separation and isolation of B cell subpopulations, such as S-specific B cells. Then, scRNA-seq permits the comparison of the transcripts of these individual antigen-specific cells, which has been useful in assessing the transcriptional similarities and differences within a population of cells, as well as rare populations of cells that may have gone undetected in analyses with a larger population[366,367]. In addition to transcriptomic analysis, scRNA-seq can also allow for BCR sequencing from individual B cells, which can track clonal expansion, SMH, and CSR[368,369]. Therefore, scRNA-seq would be an ideal technique to analyze the immunoglobulin genes during affinity maturation and other potential genes expressed during the process. This method has already been used to analyze the expression of B cell receptor sequences, their evolution over time, and the expressed antibody class of an individual cell over time[370]. Additional research has managed to use scRNA-seq to measure the single-nucleotide differences between individual primary follicular lymphoma cells, a cell type that retains the GC B-cell phenotype, as well as detect the different genes and pathways that are expressed during SHM[371]. GC B cells have also been analyzed using scRNA-seq to identify B cell subpopulations, the gene expression signatures of these cells, and the molecular mechanisms that drive differentiation into mature B cells[372,373]. This previous research indicates that scRNA-seq would be an ideal future research project to determine how different dose intervals of the SARS-CoV-2 vaccine impact the SHM process at the genetic level.

4.5.2. Germinal Centre Cell Dynamics

This project analyzed B cells that were present in the blood. However, B cells found in the blood have already left the GC, so any information gathered, while beneficial, does not reveal the processes occurring directly in the GC at that specific time. The results from this study are based on peripheral blood sampling and therefore do not capture B cells that may still be undergoing maturation in the LNs. Therefore, it may be possible that differences in antigen-specific B cell frequencies reflect differences in cellular trafficking or retention within LNs rather than changes in total proportions. Future projects could analyze B cells directly harvested from GCs, so that the differentiation process could be measured over time directly from the regions where SHM occurs. The previous section already touched on research projects that have isolated B cells directly from GCs, which provides them with cells directly undergoing SHM[372,373]. Using flow cytometry cell sorting methods, GC B cells can be measured and isolated from the B cell population. Prominent targets for probes to bind include CD38, as well as CXCR4 and CD83 to distinguish between dark zone and light zone GC B cells, respectively[374–376]. However, because GCs are found in secondary lymphoid organs, this isolation would require tonsil samples from participants, which are generally removed following tonsillectomies[374,375]. This would prevent longitudinal assessment of the GC B cells and would restrict the number and age of participants that could be recruited. Fine needle aspiration of lymph nodes can also be performed on animal or human participants to collect lymph node cells, which can allow for the collection of cells without destroying the LN, providing longitudinal assessment of GC B cells[377,378]. There are protocols which can create an *in vitro* environment that can induce a massive expansion of B cells that phenotypically and functionally resemble GC B cells, although further work will be needed to determine how to apply this to an experiment analyzing GC B cells after an extended dose interval of a vaccine[379].

