Characterising the T cell responses to SARS-CoV-2 including variants of concern induced by natural infection and vaccines

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The COVID-19 pandemic, caused by SARS-CoV-2, has profoundly impacted global health, prompting extensive research into vaccine responses and the immune dynamics of SARS-CoV-2 variants of concern (VOCs). This thesis investigates the breadth and durability of T cell responses following COVID-19 vaccination, focusing on immune responses against VOCs, including Alpha, Beta, Gamma, Delta, and Omicron, in previously infected and SARS-CoV-2-naïve individuals. T cells, as a central component of adaptive immunity, can target a broader array of SARS-CoV-2 proteins compared to antibodies, providing robust and potentially long-lasting immunity even in the face of viral mutations.

Chapter 1 provides a literature review on SARS-CoV-2 epidemiology, immune responses and variants. Chapter 2 provides an overview of the methods used in this thesis. In Chapter 3, I examine the impact of extended dosing intervals on T cell responses in both previously infected and naïve individuals. Results suggest that extended intervals enhance the polyfunctional T cell response, contributing to higher durability of the immune response, especially in naïve participants. Chapter 4 expands on T cell responses to VOCs, showing that vaccinated individuals maintain significant T cell reactivity across VOCs despite spike protein mutations that diminish antibody neutralization efficacy. Notably, Omicron's high mutation count challenges antibody responses, yet T cell responses remain comparatively maintained, likely mitigating severe outcomes during Omicron waves.

Chapter 5 utilizes peptide mapping to further dissect T cell responses across SARS-CoV-2 epitopes, revealing that vaccinated individuals with prior SARS-CoV-2 exposure show an expanded breadth of T cell responses, targeting diverse viral epitopes, particularly within conserved S1 and S2 domains. This comprehensive approach demonstrates that even as SARS-CoV-2 continues to mutate, T cell responses retain broad cross-reactivity, especially against more conserved viral regions. Additionally, I identify associations between certain HLA alleles, such as HLA-DQB1*06, and enhanced T cell responses, suggesting potential genetic underpinnings for varied immune durability.

Together, these findings underscore the importance of T cell immunity in sustaining protection against SARS-CoV-2 variants. My results highlight the need for booster strategies tailored to enhance T cell breadth and magnitude, providing insights for next-generation vaccine designs that prioritise broad and long-lasting immunity across diverse populations.

Acknowledgments

Firstly, I would like to express my deepest gratitude to my supervisors, Susie, Ellie and Paul, whose guidance, expertise, and encouragement have been instrumental throughout this journey. Starting a DPhil during the most uncertain times of a global pandemic allowed me to witness their dedication to pushing boundaries in global health research. Their unwavering support throughout my DPhil have profoundly impacted both my work and personal development. I am incredibly grateful for their mentorship, which has inspired me to approach complex challenges with curiosity and rigor.

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Most people get one great love in life. I have been blessed enough to have three. To God, the one who provided His Spirit without measure.

To Chinda, the one who walked so I could run with ease. Because of you, I have excelled at everything, each time better than the last. Mama, we made it, we are making it, and we will always make it.

And finally, my third great love, my gift from my other two loves—my husband, Amen-Patrick. What an absolute honour it is to do life by your side. You wear so many hats so seamlessly, each one with grace and love. I love you deeply.

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ACE2	angiotensin- converting enzyme 2
ADCC	antibody-dependent cellular cytotoxicity
ADCC	antibody-dependent cellular cytotoxicity
AIM	activation-induced marker
anti-N	anti-Nucleocapsid
anti-S	anti-Spike
APC	antigen presenting cell
BCR	B cell receptor
CoP	correlates of protection
COVID-19	coronavirus disease 2019
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte associated protein 4
CTV	CellTrace Violet
DAMP	danger-associated molecular pattern
DC	dendritic cell
dsRNA	double-stranded RNA
E	Envelope
ERGIC	endoplasmic-reticulum–Golgi intermediate compartment
FasL	Fas ligand
GMT	Geometric mean titre
HCW	Healthcare worker
HLA	human leukocyte antigen
HMGB1	like heat-shock proteins or high-mobility group box 1
IEDB	Immune epitope database
IFN	Interferon
IgG	immunoglobulin G
IL	Interleukin
LFT	lateral flow test
M	Membrane
M1	Matrix protein
MERS-CoV	Middle Eastern respiratory syndrome coronavirus
MHC	major histocompatibility complex
N	Nucleocapsid
NAbs	neutralizing antibodies
NK	natural killer
NSP	non-structural protein
NP	nucleoprotein
ORF	open reading frame

PAMP	pattern-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PITCH	protective immunity from T cells in healthcare workers
PRR	pattern-recognition receptor
RBD	receptor binding domain
RIG-I	retinoid inducible gene I
RLR	retinoic acid-like receptor
RTC	replication-transcription complex
S	Spike
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
ssRNA	single-stranded RNA
TAP	Transporter associated antigen processing
TCR	T cell receptor
Tfh	T follicular helper
Th1	Type 1 T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
Tregs	regulatory T cells
VE	Vaccine effectiveness
VOC	variants of concern
VOI	variants of interest
VUM	variants under monitoring
WHO	World Health Organization

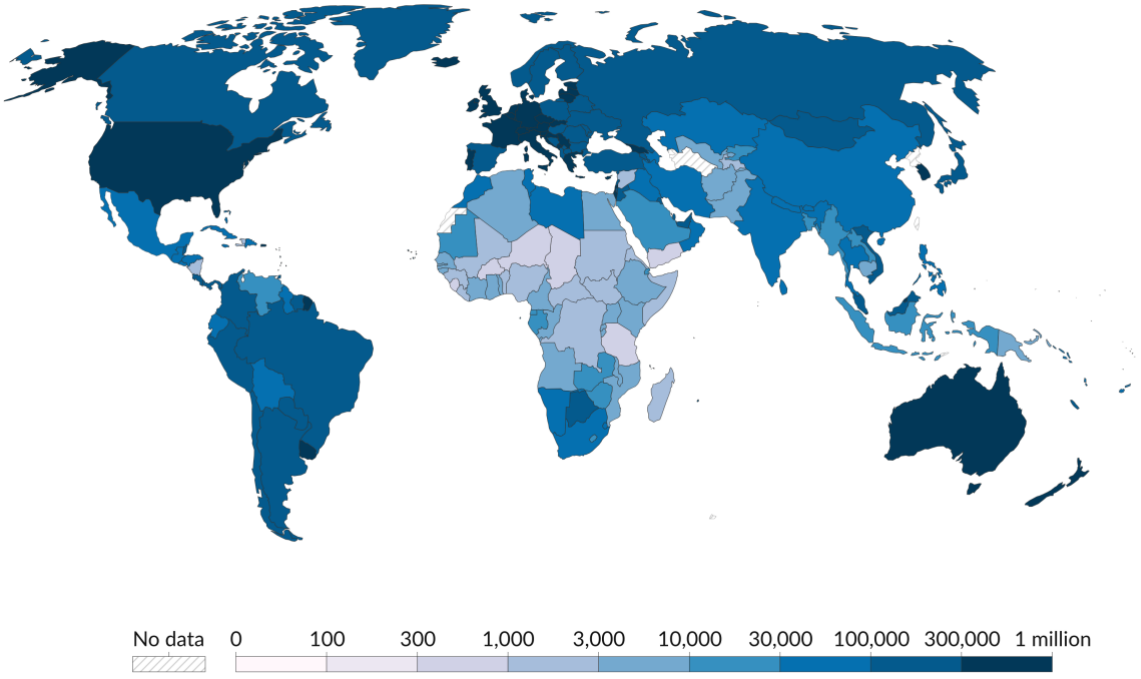
Chapter 1: Introduction

1.1 Global Epidemiology of SARS-CoV-2

Coronavirus disease 2019 (COVID-19) is a respiratory illness caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. An outbreak of COVID-19 was first reported from Wuhan, China in December 2019 [1] after which the virus spread globally, resulting in a pandemic being declared by the World Health Organization (WHO) on 11 March 2020 [2]. As of September 2024, over 776 million cases and 7 million deaths have been reported globally (Figure 1.1) [3], however, these numbers likely underestimate the true burden of the pandemic due to limited testing availability in various settings and a decline in testing interest and resources worldwide. The case fatality rate of COVID-19 has differed by country, due to factors including population structure, public health measures in place and presence of co-morbidities.

SARS-CoV-2 infection in humans ranges from asymptomatic to symptomatic, and the virus is mainly transmitted via inhaled respiratory droplets or contact with nasal, conjunctival, or oral mucosa [4]. The incubation period ranges from 2 to 14 days [5], with studies reporting a decline in incubation period with successive SARS-CoV-2 variants [6,7]. Similarly, while symptoms of COVID-19 infection primarily include pneumonia, fever, headaches, loss of smell and taste, these have been shown to differ by SARS-CoV-2 variant [8–12]. The multifaceted nature of COVID-19, stemming from the virulent SARS-CoV-2 virus, highlights the intricate interplay between viral genetics, host factors, and environmental conditions, influencing disease severity, transmission dynamics, and epidemiological outcomes, emphasizing the need for adaptive public health strategies.

A



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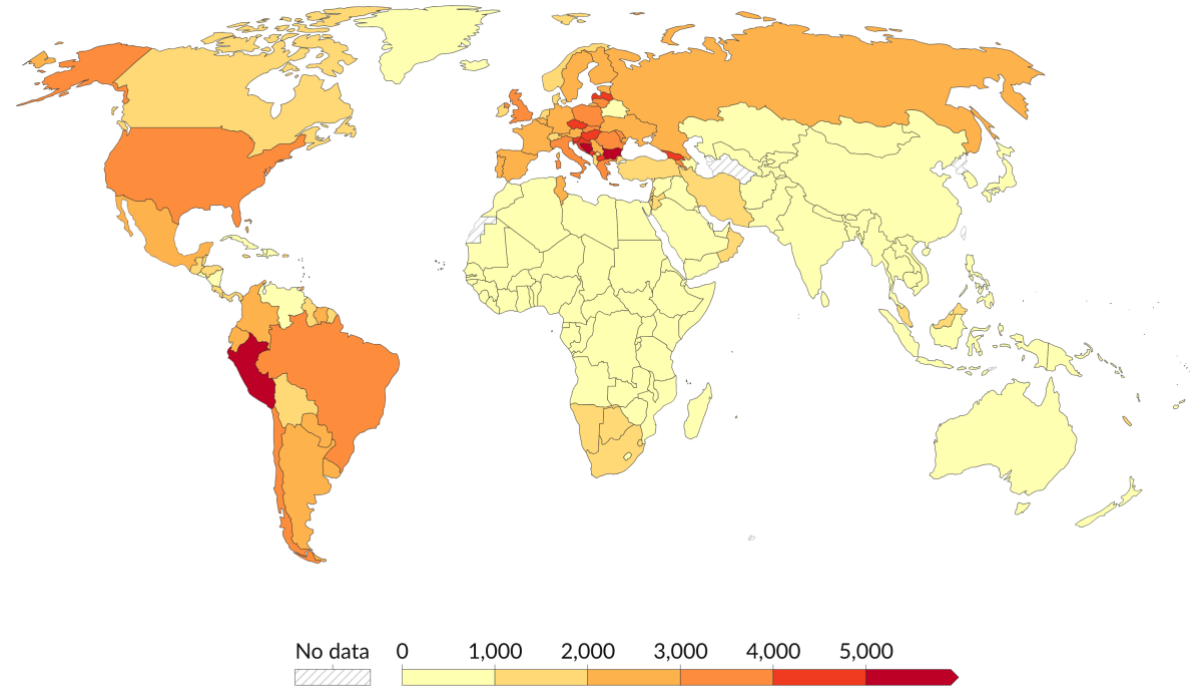


Figure 1.1. Reported COVID-19 Cumulative cases (A) and deaths (B) per 1 million people. January 2020 to September 2024. From COVID-19 data explorer, our world in data [13]

1.2 SARS-CoV-2 Virus and Viral Variants

1.2.1 SARS-CoV-2 virus

SARS-CoV-2 is an enveloped single-stranded 29.9kb RNA virus of the Beta coronavirus genus [14], made up of several viral proteins: S (S), Membrane (M), Envelope (E) and Nucleocapsid (N) (Figure 1.2). The S protein forms homotrimers which protrude from the viral surface and S is further divided into S1 and S2 subunits, which encode the receptor binding domain (RBD) and the transmembrane protein respectively. The RBD is responsible for binding to the host cell receptor, while the transmembrane protein is responsible for fusion of the viral and cellular membranes [15].

For viral entry of SARS-CoV-2, two S protein cleavage events may occur: one at the border of the S1 and S2 subunits and the other within the S2 subunit. At the junction of the S1-S2 subunits is a furin cleavage site notably absent from other coronaviruses, while the S2 site is cleaved at the target cell upon binding to angiotensin-converting enzyme 2 (ACE2), a receptor widely expressed in cells of several human organs such as the lungs, heart, and kidneys [16,17]. On the target cell, the S1 subunit binds the receptor and the S2 subunit anchors the S protein to the virion membrane and mediates membrane fusion. The E and M proteins contribute to virus assembly and budding through the interactions with other viral proteins. Assembled viruses bud into the ERGIC (endoplasmic reticulum-Golgi intermediate compartment) lumen and reach the plasma membrane via the secretory pathway, where they are released into the extracellular space after virus-containing vesicles fuse with the plasma membrane [16,18]. The ERGIC lumen is a membrane bound space that serves as a key transport hub between the endoplasmic reticulum and the Golgi apparatus, facilitating the proper folding and trafficking of viral proteins before they are assembled into mature virions [19].

There are six other coronaviruses known to infect humans: SARS-CoV-1, identified in 2003 and responsible for the 2002-2004 SARS outbreak, MERS-CoV identified in 2012, HCoV-229E and HCoV-OC43, identified in the 1960s, HCoV-HKU1 and HCoV-NL63, identified in 2004 [20–23]. MERS-CoV, or Middle East Respiratory Syndrome coronavirus, causes sporadic outbreaks with a high case fatality rate, primarily in the Middle East, and is thought to have originated from dromedary camels [24,25]. Coronaviruses that infect humans can be classified into two main genera: alpha coronaviruses, which include HCoV-229E and HCoV-NL63, and beta coronaviruses, which include HCoV-OC43, HCoV-HKU1, SARS-CoV-1,

SARS-CoV-2 and MERS-CoV [26,27]. While alpha coronaviruses tend to cause milder respiratory symptoms, beta coronaviruses, especially those classified as sarbecoviruses (like SARS-CoV-1 and SARS-CoV-2), are associated with more severe disease and have demonstrated potential for zoonotic transmission from animal hosts to humans [28,29]. The emergence of these beta coronaviruses underscores the ongoing risk of cross-species transmission, as evidenced by the SARS-CoV-2 pandemic.

The SARS-CoV-2 genome is shared among other Beta coronaviruses, with approximately 82% nucleic acid sequence identical to SARS-CoV-1 and 70% to MERS-CoV (Table 1.1) [30]. All coronaviruses share similarities in the organisation and expression of their genome, in which 16 non-structural proteins (NSPs), NSP 1 to NSP 16, are encoded by open reading frames (ORFs) 1a and 1b at the 5′ end, and are followed by the structural proteins S, E, M, and N, which are encoded by other ORFs at the 3′ end [31]. Among coronaviruses, the S2 S subunit is more conserved while the S1 subunit demonstrates more amino acid variability.

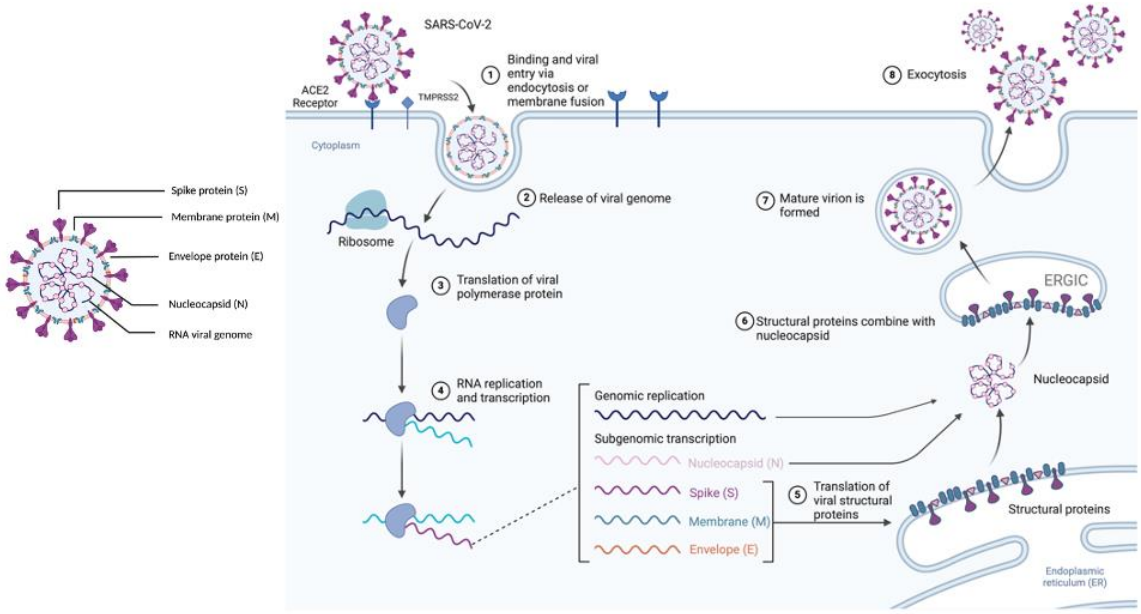


Figure 1.2. SARS-CoV-2 virus and mechanism of viral entry and replication. Created with BioRender.com.

Coronavirus	Case Fatality Rate (%)	Incubation period (days)	Genome identity with SARS-CoV-2 (%)	Genome organization
SARS-CoV-2	2	3-5	NA	
SARS-CoV-1	9	2-11	82	
MERS-CoV	36	2-13	70	
HCoV-229E*	<1	2-5	65	
HCoV-NL63*	<1	2-4	65	
HCoV-HKU1*	<1	2-4	68	

Table 1.1. Comparison across coronaviruses [20–23,31–46]. * These human coronaviruses are generally associated with mild respiratory illnesses, such as the common cold, and have a low CFR. Precise CFR data are limited due to their typically mild nature and the lack of widespread reporting.

1.2.2 SARS-CoV-2 viral variants

RNA viruses have high mutation rates, which can be associated with changes in viral characteristics, such as increased virulence or altered transmissibility. Mutation rates vary across viruses, and the mutation rate for SARS-CoV-2, while lower than some other RNA viruses such as HIV and influenza, is significant enough to have led to the emergence of new variants with potentially altered pathogenic properties. The high mutation rates in RNA viruses are driven by error-prone replication processes, which can confer a selective advantage by allowing rapid adaptation to host immune defences or new environments [47]. Unlike DNA polymerases, which possess exonuclease proofreading activity, RNA-dependent RNA polymerases lack such mechanisms, as evidenced by studies showing no detectable proofreading activity in RNA viruses like vesicular stomatitis virus [48]. This absence of

proofreading leads to higher mutation frequencies, allowing these viruses to rapidly adapt to host immune defences and environmental changes [48]. In contrast, DNA viruses such as herpes simplex virus and Epstein-Barr virus have more efficient proofreading mechanisms, significantly reducing replication errors [49].
Additionally, these mutations can affect various aspects of the biology of the virus, particularly its capacity for immune escape and infectivity [50]. For example, specific mutations such as D614G in the S protein of SARS-CoV-2 have been shown to enhance viral transmission by increasing S protein stability and binding affinity [51,52]. While some mutations may enhance the virus's fitness by improving its ability to replicate, evade the immune system, or spread between hosts, these changes do not necessarily affect the efficacy of vaccines, antivirals, or other public health interventions. [53].

The first variant of concern (VOC) to emerge was designated Alpha, with first documented samples from the United Kingdom in September 2020. During this period Beta also emerged, firstly in South Africa, before spreading globally. Following these, Gamma and Delta emerged in December 2020, with first documented samples in Brazil and India respectively. The final major VOC group to emerge to date was Omicron with first documented samples from South Africa, and further Pango lineage designations, Omicron BA.1 to Omicron BA.5. BA.1, BA.2 and BA.3 emerged in November 2021, BA.4 in January 2022 and BA.5 in February 2022 [54]. Table 1.2 highlights the WHO classification of these variants into VOCs, variants of interest (VOIs) and variants under monitoring (VUMs).

All emerged VOCs to date have shown an increase in transmissibility compared to the wildtype SARS-CoV-2 virus, while all except Omicron have shown an increase in disease severity compared to the wildtype SARS-CoV-2 [55–62]. In addition to Omicron showing a decrease in severity compared to the wildtype virus, all subsequent VOIs have also shown a decline in severity when compared to the wildtype virus. An increase in population immunity from vaccinations and infections resulting in milder disease outcomes may contribute to the observed decline in disease severity.