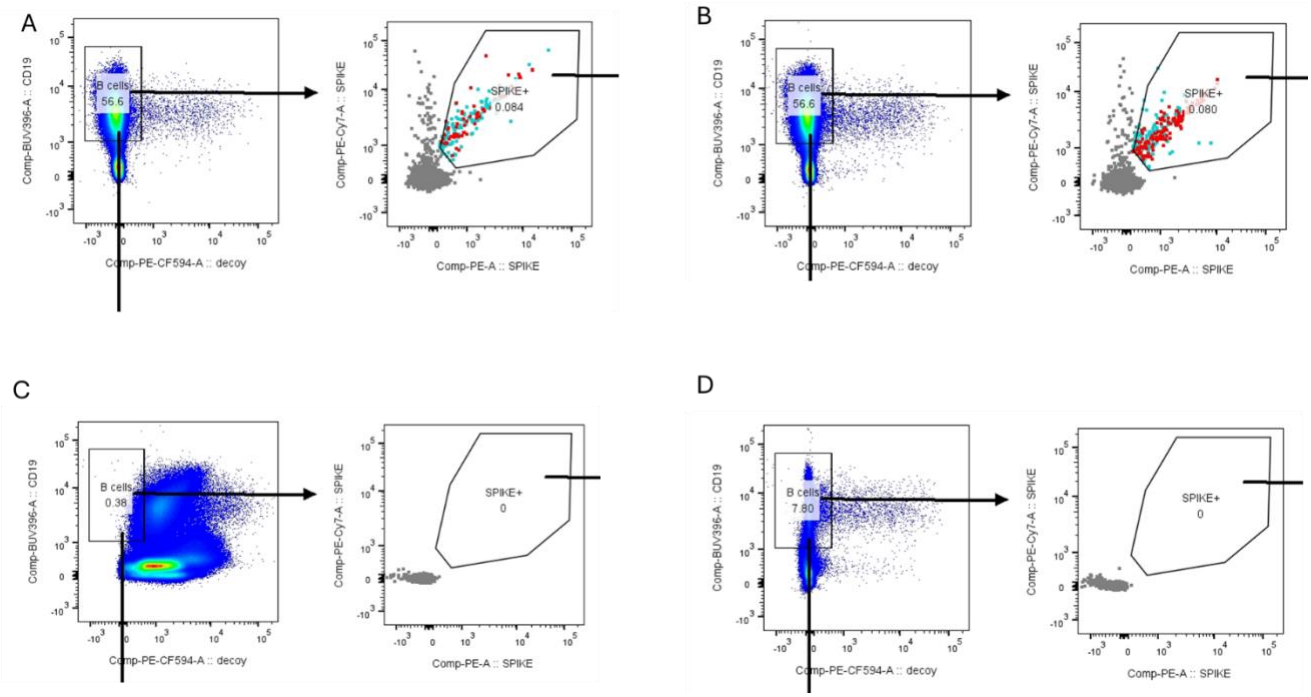
4.6. Conclusion

This study builds upon existing evidence that extended dose intervals enhance the humoral immune response by providing a detailed analysis of the underlying B cell responses to SARS-CoV-2 mRNA vaccination. Previous research has shown that extended dose intervals can improve plasma antibody titres, but the mechanisms driving these improvements have remained unclear. Here, we show that, at 10-14 days post-dose 2, an extended dose interval of the SARS-

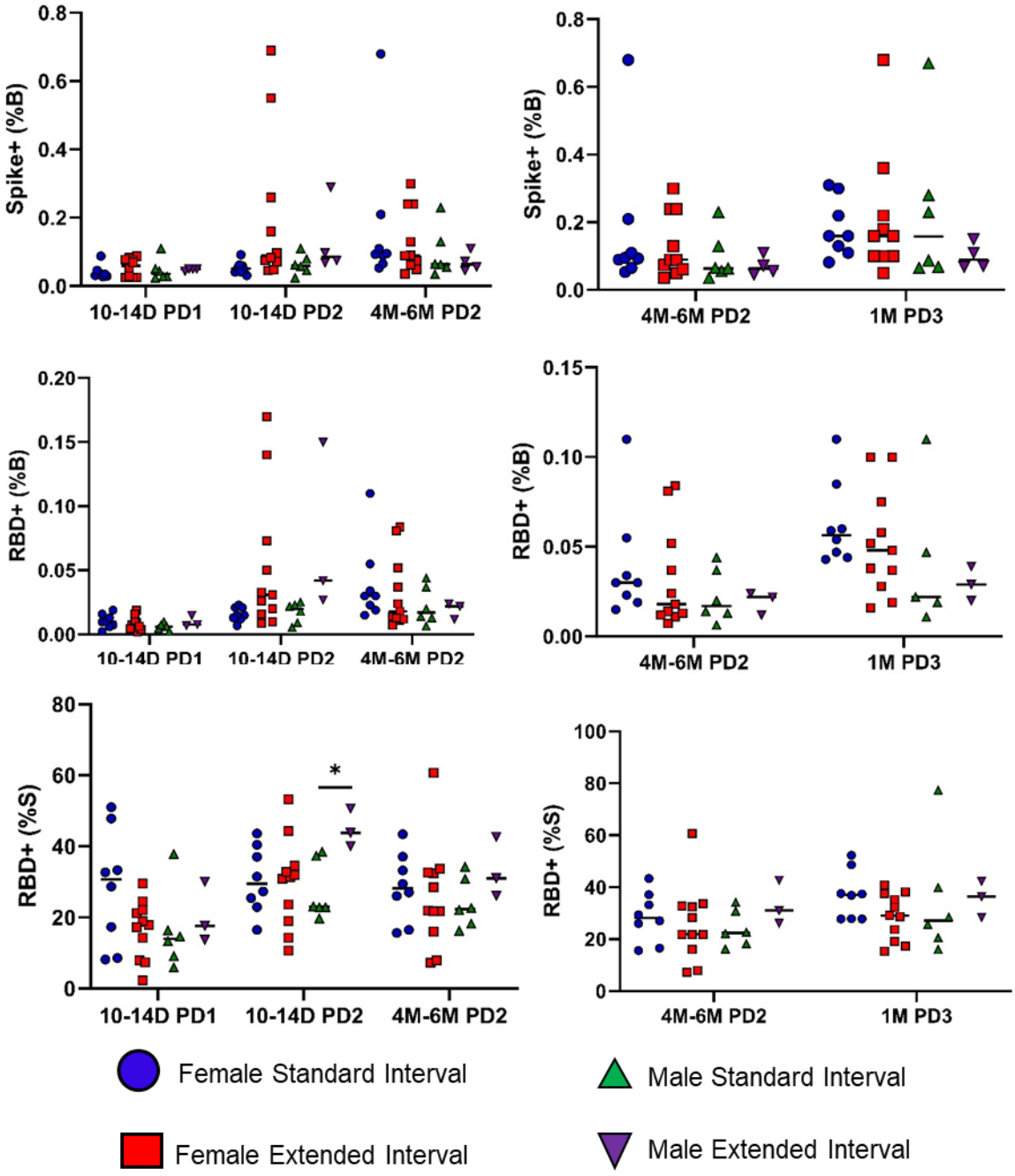
CoV-2 mRNA vaccine leads to an increase in the proportions of S-specific and RBD-specific B cells, IgG+ memory B cells and CD71+ memory B cells compared to a standard dose interval. An extended dose interval also results in the differentiation of memory B cells capable of producing antibodies with significantly greater neutralization of SARS-CoV-2 variants compared to a standard dose interval. This enhanced neutralization was correlated with higher levels of RBD-specific IgG in plasma, as well as increased frequencies of S-specific and RBD-specific B cells, IgG+ memory B cells and CD71+ memory B cells at 10-14 days and 4-6 months post-dose 2. While Omicron sublineages were not significantly impacted by the dose interval after the second and third doses, the association between B cell phenotypes and antibody function emphasizes the importance of dosing intervals in shaping humoral immunity. These findings suggest that extended intervals may promote more effective GC reactions, leading to enhanced affinity maturation and broader antibody responses, which is consistent with prior observations of SARS-CoV-2 and non-SARS-CoV-2 vaccine schedules. Together, this work provides a link between extended dose intervals and improved humoral immune quality, offering insights that can inform future vaccine policies and optimization strategies.

REFERENCES AND SUPPLEMENTARY DATA

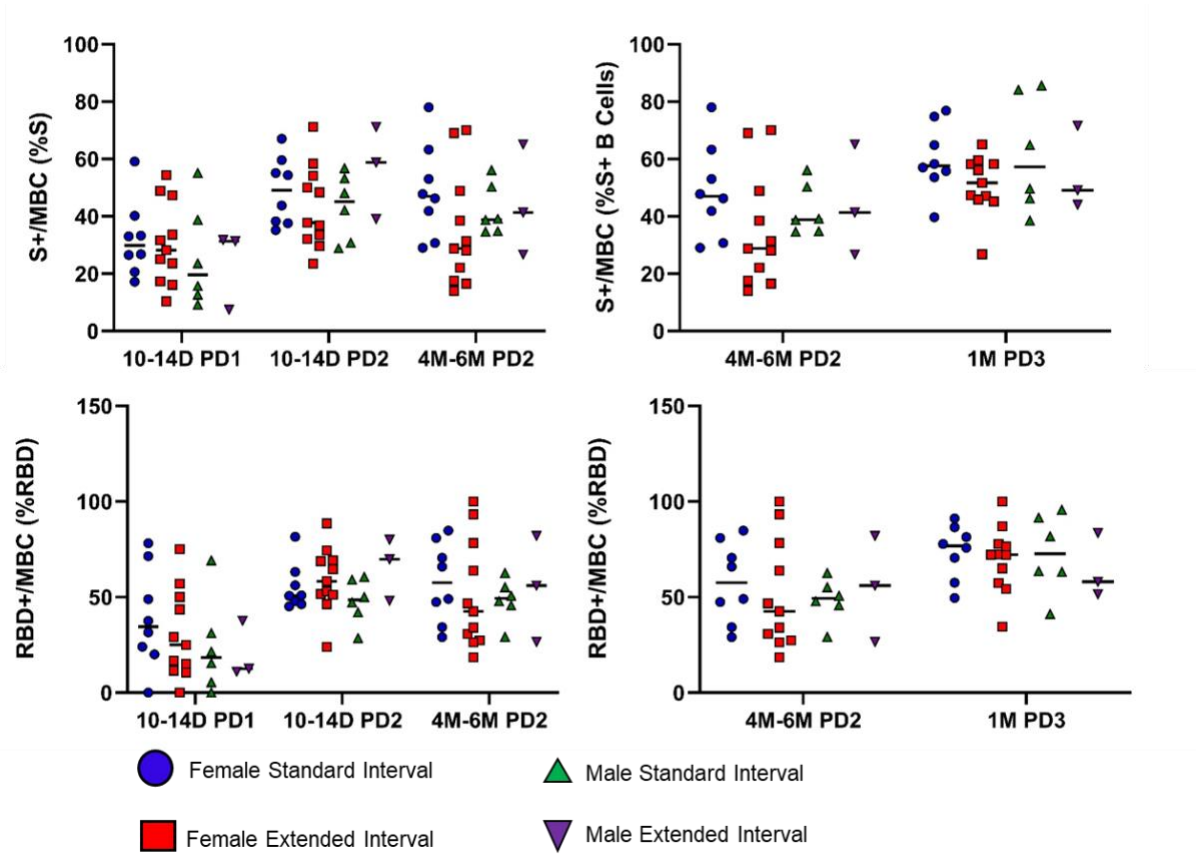
5.1. SUPPLEMENTARY DATA



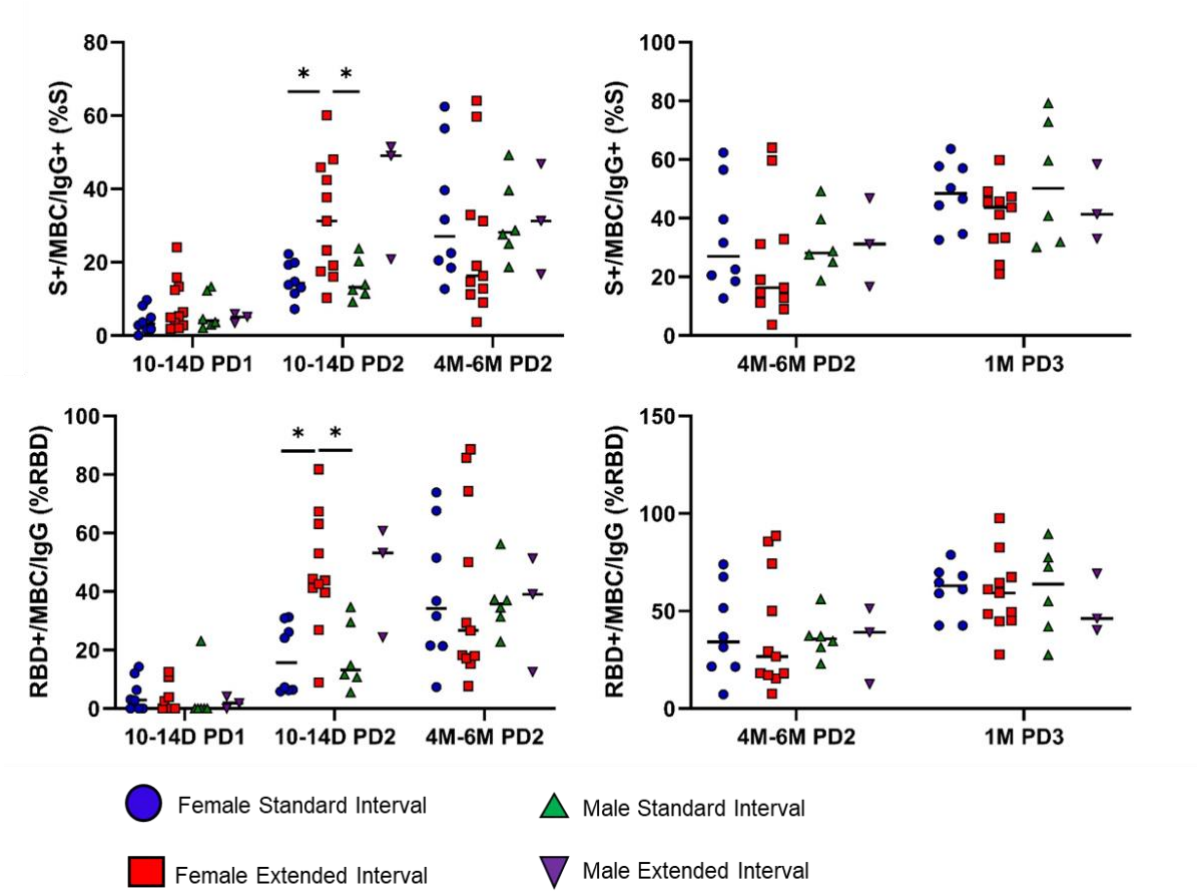
Supp. Figure 5.2-1: Gating Outputs of PBMCs to Determine Impact of Incorrect Biotin Reagent. Gating outputs show proportions of B cells and proportions of Spike-specific B cells from one sample timepoint**. (A)** Proportions of B cells and Spike-specific B cells when free D-biotin is used during flow cytometry. **(B)** Proportions of B cells and Spike-specific B cells when CD45 biotin is used during flow cytometry. **(C)** Proportions of B cells when CD45 biotin is used at the recommended volume (20 µL per test). No other fluorescent antibodies or probes were used. **(D)** Proportions of B cells when CD45 biotin is used at the volume used during the experiment (1 µL per test, 1/100 dilution). No other fluorescent antibodies or probes were used.