	WHO definition	Pango lineages
VUM	A notification to public health authorities indicating that a SARS-CoV-2 variant may warrant enhanced monitoring and prioritization.	All SARS-CoV-2 variants meeting these criteria have either been escalated to VOIs or deescalated
VOI	A SARS-CoV-2 variant with changes that could impact its behaviour or its effects on human health. This may include factors such as how easily it spreads, the severity of disease it causes, or how readily it can be detected or treated.	XBB.1.5 XBB.1.16 EG.5 BA.2.86 JN.1
VOC	A variant that qualifies as a VOI and meets at least one of the following criteria when compared to other variants: • It causes a significant worsening in disease severity. • It poses a considerable challenge to health systems, impacting their ability to care for COVID-19 patients or manage other illnesses, necessitating major public health interventions. • It leads to a marked reduction in the effectiveness of available vaccines in preventing severe disease.	B.1.1.7 (Alpha) B.1.351 (Beta) P.1 (Gamma) B.1.617.2 (Delta) B.1.1.529 (Omicron)

Table 1.2. WHO classification of SARS-CoV-2 variants [63]. VUM, variant under monitoring, VOI, variant of interest, VOC, variant of concern.

Key mutations in VOCs and their clinical implications

Since the emergence of SARS-CoV-2, several mutations have occurred, sometimes leading to variants with altered characteristics from the wildtype SARS-CoV-2 virus. As several mutations arose in SARS-CoV-2 S and non-S regions, some key mutations were common across several VOCs, particularly within the RBD. Notably, D614G, N501Y and E484K/A. Table 1.3 highlights the number of total mutations in the VOCs and the percentage of these mutations that lie within S, and Figure 1.3B shows all the mutations across S in the VOCs, highlighting mutations shared across the VOCs. The D614G mutation is present in all VOCs,

N501Y is present in all except Delta, and E484K is present in beta and Gamma while E484A is present in Omicron, BA.1 to BA.5. From December 2020 to November 2024, approximately 17 million sequences have been submitted globally to GISAID [64], an international database that facilitates the sharing of genetic data on influenza viruses and coronaviruses, including SARS-CoV-2, to support public health and research efforts. Of all the SARS-CoV-2 sequences submitted to GISAID, 98% of these sequences include the D614G mutation, 59% the N501Y mutation and 53% the E484K/A mutation.

Studies on these mutations have shown they play crucial roles in viral replication, immune evasion, and pathogenesis. The D614G mutation, which results in an aspartic acid (D) to glycine (G) substitution at position 614 of the S protein, has been shown to increase viral entry into cells by altering the S protein's conformation. This change enhances its binding to ACE2, the host receptor, by stabilizing the open form of the S protein, making it more accessible to ACE2. This leads to increased replication of SARS-CoV-2 in human lung epithelial cells and primary human airway tissues [65–67]. The N501Y mutation, a switch from asparagine (N) to tyrosine (Y) at position 501, increases the binding affinity of the receptor-binding domain (RBD) for ACE2 by approximately six-fold. This is due to the ability of tyrosine to form stronger hydrogen bonds with ACE2 compared to the wild-type asparagine, enhancing viral attachment and facilitating infection [68–70]. Similarly, the E484K/A mutations, which replace glutamic acid (E) with lysine (K) or alanine (A) at position 484, contribute to immune evasion. These mutations alter the structure of the RBD, reducing the ability of neutralizing antibodies to bind effectively. This leads to decreased neutralization by both convalescent serum and vaccine-elicited antibodies, posing challenges for immune control of the virus. [71–75].

While these studies highlight the altered viral characteristics and potential immune implications of these mutations, all mutations within a variant should be considered together, and consideration for the continuously evolving population immune landscape of the virus. SARS-CoV-2 VOCs often consist of multiple mutations, some of which are major drivers of viral fitness, while others are considered minor variations. These minor mutations can occur alongside hallmark changes, adding layers of complexity to the viral genome. Minor variations within a VOC may not individually alter the behaviour of the virus significantly, but their cumulative effects can lead to subtle changes in transmissibility, immune evasion, or resistance to treatments. Moreover, these variations can make it challenging to study specific mutations

in isolation, as combinations of mutations might interact in unpredictable ways. To address this complexity, researchers often rely on consensus sequences, which represent the most common sequence across a set of viral genomes. By using consensus sequences, scientists can filter out low-frequency variations and focus on mutations that are more likely to be biologically significant. This is crucial for ensuring that vaccines, diagnostics, and therapeutics are designed to target the dominant viral forms while accounting for genetic diversity within VOCs.

Variant of Concern	**Number of mutations relative to wildtype**	**Percentage of mutations in S**	**Date first reported**	**Place first reported**
Alpha	22	41%	September 2020	United Kingdom
Beta	19	42%	May 2020	South Africa
Gamma	23	52%	November 2020	Brazil
Delta	29	28%	October 2020	India
Omicron BA.1	49	61%	November 2021	South Africa
Omicron BA.2	53	55%	November 2021	South Africa
Omicron BA.3	34	44%	December 2021	South Africa
Omicron BA.4	57	54%	January 2022	South Africa
Omicron BA.5	54	57%	February 2022	South Africa

Table 1.3. SARS-CoV-2 Variants of concern. Number and percentage of mutations in S across SARS-CoV-2, with date and place of first reporting. Data from GISAID.org [64], using 75% of sequences submitted globally for each variant [76–80]

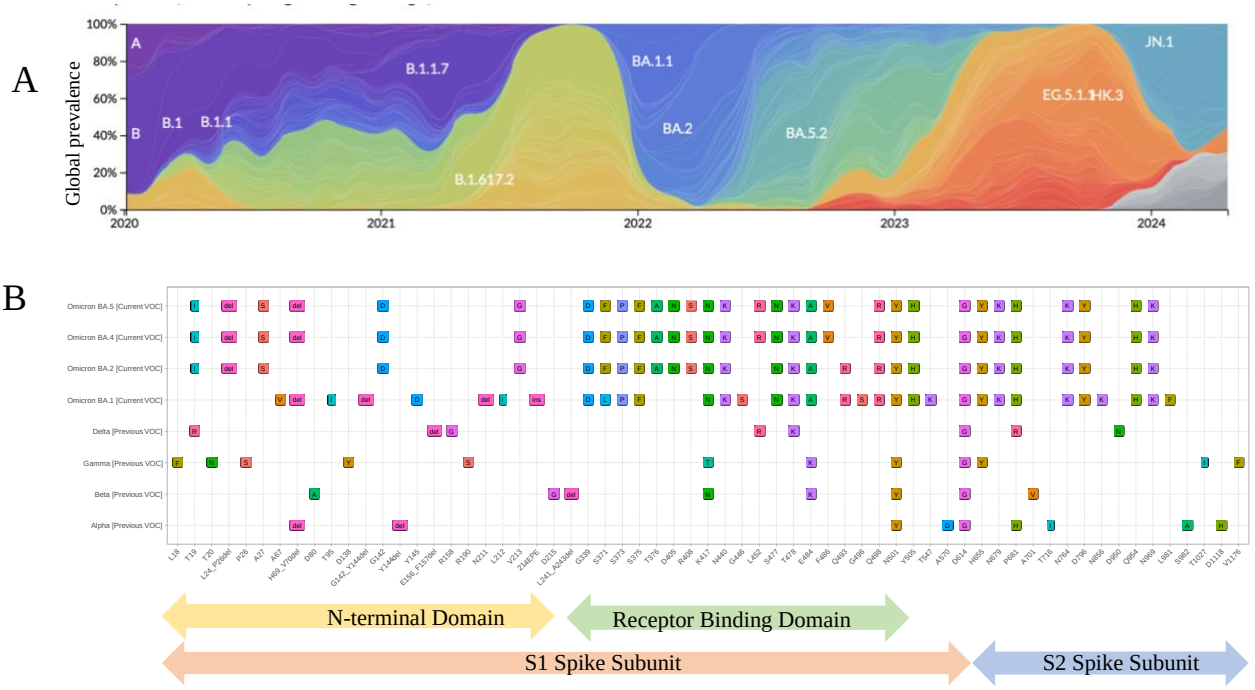


Figure 1.3 Timeline of global prevalence of SARS-COV-2 variants (A) and key mutations in SARS-CoV-2 S in variants of concern (B). A, B, B.1, B.1.1 represent wildtype/ancestral SARS-CoV-2, Alpha/B.1.1.7, Delta/B.1.617.2, Omicron/BA.1.1, BA.2, BA.5.2, EG.5.1, HK.3 and JN.1 [81–83]

1.3 The Immune Responses to SARS-CoV-2

The immune responses to SARS-CoV-2 can be divided into innate and adaptive immunity. The innate immune system restricts viral replication within infected cells and primes the adaptive immune response, governed by B cells and T cells. The adaptive immune system provides a more specific response that takes several days to initiate after priming due to selection and expansion of naïve cells. Unlike the adaptive immune system, the innate immune system is non-specific and considered not to result in immunological memory [84]. However, studies have shown that innate immune cells can undergo "trained immunity," where prior infections or vaccinations induce long-lasting epigenetic changes, allowing them to mount a stronger and more effective response upon subsequent infections [85].

1.3.1 Innate immune response to SARS-CoV-2

The innate immune system acts as the first line of defence against invading pathogens. Using innate immune cells such as macrophages, natural killer (NK) cells and dendritic cells (DCs), the innate immune system carries out its functions of promoting viral clearance and activating inflammatory pathways.

Upon viral entry, innate immune cells recognise pathogen-associated molecular patterns (PAMPs), such as viral RNA or DNA, and danger-associated molecular patterns (DAMPs), such as host cell-derived proteins released during infection, using pattern-recognition receptors (PRRs). Examples of viral PAMPs include double-stranded RNA (dsRNA) and viral single-stranded RNA (ssRNA), while DAMPs can include molecules like heat-shock proteins or high-mobility group box 1 (HMGB1) released from infected or damaged cells. There are several classes of PRRs; retinoic acid-like receptors (RLRs), including retinoid inducible gene I (RIG-I), and Toll-like receptors (TLRs), which trigger interferon (IFN) pathways and promote cytokine production [86–89]. Ten functional TLRs have been identified in humans designated as TLR1 to TLR10 [90]. TLRs are expressed throughout the human respiratory tract but show heterogeneous expression across innate immune cell populations; for example, TLR3 is more abundant in NK cells, whereas TLR4 is more common in macrophages [91].

Recognition of PAMPs and DAMPs by PRRs begins an antiviral state that limits viral replication within the cell and reduces the susceptibility of surrounding cells to the infection. Activation of PRRs causes downstream activation of kinases and consequently transcription factors, which lead to the production and release of type I and type III IFNs, chemokines, and surfactant proteins which promote the migration of NK cells, neutrophils, and macrophages to the site of infection [86,92,93]. Studies have shown that in SARS-CoV-2 infection, the virus is able to evade the mechanisms involved in the innate immune response, for example by evading the antiviral effects type I and type III IFNs [94,95]. Additionally, a delayed type I IFN response has been shown to increase the risk of severe COVID-19 in patients, further underscoring the importance of the innate immune system in limiting viral replication and promoting antiviral activity [96].

Macrophages and monocytes play key roles in the control of SARS-CoV-2. Following SARS-CoV-2 virus entry, TLRs expressed by macrophages detect the SARS-CoV-2 S and N

proteins [92], triggering the release of proinflammatory cytokines such as interleukins (IL) 1 and 6. Macrophages also play a role in phagocytosis and in secreting chemokines to recruit other immune cells to the site of infection, for example monocytes, which can differentiate into macrophages or DCs [97].

Dendritic cells are antigen presenting cells (APCs) which support both innate and adaptive immune responses. DCs play a role in cytokine production, control of inflammatory responses and recruitment of immune cells [98]. Upon SARS-CoV-2 viral entry and TLR activation, immature DCs become mature DCs through secretion of cytokines and expression of surface and costimulatory molecules. Mature DCs migrate to lymph nodes for activating naive T cells by presenting processed antigens on major histocompatibility complex (MHC) molecules [99]. The SARS-CoV-2 S protein induces the maturation and activation of dendritic cells [100].

Natural killer cells are innate immune cells that contribute to viral defence by killing virus-infected cells. NK cells can be sub-grouped based on their expression levels of the CD56 and CD16 cell surface molecules [92]. Their expression levels determine their role in antiviral host defence; cell-mediated cytotoxicity or cytokine production. $CD56^{bright}$ ($CD56^{bright}$ / $CD16^{neg}$) NK cells have more cytokine production ability while $CD56^{dim}$ ($CD56^{dim}$ / $CD16^{pos}$) NK cells have more cytotoxic ability [92,101]. In SARS-CoV-2, NK cells can induce apoptosis in infected cells via the Fas ligand (FasL) or by the release of lytic granules, and they can also play a role in the recruitment of other immune cells via cytokine production [102].

1.3.2 Adaptive immune response to SARS-CoV-2

The innate immune system plays a critical role in initiating the adaptive immune response. Evasion of the innate response by the SARS-CoV-2 virus can delay the priming of the adaptive immune system, potentially leading to increased COVID-19 severity [96]. The adaptive immune system consists of CD4+ T cells, CD8+ T cells and B cells. The process of priming the cells of the adaptive immune response takes several days, due to the process of selection and expansion of virus-specific cells.

B cell and T cell development

T cell development starts when bone marrow-derived lymphocyte precursor cells enter the thymus, complete their primary development as naïve T cells, and enter the bloodstream as

mature T cells, while the maturation process for B cells occur in the bone marrow [103,104]. This process aims to generate a diverse repertoire of B cell receptors (BCRs) and T cell receptors (TCRs) on circulating B and T cells, enabling the immune system to recognise a wide range of antigens [103].

In the thymus, the T cells develop to express either αβ TCRs or γδ TCRs [105]. T cells expressing αβ receptors recognise peptide antigens bound to a human leukocyte antigen (HLA) class I or class II molecule, while γδ T cells recognise different types of surface molecules whose expression may indicate infection or cellular stress [103]. Mature T cells undergo positive and negative selection, where T cells that bind with moderate affinity to the MHC are driven to survive and those that react strongly to self-receptors are killed respectively [106,107].

Antigen presentation and activation

Antigens are presented by class I or class II molecules to T cells. CD8+ T cells recognise antigens bound to class I molecules, and CD4+ T cells recognise antigens bound to class II molecules. Once bound to a peptide, they exhibit a range of effector functions. CD4+ T cells differentiate into specialized subsets and have a broad range of effector and helper functions. These include T follicular helper cells (Tfh) that support B cells with affinity maturation and antibody production, Type 1 T helper cells (Th1) which play a role in cytokine secretion and recruitment of innate cells, CD4+ T cells that help in the differentiation and proliferation of CD8+ T cells and cytotoxic CD4+ T cells (CD4 CTL) which exhibit direct cytotoxic activity against virally infected cells [104,108].

MHC class I molecules are expressed on all nucleated mammalian cells while MHC class II molecules are selectively expressed on APCs such as DCs, macrophages, and B cells. The expression of both MHC class I and class II can be upregulated by cytokines, particularly interferons [103,109]. IFNα and IFNβ increase the expression of MHC class I molecules on all types of cells, whereas IFNγ increases the expression of MHC class I and II molecules[103]. MHC class I molecules bind short peptides, 8 to 10 amino acids in length, while MHC class II molecules bind peptides of variable lengths as their anchor residues lie at various distance from the ends of the peptide [103].

Foreign antigens presented by class I molecules are derived from intracellular pathogens such as viruses while those presented by class II molecules are derived from pathogens present in the extracellular space (Figure 1.4) [105]. For class I processing, antigens in the cytosol are degraded by proteasomes into smaller peptide fragments and transported via Transporter associated antigen processing (TAP) to the lumen of the ER. Here, the peptides form complexes with class I molecules, leaving the ER for the plasma membrane where they are recognised by CD8+ T cells [110]. CD8+ T cells are then activated and can stop viral replication by killing virus-infected cells. In addition to antigen presentation, full activation of CD8+ T cells requires costimulatory signals provided by molecules such as CD80 and CD86 on APCs. These key costimulatory molecules bind to the CD28 receptor on naïve T cells, providing a crucial signal that enhances T cell survival, proliferation, and differentiation [103]. Without these costimulatory signals, CD8+ T cells may become anergic, failing to proliferate or exert their cytotoxic functions effectively. This dual-signal requirement ensures that T cells are selectively activated by genuine pathogenic threats rather than by incidental self-antigens, which helps to prevent autoimmunity. Costimulatory signals not only support the activation of CD8+ T cells, enabling them to proliferate and carry out cytotoxic activities, but also play a significant role in the activation and differentiation of CD4+ T cells into specialized subsets, including Th1, Th2, Th17, and regulatory T cells (Tregs), each of which performs distinct roles in coordinating and modulating immune responses [111,112].

For class II molecules, extracellular proteins are taken into the cell by endocytosis or phagocytosis and are degraded to peptides within endosomes [113]. The invariant chain, which prevents peptides from binding to the class II molecule is degraded in MIIC vesicles, allowing peptides to bind [114,115]. MIIC, MHC class II-containing compartment, are specialized endosomal compartments in APCs where the processing and loading of peptides onto MHC class II molecules occurs. The ensuing HLA Class II and peptide complex is taken to the plasma membrane for recognition by CD4+ cells. Activated CD4+ T cells carry out a range of functions such as cytokine secretion and B cell activation for antibody production [116].

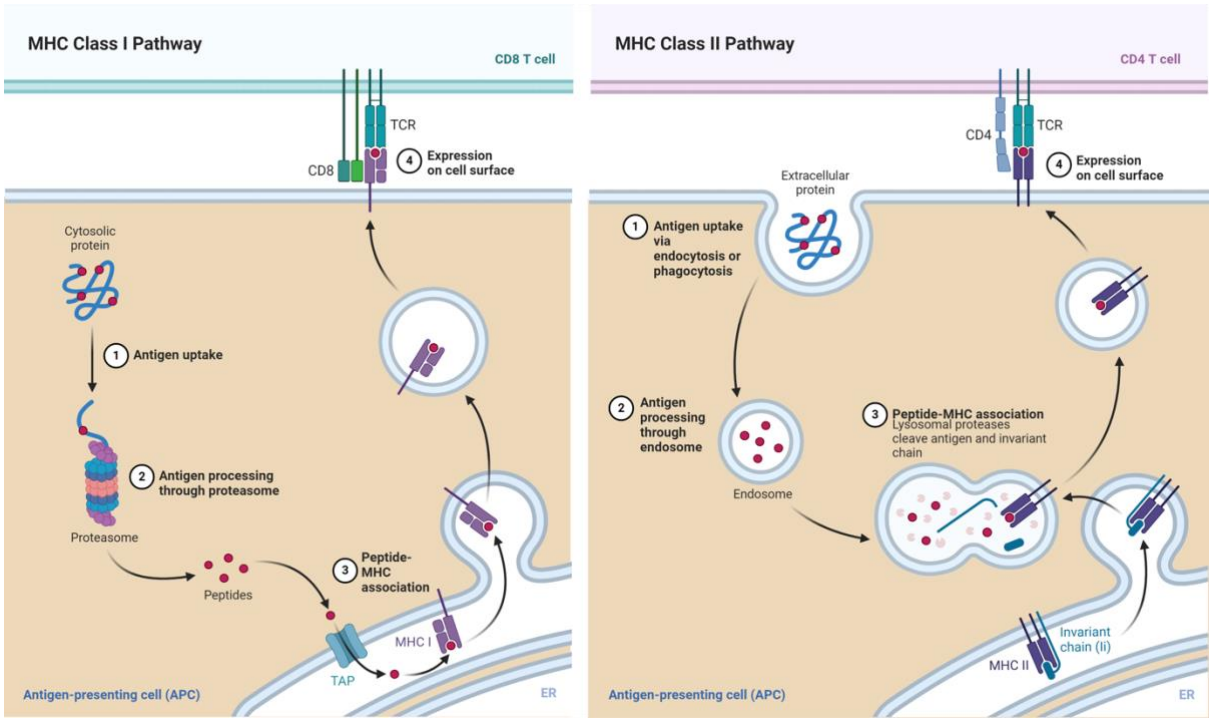


Figure 1.4. Schematic showing how antigens are processed via the MHC class I and class II pathways. Created with BioRender.com

Antibodies are critical components of the adaptive immune response, produced by B cells upon activation. One of their primary functions is neutralization, where antibodies bind directly to pathogens, such as viruses or bacteria, blocking their ability to enter host cells and thereby preventing infection [103]. Beyond neutralization, antibodies also engage in opsonization, a process where antibody-coated pathogens are tagged for phagocytosis by immune cells like macrophages and neutrophils [117]. Additionally, antibodies can activate the complement system, a series of proteins that work together to lyse pathogens and enhance inflammation, further promoting the clearance of infections [112]. Certain antibody subclasses, such as IgG, also trigger antibody-dependent cellular cytotoxicity (ADCC) by binding to Fc receptors on NK cells, facilitating the destruction of infected or malignant cells [111]. Together, these mechanisms make antibodies versatile defenders that play a central role in both neutralizing pathogens and enhancing other immune processes to ensure efficient pathogen clearance.