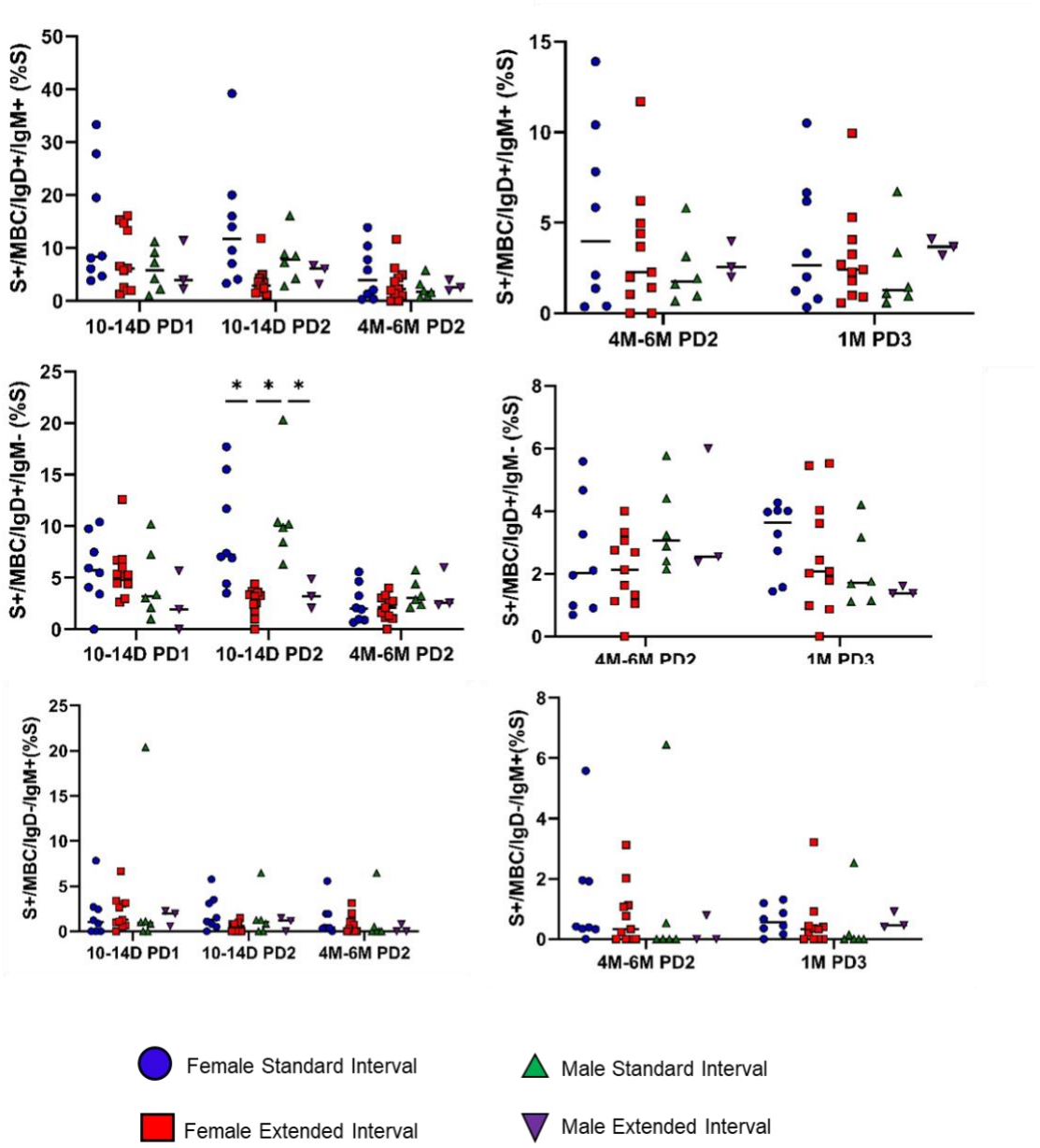
Supp. Figure 5.2-2 Assessment of S-Specific and RBD-Specific B Cells as a Percentage of B cells and Specific B cells in Males and Females. Comparison of S-specific B cells and RBD-specific B cells as a percentage of B cells, and RBD-specific B cells as a percentage of S-specific B cells, between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.