1.4 SARS-CoV-2 Vaccines

The first COVID-19 vaccines were introduced in December 2020, and by December 2023, over 5 billion vaccine doses had been administered globally [118]. As of March 2023, there were 183 vaccine candidates in clinical development and 199 in pre-clinical development [119]. In the United Kingdom, some of the COVID-19 vaccines used to date include the Pfizer BioNTech mRNA vaccine (BNT162b2), the Oxford-AstraZeneca viral vector vaccine (AZD1222) and the Moderna COVID-19 vaccine (mRNA-1273) [120]. Initially, the vaccine formulations were monovalent, targeting the wildtype/ancestral SARS-CoV-2 virus, however, as variants emerged, vaccine formulations were updated to include emerging viral strains, leading to bivalent formulations.

mRNA vaccines, delivered through lipid nanoparticles, encode the SARS-CoV-2 S protein which is taken up by the host's cells upon administration. Within the cell cytoplasm, the mRNA is translated, and the resulting S protein produced is displayed on the surface of cells and able to trigger an immune response, such as the activation of the innate immune system and subsequent activation of B and T cells [121]. More specifically, when cells express the S protein after mRNA vaccination, intracellular processing leads to fragments being presented on MHC class I molecules, activating CD8+ T cells and promoting cytotoxic responses [122]. Concurrently, APCs can capture the S protein released from vaccinated cells, processing it via the MHC class II pathway to activate CD4+ T cells. This supports B cell antibody production and amplifies CD8+ T cell responses [123]. Viral vector vaccines use modified viruses to deliver genetic material, which is expressed inside the host's cell, using the host cells' machinery to produce the target protein. The Oxford-AstraZeneca vaccine uses a chimpanzee adenovirus (ChAdOx1) as the vector, while some other viral vector COVID-19 vaccines use a human adenovirus such as Ad26 (Janssen, Ad26.COV2.S) and Ad5 (Sputnik V) [124–126]. Similarly, viral vector vaccines deliver the S protein gene directly to cells, resulting in expression and presentation on both MHC class I and II molecules, which elicits a broad immune response encompassing cytotoxic T cells, helper T cells, and antibody-producing B cells [127].

1.4.1 T cell responses to SARS-CoV-2

T cell studies have shown the presence of SARS-CoV-2 specific T cell responses in individuals infected with SARS-CoV-2 as well as vaccinated individuals. In patients who had recovered from SARS-CoV-2 infection, SARS-CoV-2 specific T cells were present, however, T cell responses were also present in individuals not exposed to SARS-CoV-2, suggesting the presence of T cells from other coronaviruses that are cross reactive [128,129], with 40% to 60% of unexposed individuals showing T cell responses to SARS-CoV-2 [130].

To further understand these observations, it is important to consider the variability in T cell assays used to detect SARS-CoV-2-specific T cell responses. Different assays can yield different results due to their sensitivity and the types of immune responses they measure. For example, activation-induced marker (AIM) and CellTrace Violet (CTV) proliferation assays tend to be highly sensitive, often detecting low-frequency T cell populations, which may account for the detection of cross-reactive T cells in unexposed individuals [131,132]. These assays can capture both effector and memory T cell responses over an extended period of stimulation. In contrast, IFNγ enzyme-linked immunospot (ELISpot) assays, while widely used to measure T cell effector function, are often less sensitive in detecting low-frequency responses and may fail to detect cross-reactive T cells in unexposed individuals, as seen in some studies [133]. The choice of assay is therefore critical when interpreting the presence and magnitude of SARS-CoV-2-specific T cell responses, particularly in the context of cross-reactivity with other coronaviruses.

CD4+ T cells and CD8+ T cells have been detected in 100% and 70% of convalescent COVID-19 patients respectively [130]. In patients with acute COVID-19, studies have shown that those with severe disease often exhibit lower circulating CD4+ and CD8+ T cell responses compared to individuals with mild disease, and this reduced T cell response is associated with worse clinical outcomes [128]. However, during the recovery phase, survivors of severe COVID-19 tend to exhibit higher T cell responses compared to those with mild disease [133–135]. This discrepancy likely arises from the prolonged and higher levels of viral antigen exposure in severe cases, leading to a more robust T cell response post-infection. This suggests that while T cells are critical for controlling the virus during the acute phase, the magnitude of T cell responses during recovery reflects the degree and duration of viral exposure.

Additionally, in severe COVID-19 patients, there was an increase in inflammatory cytokines such as IL-6, IL-10 and TNF and recovery was associated with a reduction in these inflammatory cytokines [136]. Sekine et al. showed robust memory T cell responses in convalescent patients who had experienced either asymptomatic or mild COVID-19, suggesting that the severity of symptoms is not necessarily indicative of the strength of the T cell response [137]. However, other studies reveal a more complex relationship between disease severity and immune response. For example, Lucas et al. reported that patients with severe COVID-19 exhibited strong SARS-CoV-2-specific T cell responses, but these T cells often showed signs of functional impairment, including reduced cytokine production and increased expression of exhaustion markers such as PD-1 (programmed cell death protein 1) and CTLA-4 (cytotoxic T-lymphocyte associated protein 4) [138]. Similarly, Mathew et al. found that while severe patients had high levels of virus-specific CD4+ and CD8+ T cells, their functionality was limited, likely contributing to poor viral clearance [134]. This suggests that T cell responses in severe COVID-19 may be robust but are often dysregulated or exhausted, contributing to disease pathology.

A study of the immune responses following a single dose of BNT162b2 vaccine in healthy healthcare workers (HCWs) with and without prior SARS-CoV-2 infection showed an increase in the magnitude of T cell responses compared to baseline [139], demonstrating an immune benefit of vaccination even in those with prior infection. Additionally, T-cell responses were stronger in previously infected individuals after the first vaccine dose compared to infection-naive individuals. Also, SARS-CoV-2 S-specific T-cell responses in previously infected individuals after one dose were similar to those in infection-naive individuals after two doses [139]. This evidence suggests that SARS-CoV-2 infection in healthy individuals elicits a T cell response comparable to a single vaccine dose. A study exploring the role of breakthrough infections in participants who had received two to four vaccine doses showed that breakthrough infections enhance T cell responses, both in magnitude and breadth of antigens recognised [140]. CD4+ T cell responses to S and non-S antigens were boosted following breakthrough infections while CD8+ T cell responses were enhanced to non-S antigens, including M (membrane) and N (nucleocapsid) antigens. The study also showed that breakthrough infections do not lead to increased exhaustion in S-specific CD4+ or CD8+ T cells. Instead, there is a trend toward reduced expression of exhaustion markers, indicating improved T cell functionality and enhanced polyfunctionality in CD4+ T cells. These findings contrast with a study on breakthrough infections which showed that while hybrid immunity confers a more

robust T cell response by inducing more polyfunctional S CD4+ T cells compared to vaccination only, repeated S exposures do not enhance the frequency of S-specific T cell responses [141], with responses plateauing after three exposures either via vaccination or infection.

Antibodies, particularly neutralizing antibodies (NAbs), play a crucial role in protection against SARS-CoV-2 by binding to viral particles, thereby preventing them from infecting host cells. High levels of anti-S and RBD IgG have been associated with lower risks of breakthrough infections, especially in the Delta variant [142]. These antibodies, produced in response to vaccination or prior infection, serve as partial correlates of protection (CoP), where higher titres generally correlate with better protection against symptomatic infection. However, while antibody levels tend to wane over time, breakthrough cases often display lower neutralizing antibody titres at diagnosis compared to controls who remain uninfected, highlighting the importance of maintaining robust humoral immunity [142]. This protective effect is augmented by memory B cells, which can rapidly respond to reinfection by producing specific antibodies, although this response may vary depending on the viral variant involved.

Unlike antibody responses which wane more rapidly, T cell responses are maintained following COVID-19 infection and vaccination [143,144]. Six months post infection, SARS-CoV-2 specific T cell responses are robust and maintained, with both CD4+ and CD8+ T cells present in individuals post recovery [145]. Additionally, many SARS-CoV-2 T cells exhibit a central memory phenotype, indicating their potential long-term persistence and readiness to respond to re-infection [145], with SARS-CoV-2 T cells detected 12 months post infection [146].

Similar to infection, following a second COVID-19 vaccine dose, there is a decline in NAbs while T cell responses are maintained [143]. In addition to persisting for at least six months, the induced T cells survived the physiological contraction of the immune response, highlighting the potential for long-lasting cellular immunity even as antibody levels wane [147]. These T cells also showed markers of immunological maturity and stem cell memory such as CD45RO, CD57 and CD127, highlighting the potential for long-term persistence and quick reactivation upon re-exposure to the virus [147]. Interestingly, booster vaccination significantly enhances antibody responses while the effect on T cell responses is not as pronounced. Following a third vaccine dose, the frequency and magnitude of T cell responses were not significantly boosted,

while there was a significant increase in antibody titres [148], though T cell responses do not exhibit a significant decline compared to antibody responses.

When this project began in January 2021, the SARS-CoV-2 Alpha variant had just emerged, and little was known about the cross-reactivity of T cell responses across SARS-CoV-2 variants. At the time, it was unclear whether T cell immunity induced by the ancestral strain would recognise or respond to emerging variants. This uncertainty paralleled findings from studies on influenza, where cross-reactive T cells targeting conserved internal proteins, like nucleoprotein (NP) and matrix protein (M1), demonstrated protection across different influenza strains, including avian H5N1 and seasonal human H3N2 strains [149]. These cross-reactive T cells in influenza provided partial protection by enhancing viral clearance and reducing illness severity, even without preventing infection entirely. This concept of cross-reactive T cell immunity provided a theoretical framework for hypothesizing that SARS-CoV-2 T cell responses might similarly show resilience to mutational changes in VOCs, a hypothesis that was later confirmed as studies emerged. Although research quickly identified binding and NAbs as CoPs against SARS-CoV-2, as VOCs emerged, particularly Omicron, existing NAbs generated by infection or vaccines based on the ancestral SARS-CoV-2 strain showed significant reductions in neutralization capacity, sparking concerns about vaccine efficacy and the potential for a global surge in COVID-19 mortality [150]. Omicron's extensive immune evasion led to high rates of breakthrough infections despite vaccination, yet mortality rates remained lower compared to earlier in the pandemic, when high infection rates in COVID-naive populations led to substantial mortality. This disparity suggested that factors beyond neutralizing antibodies, such as cross-reactive T cells, might contribute to protection against severe disease and death.

1.5 SARS-CoV-2 T cell Epitopes

Identifying SARS-CoV-2 T cell epitopes is important as these findings have implications for vaccine design, understanding cross-reactivity and immune escape. The repertoire of epitopes identified can inform the design of vaccines that elicit broad and effective T cell responses, understanding of cross-reactivity with other coronaviruses can help study immune responses in diverse populations and knowledge of immunodominant regions. Additionally, knowledge of immunodominant regions and T cell responses is crucial for anticipating and addressing potential immune escape by SARS-CoV-2 variants.