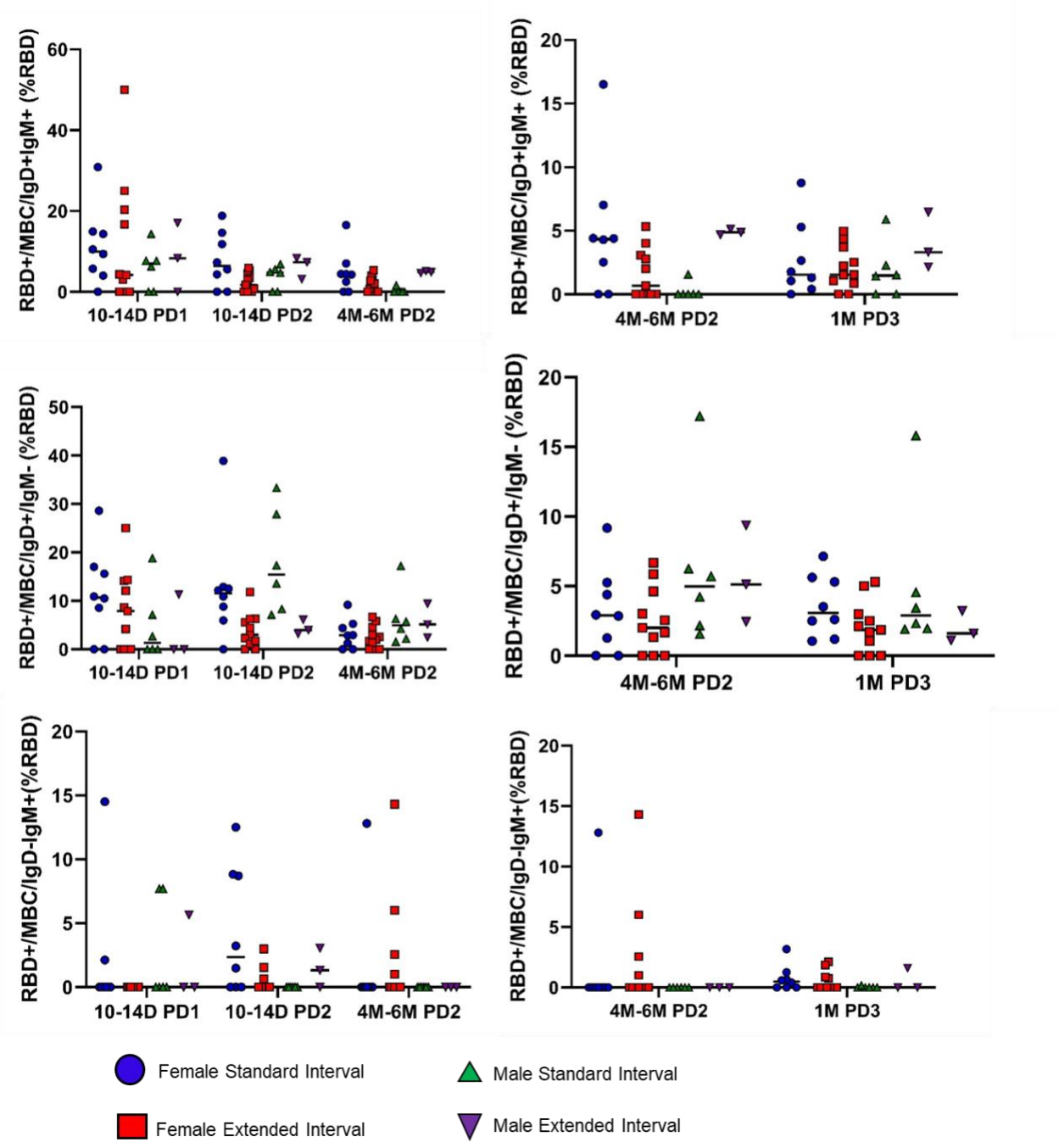
Supp. Figure 5.2-3: Assessment of Memory B Cells as a Percentage of S-Specific and RBD-Specific B Cells in Males and Females. Comparison of memory B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.



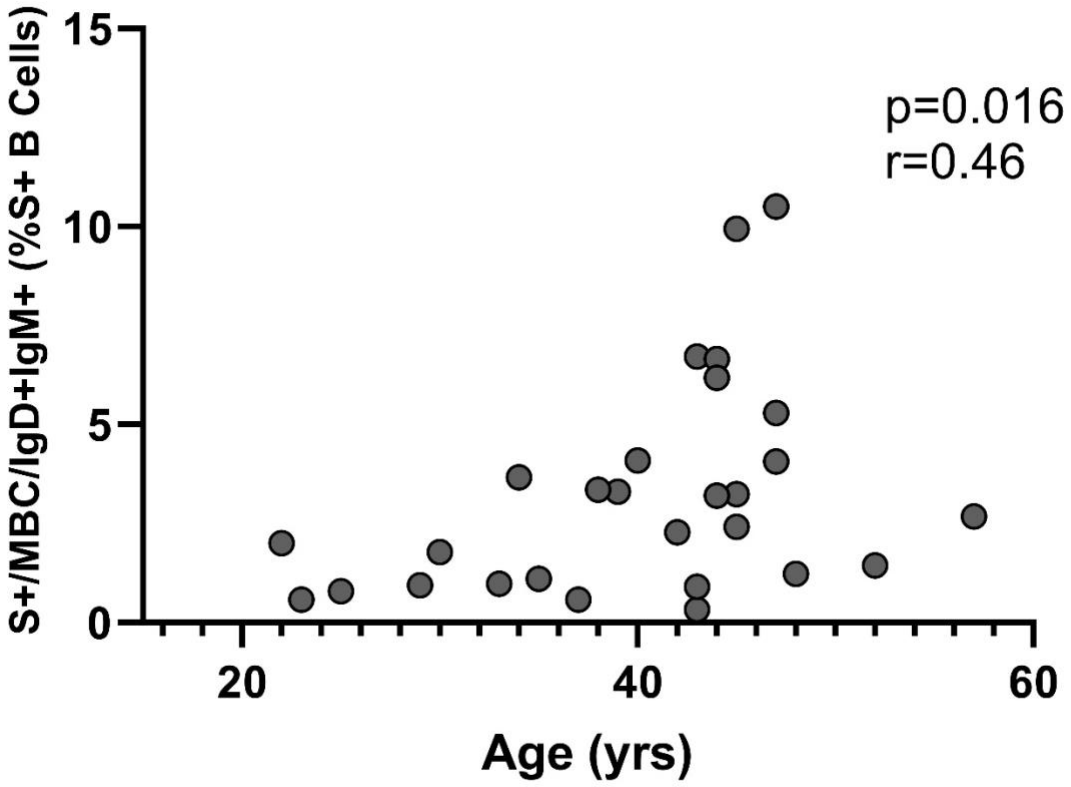
Supp. Figure 5.2-4: Assessment of IgG+ Memory B Cells as a Percentage of S-Specific and RBD-Specific B Cells in Males and Females Comparison of IgG+ memory B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.