As discussed in previous sections, T cells can recognise epitopes only when these are presented by MHC molecules. During their development in the thymus, T cells undergo positive selection to ensure they can bind to self-MHC molecules [151]. MHC class I molecules present endogenous peptides to CD8+ T cells, while MHC class II molecules on APCs present exogenous peptides to CD4+ T cells. Correspondingly, there are two main types of T cells equipped for binding these MHC classes: CD8+ T cells bind to MHC class I, while CD4+ T cells bind to MHC class II. In humans, HLA is the name for the human version of the MHC. It refers specifically to the gene complex located on chromosome 6 in humans.

HLA molecules are involved in the presentation of peptide antigens to T cells. The HLA system is highly polymorphic, which allows for a diverse range of peptide presentations and a broad immune response [152]. The HLA complex is divided into three regions; Class I HLA includes HLA-A, HLA-B, and HLA-C, which are MHC Class I molecules. Class II HLA includes HLA-DP, HLA-DQ, and HLA-DR, which are MHC Class II molecules. Class III HLA encodes components of the complement system and other immune molecules, but not directly involved in antigen presentation [103,105].

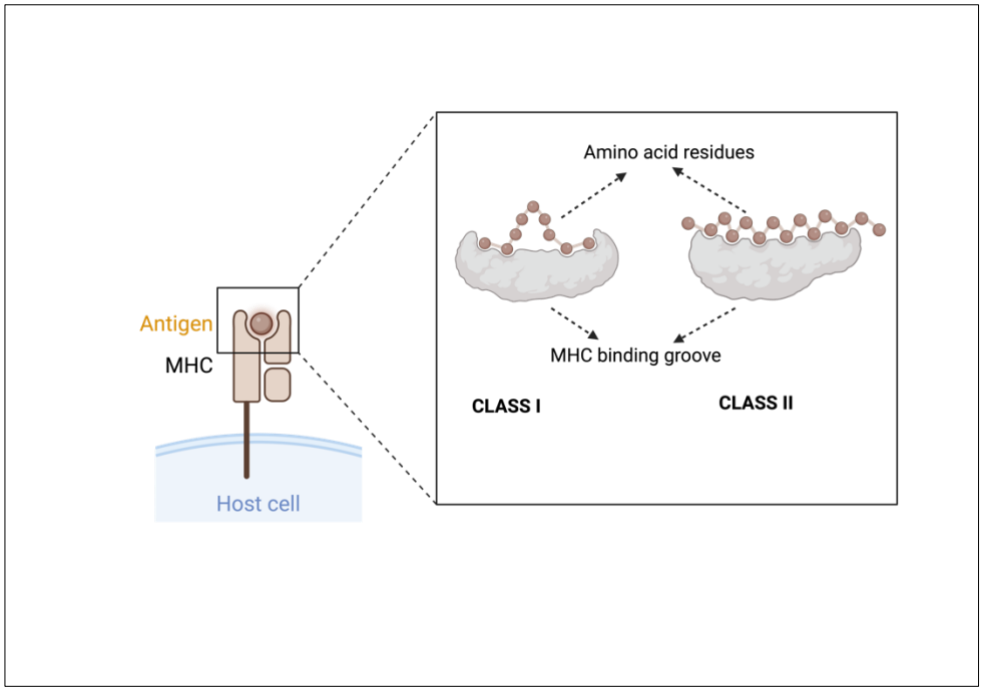


Figure 1.5. Schematic representation of peptides occupying HLA binding sites in class I and class II molecules. Created with Biorender.com

In HLA class I, the terminal ends of the peptide are buried within the MHC binding groove while in class II, the peptide binding site has open ends which allow the peptide to hang out of the groove at both ends as shown in Figure 1.5 [105]. As a result of this, peptides bound by HLA class I are limited to lengths of 9-10 amino acid residues, with several also being 8 or 11 residues depending on HLA restriction [153]. The peptides bound to HLA class II can vary between 12-24 amino acids and sometimes longer. This is because peptides bind to HLA class II by being gripped in the middle, allowing for much longer and more variability in their length [105]. A single complex formed by an HLA molecule and a peptide can be recognised by many different TCRs. These TCRs often have sequences that are similar to each other, indicating a pattern in how they can recognise and bind to the HLA-peptide complex. This implies that there is a level of commonality in the TCRs' sequences that allows them to identify the same HLA-peptide complex [154].

TCRs are composed of two different protein chains, typically referred to as the alpha (α) and beta (β) chains. Each chain has a variable (V) region and a constant (C) region. The V regions of the α and β chains form the antigen-binding site which recognises and bind to the HLA-peptide complex [103]. Despite their specificity, a single TCR can sometimes recognise

multiple HLA-peptide complexes, a phenomenon known as cross-reactivity. This is due to the flexibility in the binding regions and the ability of TCRs to tolerate some variability in the peptides they recognise [155,156]. As mentioned earlier, TCRs can also exhibit degenerate recognition, where different TCRs recognise the same HLA-peptide complex. This often involves shared sequence motifs in the TCRs that allow them to bind similarly to the same complex. The functional implications of recognition of HLA-peptide complexes by TCRs is critical for initiating an immune response. Upon binding, TCRs transmit signals into the T cell, leading to its activation, proliferation, and differentiation into effector cells. Additionally, proper TCR recognition is essential for immune tolerance. Misrecognition or inappropriate activation of T cells can lead to autoimmunity, where the immune system attacks the body's own cells [103,157,158].

An epitope is the specific part of an antigen that is recognised by the TCR. Table 1.4 provides a summary of some studies identifying SARS-CoV-2 epitopes across the SARS-CoV-2 proteome. Most commonly, T cell epitopes are predicted using bioinformatic tools which need to be validated using wet lab T cell experimental techniques. Tools such as NetMHC and those accessible through the IEDB (Immune Epitope Database) platform are widely used for predicting binding affinity and immunogenicity. IEDB provides access to several prediction tools, including NetMHCpan 4.1 EL, NetMHCpan 4.1 BA and others, each offering unique approaches to epitope analysis. For example, these tools can help to predict binding affinity, predict elution rank and immunogenicity. High binding affinity does not always correlate with immunogenicity, which depends on multiple factors including T-cell receptor recognition and peptide processing. Thus, predictions are made in isolation, without considering the biological context, such as the processing and presentation of peptides in the cell. Both NetMHC and IEDB rely on computational predictions, which, while useful, are inherently limited in capturing the true in vivo complexity of the immune system. These models cannot fully account for prior antigen exposure, immune memory, or the nuances of cross-reactivity, leading to potential false positives or negatives. As static models, they may fall short of reflecting the dynamic and adaptive nature of immune responses. Therefore, it is important for bioinformatically predicted epitopes to be validated using in vitro methods such as T cell ELISpot and intracellular cytokine staining assays. In Table 1.4, the number of CD4+ and CD8+ epitopes predicted bioinformatically are shown, the percentage of these epitopes which have been validated using a T cell assay, and the T cell assay used. Additionally, the predicted HLA types from the studies are also highlighted.

Predicted CD4+ epitopes	Predicted CD8+ epitopes	SARS-CoV-2 proteome region	Percentage of epitopes validated using T cell assay	T cell assay used for validation	HLA types identified	Reference
32	35	S, N	4%	T cell line culture Intracellular cytokine staining (ICS)	HLA-A*02:01	Lee et al. 2021[159]
NA	36	S, N	61%	T cell line culture T cell IFNg ELISpot Single cell sequencing Tetramer assays	HLA-A*02:01 HLA-B*07:02	Weingarten-Gabbay et al. 2021[160]
68	48	S, N, E, M	21%	T cell IFNg ELISpot Cytokine release assay Flow cytometry	HLA-A*02:01 HLA-B*07:02 HLA-DRB1*01:01 HLA-DRB1*15:01	Devi et al. 2021[161]
3,372	11,897	S, N, E, M, ORF1a, ORF1b, ORF3a, ORF6, ORF7a, ORF7b, ORF8,ORF10	0.2%	T cell multimer assay Flow cytometry	74 HLA class I alleles HLA-A: 21 alleles HLA-B: 35 alleles HLA-C: 18 alleles 83 HLA class II alleles HLA-DR: 46 alleles HLA-DP: 17 alleles HLA-DQ: 20 alleles	Poran et al. 2020[162]
NA	101	S, E, ORF1a, ORF3a	58%	T cell activation-induced marker (AIM) assay Flow cytometry	15 HLA class I alleles HLA-A: 5 alleles HLA-B: 6 alleles HLA-C: 4 alleles	Federico et al. 2023[163]
284	292	S,M,N,ORF1ab, nsp3, nsp12, nsp13	2%	T cell IFNg ELISpot	16 HLA class I alleles HLA-A: 5 alleles HLA-B: 5 alleles HLA-C: 6 alleles 22 HLA class II alleles HLA-DR: 7 alleles HLA-DQ: 15 alleles	Smith et al. 2021[164]

221	628	S,M,N, nsp3, nsp4, ORF3a, ORF8	NA	T cell activation-induced marker (AIM) assay Flow cytometry Intracellular cytokine staining (ICS)	12 HLA class I; HLA-A and HLA-B	Grifoni et al. 2020[130]
NA	29	S,N,M,E, ORF3a	45%	T cell line culture Flow cytometry Multimer staining assay	5 HLA class I alleles HLA-A: 3 alleles HLA-B: 2 alleles	Meyer et al. 2023[165]
NA	27	S,N,M,E, ORF1ab	33%	T cell IFNg ELISpot Flow cytometry Tetramer staining	HLA-A*02:01 HLA-B*40:01	Agerer et al. 2021[166]
100	20	S,N,E	41%	T cell IFNg ELISpot Flow cytometry T cell line culture	10 HLA class I alleles HLA-A: 5 alleles HLA-B: 4 alleles HLA-C: 1 allele 6 HLA class II alleles HLA-DR: 6 alleles	Nelde et al. 2021[167]
20	25	S,N,M,	22%	T cell IFNg ELISpot Flow cytometry Intracellular cytokine staining (ICS)	HLA-A*02:01 HLA-B*07:02 HLA-DRB1*15:01	Joag et al. 2021[168]
118	53	S,N,M,ORF3a,ORF7a, ORF8	83%	T cell IFNg ELISpot Intracellular cytokine staining (ICS) Tetramer staining	7 HLA class I alleles HLA-A: 4 alleles HLA-B: 3 alleles 7 HLA class II alleles HLA-DR: 5 alleles HLA-DQ:2 alleles	Peng et al. 2020[131]
91	51	S,N,M	32%	T cell IFNg ELISpot Intracellular cytokine staining (ICS) Tetramer staining	20 HLA class I alleles HLA-A: 6 alleles HLA-B: 8 alleles HLA-C: 6 alleles 13 HLA class II alleles HLA-DR: 8 alleles HLA-DQ:5 alleles	Le Bert et al. 2020[169]

Table 1.4. Summary of key studies highlighting identified T cell Epitopes. SARS-CoV-2 T cell studies highlighting the percentage of epitopes validated using T cell assays, the region of the SARS-CoV-2 proteome where the identified epitopes lie, the T cell assays used, and the HLA associations identified.