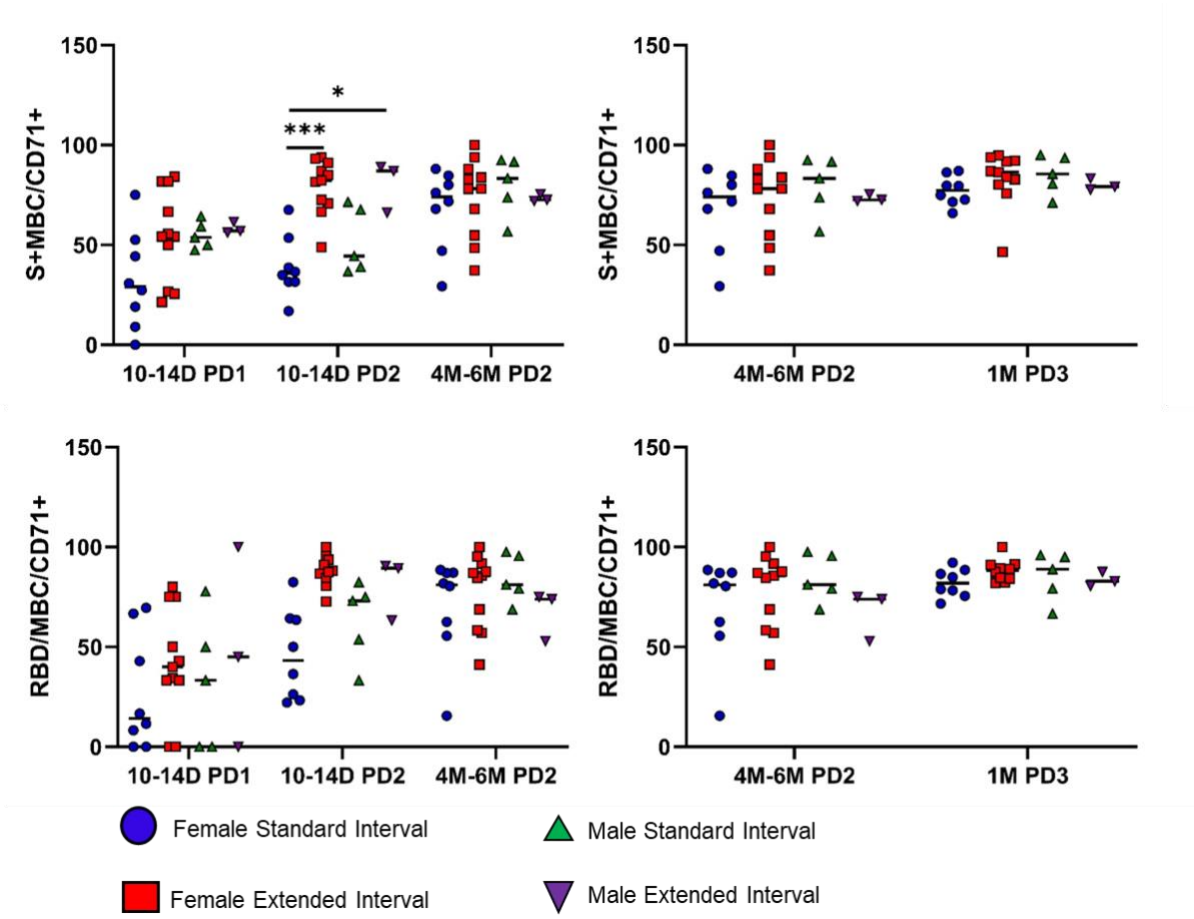
Supp. Figure 5.2-5: Assessment of IgD/IgM Memory B Cells as a Percentage of S-Specific B Cells in Males and Females. Comparison of IgD/IgM memory B cells as a percentage of S-specific B cells between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.