1.6 Overall Aims of this Thesis

In this thesis, I aimed to investigate how prior SARS-CoV-2 infection influences vaccine-induced T cell responses using peripheral blood samples collected from a cohort of COVID-19 vaccinated individuals. Additionally, I hypothesised that T cell responses would demonstrate cross-reactivity to SARS-CoV-2 variants of concern (VOCs), even in cases where neutralizing antibody responses may wane or be less effective. Furthermore, I sought to identify specific T cell epitopes associated with enhanced immune protection, particularly within certain HLA types. The overall objectives of the thesis are outlined below.

1. Understand T cell responses to SARS-CoV-2 vaccines in naïve and previously infected individuals (Chapter 3)

2. Define the impact of SARS-CoV-2 variants on T cell responses in SARS-CoV-2 vaccinated cohorts (Chapter 4)

3. Explore the breadth of SARS-CoV-2 responses in SARS-CoV-2 vaccinated cohorts and identify T cell epitopes associated with a protective Human Leukocyte Antigen allele (Chapter 5)

Chapter 2: Materials and Methods

2.1 Study Cohort and Ethics Approval

Ethics statement

Ethical approval was obtained for all work described in this thesis, as reported in the corresponding publications [143,170]. The protective immunity from T cells in healthcare workers study (PITCH) is a sub-study of the SARS-CoV-2 immunity and reinfection evaluation (SIREN) study, which was approved by the Berkshire Research Ethics Committee, Health Research 250 Authority (IRAS ID 284460, REC reference 20/SC/0230), with PITCH recognised as a sub-study on 2 December 2020. SIREN is registered with ISRCTN (Trial ID:252 ISRCTN11041050). The PITCH study recruits participants from across 6 study sites in the United Kingdom: Oxford, Newcastle, Birmingham, Sheffield, Liverpool and Cambridge. In Oxford, participants were recruited under the GI Biobank Study 16/YH/0247, approved by the research ethics committee (REC) at Yorkshire & The Humber - Sheffield Research Ethics Committee on 29 July 2016, which has been amended for this purpose on 8 June 2020. The study was conducted in compliance with all relevant ethical regulations for work with human participants, and according to the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) guidelines. Written informed consent was obtained for all participants enrolled in the study.

Study cohort

Healthcare workers (HCWs), including students and volunteers in PITCH study sites were recruited primarily by word of mouth, hospital e-mail communications and hospital-based staff screening programs. Participant samples were collected from December 2020 and at successive timepoints following SARS-CoV-2 infection and vaccination. Figure 2.1 shows the demographic breakdown of the PITCH Oxford study cohort. There is likely to be a sampling bias in the study cohort as the recruitment methods used may not have reached a representative sample of all HCWs, potentially favouring those who are more engaged with hospital communications or who have easier access to the recruitment channels. This could result in an overrepresentation of certain groups within the healthcare workforce (for example those interested in vaccination and SARS-CoV-2 prevention) and underrepresentation of others (for

example those with the busiest front-line jobs), which may impact the generalisability of study findings.

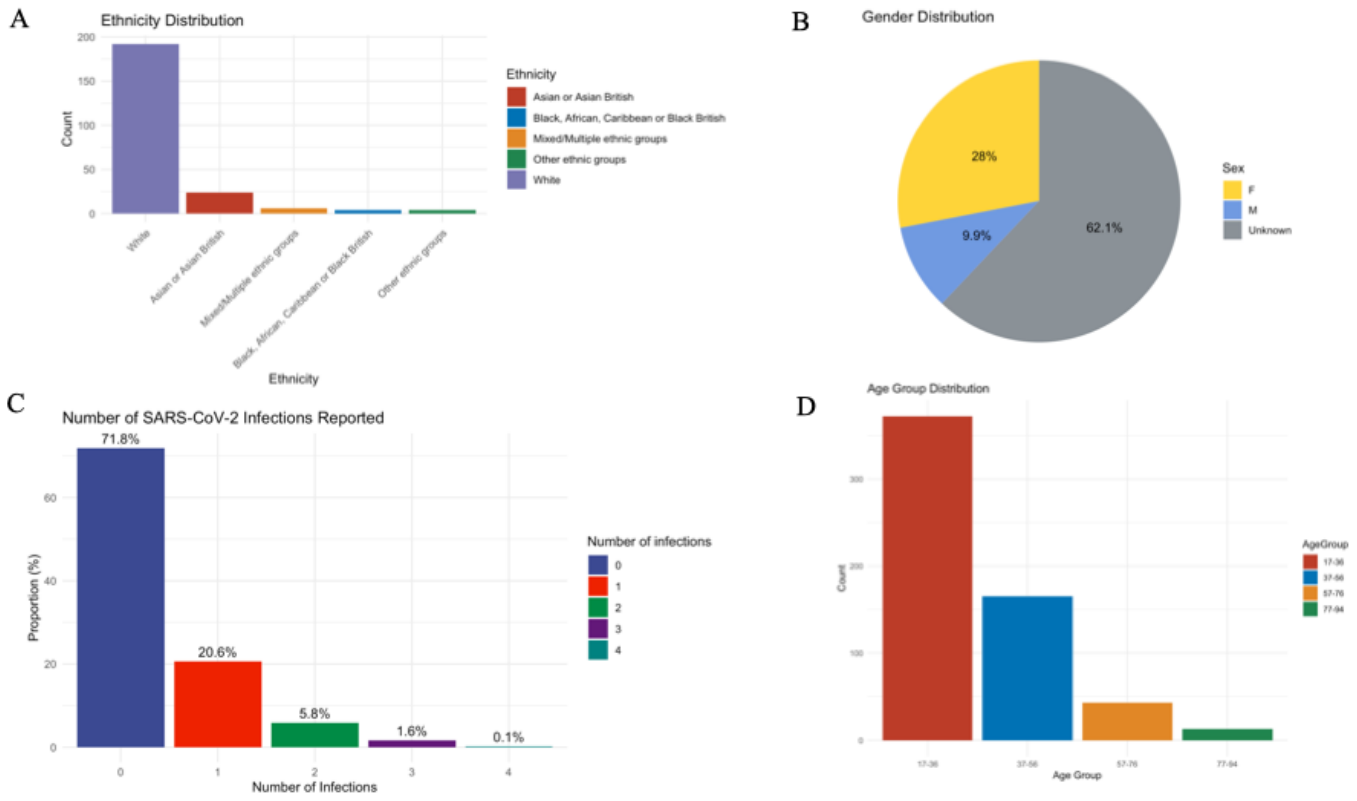


Figure 2.1. Demographics of PITCH Oxford study cohort. (A) ethnicity distribution of study cohort (B) gender distribution (C) number of reported SARS-CoV-2 infections and (D) age group distribution.

2.2 Sample Collection and Processing

Sample collection and processing have previously been described [133]. Briefly, whole blood samples were collected from study participants at designated timepoints, transferred to the laboratory at room temperature and processed within 4 hours of blood draw where possible (and always within the same working day). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using LymphoprepTM (1.077 g/ml, Stem Cell Technologies). Plasma was collected and spun at 2000 × g for 10 min to remove platelets before freezing at −80 °C for later use. PBMCs were collected and washed twice with pre-warmed R10 media: RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated FCS (Sigma), 1 mM Pen/Strep and 2mM L-Glutamine (both from Sigma). After the second

centrifugation, cells were resuspended in freezing media, 10% DMSO and 90% heat-inactivated FCS and stored at −80 °C overnight and transferred to liquid nitrogen. Prior to assays, frozen cells were thawed at 37 °C and left to rest for 1-2 hours at 37 °C in R10 media supplemented with benzonase.

2.3 SARS-CoV-2 Peptides and Matrix Design

Peptide design

Peptides corresponding to SARS-CoV-2 wildtype (ancestral strain), Beta, Gamma, Delta and Omicron peptides, 15–18 amino-acids overlapping by 10 amino-acids and spanning the entire S region, were used in T cell assays. S peptides were deconvoluted into in two pools, S1 and S2 (Mimotopes, Australia). In total, there were 178 S peptides. The S sequence for the peptides used in this thesis are shown in Appendix 1. To design variant peptides, SARS-CoV-2 wildtype sequences were download from GenBank, the National Institute of Health (NIH) genetic sequence database. Using the bioinformatic tools MAFFT and AliView, mutations as reported by WHO were included, and a variant sequence generated.

This alignment process allowed for the identification of mutations affecting T cell epitopes, and adjustments were made to ensure robust T cell engagement. For each peptide, toggling rules were applied to modify the C-terminal end if necessary; peptides were truncated down to 15 amino acids when they ended with non-binding residues to optimize HLA class I binding potential. Additionally, regions with significant deletions or insertions were carefully analysed, and extra peptides were designed to fully capture these changes without altering the overlapping pattern of 10 amino acids, ensuring comprehensive representation of variant-specific epitopes. The resulting peptide libraries aimed to balance the need for mutation coverage with structural requirements for effective immune recognition.

2.4 T cell ELISpot Assays

The T cell interferon-gamma (IFNγ) ELISpot assay has been described previously in [133,143,171]. Briefly, IFNγ ELISpot assays were set up from cryopreserved PBMCs using the Human IFNγ ELISpot Basic kit (Mabtech 3420-2A). PBMCs were thawed and rested for 2 hours in R10 media: RPMI 1640 (Sigma) supplemented with 10% (v/v) Fetal Bovine Serum (Sigma), 2mM L-Glutamine (Sigma) and 1mM Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C, 5% CO_2, prior to stimulation with peptides. PBMCs were then plated in duplicate or triplicate at 200,000 cells/well in a MultiScreen-IP filter plate (Millipore, MAIPS4510) previously coated overnight with capture antibody (clone 1-D1K) and blocked with R10. PBMCs were then stimulated with the relevant peptide stimulants at a final concentration of 2 mg/ml for 16 to18 hours in a humidified incubator at 37 °C, 5% CO_2. Pools consisting of CMV, EBV and influenza peptides at a final concentration of 2mg/ml (CEF; Proimmune) and concanavalin A were used as positive controls. DMSO was used as the negative control at an equivalent concentration to the peptides. After the incubation period as well as all subsequent steps wells were washed with PBS/0.05% (v/v) Tween20 (Sigma). Secreted IFNγ was detected by adding 1 μg/ml anti-IFNγ biotinylated mAb (7-B6-1-biotin, Mabtech) for 4 hours, followed by 1 μg/ml streptavidin alkaline phosphatase for 1–2 h (SP-3020, Vector Labs). The plates were developed using BCIP/NBT substrate (Pierce) for 5 minutes at room temperature. Colour development was stopped by washing the wells with cold tap water. Plates were dried for a minimum of 24 hours in the dark and dried plates were scanned and analysed with either the AID Classic ELISpot reader (version 4.0) or the CTL ImmunoSpot analyser. Antigen-specific responses were quantified by subtracting the mean spots of the negative control wells from the peptide stimulant wells and the results were expressed as spot-forming units (SFU)/10^6 PBMCs. Samples with a mean spot value greater than 50 SFU/10^6 spots in the negative control wells were excluded from the analysis. This was done to ensure the reliability and accuracy of the assay results, as elevated background responses may indicate nonspecific activation potentially confounding the interpretation of antigen-specific T cell responses.