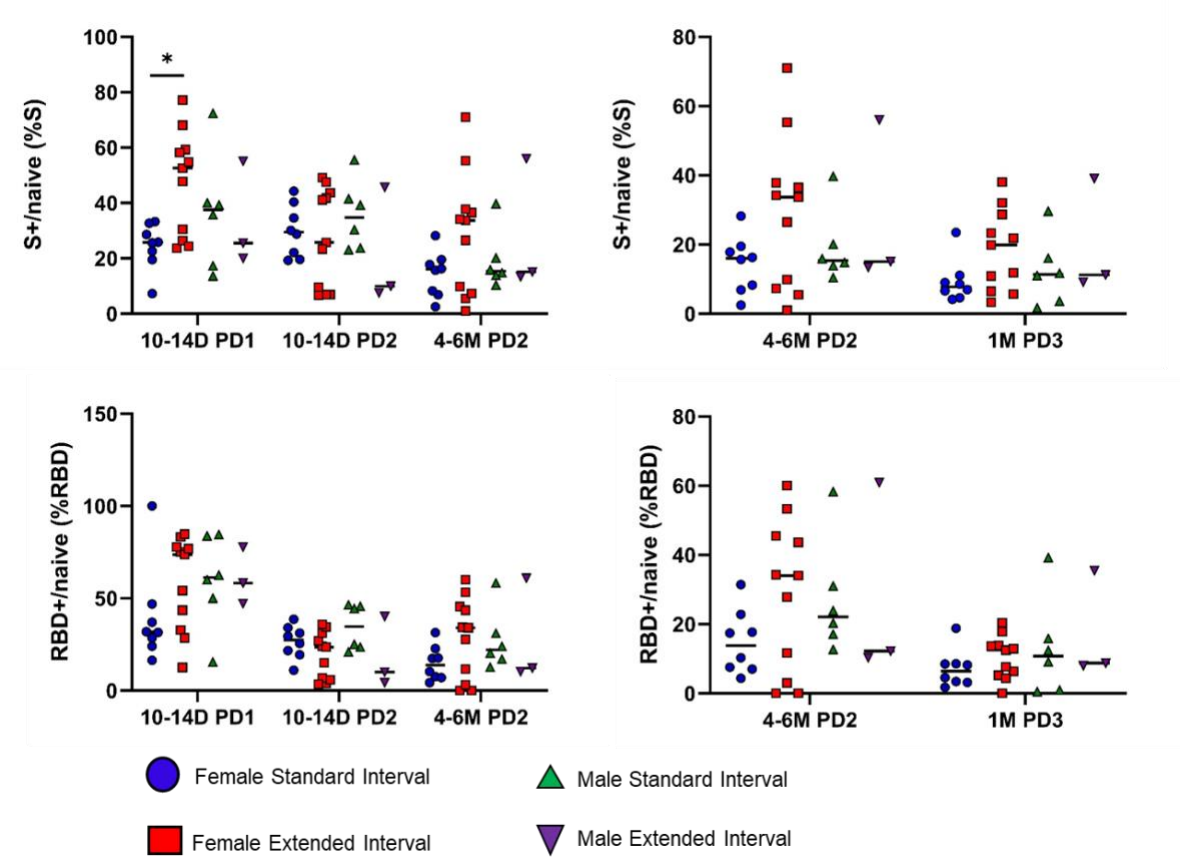
Supp. Figure 5.2-6: Assessment of IgD/IgM Memory B Cells as a Percentage of RBD-Specific B Cells in Males and Females. Comparison of IgD/IgM memory B cells as a percentage of RBD-specific B cells between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.



Supp. Figure 5.2-7: Correlation between Age of Participants and SARS-CoV-2 Specific B cell Proportions at 1-Month Post-Dose 3. The age of the participants in years were compared to S-specific B cells that were IgD+/IgM+ proportions at 1-Month Post-Dose 3. Spearman Correlation tests were performed to assess correlations.



Supp. Figure 5.2-8: Assessment of CD71+ B Cells as a Percentage of S-Specific and RBD-Specific Memory B Cells in Males and Females. Comparison of CD71+ B cells as a percentage of S-specific and RBD-specific memory B cells between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.



Supp. Figure 5.2-9: Assessment of Naïve B Cells as a Percentage of S-Specific and RBD-Specific B Cells in Males and Females. Comparison of naïve B cells as a percentage of S-specific and RBD-specific B cells between the standard and extended dose interval groups in males and females after the second and third doses of the SARS-CoV-2 vaccine. Groups were compared using two-way ANOVA tests with multiple comparisons. Lines show median values.

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