Matrix design

A peptide matrix was used to efficiently screen all 178 SARS-CoV-2 peptides and identify specific peptide sequences that are recognised by T cells (Chapter 5). The matrix design

included 10 columns-pools 1 to 10 and 18 rows, pools 11 to 38. Each pool contained 8-10 individual peptides, and each peptide appears twice in the matrix and never in the same pool to ensure optimal coverage and minimize redundancy. Table 2.1 shows a sample peptide matrix. Here, a positive T cell ELISpot response in pools 3 and 6 denote peptide 5 is likely contributing to that response as it is the only peptide common to both pools. A positive response was set as the background value (negative control, DMSO) + 2 standard deviations.

Pool 1	Pool 2	Pool 3	Pool 4
Pool 5	Peptide 1	Peptide 2	Peptide 3
Pool 6	Peptide 4	Peptide 5	Peptide 6
Pool 7	Peptide 7	Peptide 8	Peptide 9

Table 2.1. Sample peptide matrix design

2.5 Proliferation Assays

The proliferation assay has been described in [143,172]. Briefly, PBMCs from cryopreserved samples were washed twice with 1× phosphate buffered saline (PBS) and stained using CellTrace® Violet (CTV, Life Technologies) at a final concentration of 2.5 μM for 10 min at room temperature. The reaction was quenched by adding cold fetal bovine serum (FBS). CTV-labelled PBMC in RPMI containing 10% human AB serum (Sigma), 1 mM Pen/Strep and 2mM L-Glut were plated in 96-well round-bottom plates at 250,000 cells and stimulated with SARS-CoV-2 peptide pools (1 μg/ml per peptide). Media containing 0.1% DMSO (Sigma) representing DMSO content in peptide pools were used as negative control and 2 μg/ml phytohemagglutinin L (PHA-L, Sigma) as used as a positive control. Cells were subsequently incubated at 37 °C, 5% CO_2, 95% humidity for 7 days with media change on day 4. At the end of incubation, cells were subjected to flow cytometry staining. The staining panel is shown in Table 2.2. Responses above 1% were considered true positive. To determine the magnitude of the total response to SARS-CoV-2 proteins, the number of cells proliferating in response to any of the peptides was obtained as a function of their respective CD4+ or CD8+ T cell population and then expressed as a percentage. The background was then subtracted from the total response for each volunteer.

Fluorochrome	Marker	Concentration
FITC	CD3	1:50
PECy7	CD8	1:200
APC	CD4	1:200
NiR	Live/dead	1:1000

Table 2.2. CTV proliferation assay staining panel

2.6 Intracellular Cytokine Staining

On Day 0, PBMCs were thawed and rested for 4 hours at 37°C with 5% CO_2. During this period, SARS-CoV-2 peptides and co-stimulants, CD28 and CD49d and positive controls, 1x PMA/Ionomycin are prepared and equilibrated at 37°C. After the rest period, peptides and co-stimulants are added to the appropriate wells of a 96-well plate and the PBMCs adjusted to the desired concentration (1x 10^6 PBMC/well per peptide pool and 0.5-1x10^6 PBMC/well for positive and negative controls (DMSO, PMA/I) and plated. One hour after plating, Brefeldin A (BFA) is added to block cytokine secretion, and the cells are incubated overnight.

The following day, the cells are stained extracellularly (ECS) and intracellularly (ICS). Firstly, the cells are first stained with a live/dead stain and surface markers, followed by incubation and washing steps with cell staining buffer. The stained cells are incubated in the dark at 4°C for 20 minutes. Next, the ICS process involves fixing and permeabilizing the cells, using 1x BD Cytofix/Cytoperm and 10x BD Perm/Wash. Fixed cells are stained with intracellular markers, for 20 minutes at 4°C. The ECS/ICS staining panel is shown in Table 2.3. Stained cells were then acquired using the MACSQUANT flow cytometer.

Fluorochrome	Marker	Concentration
NiR	Live/dead	1:1000
APC-Fire 750	CD14	1:200
BV421	CD154	1:100
PerCP	CD3	1:100
APC	CD4	1:200
BV510	CD8	1:600

FITC	TNF	1:40
PE	IFNg	1:50
PECy7	IL-2	1:100

Table 2.3. Intracellular cytokine staining panel

2.7 In Vitro T cell Lines

T cell lines were cultured for 14 days. On day 0, cryopreserved PBMCs were thawed and rested in cell media for 2 hours. 5ug/ml of SARS-CoV-2 peptides were used, and cells were plated at 5 x 10^6 cells in a 12 well plate or 2.5 x 10^6 cells in a 24 well plate. 10ug/ml IL-2 (Roche) was added to the cell media. Cell media was changed on days 3, 7 and 11, by removing 500ul of cell media and replacing with an equal volume of cell media containing IL-2. On day 14, cells were washed thrice in cell media without IL-2 and rested overnight in cell media without IL-2 and harvested.

2.8 Statistical Analysis

Data were analysed using GraphPad prism, version 10 and statistical test differences were considered significant if $p < 0.05$ (two-tailed). Nonparametric tests were used to compare differences between groups. For pairwise comparisons, the Mann-Whitney test was used to compare differences between two independent groups and Wilcoxon signed-rank tests for paired data. Additionally, one-way ANOVA was employed to compare differences across multiple groups when parametric assumptions were met, and chi-squared tests were used to evaluate categorical variables. Flow cytometry data was analysed using FlowJo version 10.

Chapter 3: Impact of Dosing Intervals on T cell Responses in Naïve and Previously Infected Individuals

Aim: To understand how varying dosing intervals impact T cell responses to SARS-CoV-2 vaccines in naïve and previously infected individuals

Hypothesis:

- Extending the dosing interval of SARS-CoV-2 vaccines can lead to increased polyfunctional T cell responses.

- Prior SARS-CoV-2 infection has a lasting impact on T cell responses, contributing to an increase in magnitude of T cell responses.

Abstract

This chapter investigates the impact of varying dosing intervals on T cell responses to the SARS-CoV-2 BNT162b2 mRNA vaccine in both naïve and previously infected individuals. Specifically, the objective was to determine if extending the dosing interval influences T cell polyfunctionality and magnitude, and how prior SARS-CoV-2 infection modulates these responses.

T cell responses in a cohort of healthcare workers receiving the BNT162b2 vaccine, divided into short (2–5 weeks) and long (6–14 weeks) dosing interval groups were analysed. T cell responses to SARS-CoV-2 spike and other viral peptides were measured using IFNγ ELISpot and intracellular cytokine staining assays. Additionally, T cell proliferation was assessed with a CTV assay six months after the second dose to evaluate memory response durability.

In naïve individuals, extending the dosing interval resulted in a more robust and polyfunctional CD4+ T cell response, marked by higher levels of IFNγ and IL-2 production, which may contribute to improved humoral immunity. However, it is associated with slightly lower overall T cell response magnitude as measured by ELISpot and reduced CD8+ T cell IFNγ responses compared to the shorter interval. T cell responses were detectable four weeks after the first dose in naïve individuals, increasing further after the second dose. In contrast, previously infected individuals exhibited a higher baseline of T cell responses, with minimal differences observed between short and long dosing intervals. At six months post-second dose, T cell responses remained strong across all groups, with previously infected individuals maintaining higher response levels than naïve individuals.

This study demonstrates that extending the dosing interval of the BNT162b2 vaccine enhances T cell polyfunctionality in naïve individuals, potentially contributing to more durable immune memory, although by six months after the second dose, differences in T cell magnitude or functionality were no longer detectable. Prior infection mitigates the impact of dosing intervals, suggesting that hybrid immunity provides a robust foundation for maintaining T cell responses. These findings underscore the importance of considering dosing intervals in vaccination strategies, particularly for naïve individuals, to optimize cellular immune responses.

Contributions

Work presented in this chapter has previously been published in the following papers;

Payne, Rebecca P., Stephanie Longet, James A. Austin, Donal T. Skelly, Wanwisa Dejnirattisai, **Sandra Adele**, Naomi Meardon et al. "Immunogenicity of standard and extended dosing intervals of BNT162b2 mRNA vaccine." *Cell* 184, no. 23 (2021): 5699-5714.

In this study, I contributed significantly to the T cell analysis as part of our role as the lead site for T cell work. I was responsible for conducting ELISpot assays within the Oxford PITCH site on samples from our cohort to assess T cell responses to varying dosing intervals. Additionally, I performed the initial data analysis and prepared figures for inclusion in the manuscript. In particular, Figures 3.2 , 3.3 and 3.4 were derived from the data I generated and analysed.

Moore, Shona C., Barbara Kronsteiner, Stephanie Longet, **Sandra Adele***, et al. "Evolution of long-term vaccine-induced and hybrid immunity in healthcare workers after different COVID-19 vaccine regimens." *Med* 4, no. 3 (2023): 191-215.

**Joint first author*

In this publication, I played a central role in the T cell response component of the research, with our site designated as the lead for T cell analysis. My specific contributions included conducting ELISpot and CTV assays, performing peptide design, and leading both site-wide and site-specific analyses within the PITCH cohort. I independently analysed resulting T cell data with Figures 3.5, 3.6 and 3.7 included in the publication.

3.1 Introduction

The 2019 SARS-CoV-2 pandemic led to the development of several vaccines at a rapid pace. The first vaccines were administered in December 2020, with several cohort studies running concurrently to further understand the characteristics of the vaccines such as magnitude of antibody and T cell responses, vaccine effectiveness and adverse effects. In the United Kingdom, three vaccines were being administered: BioNTech/Pfizer's BNT162b2 vaccine, Moderna's mRNA-1273 vaccine and AstraZeneca's AZD1222 vaccine. Determining the optimal dosing interval of the vaccines was crucial as the dosing interval can significantly affect the immune response and overall vaccine efficacy. Different vaccines have recommended intervals: the BNT162b2 vaccine initially recommended a three-week interval, the mRNA-1273 vaccine suggested four weeks and the AZD1222 vaccine suggesting four to twelve weeks [173–175]. Furthermore, with an increasing number of SARS-CoV-2 infections occurring, there was a need to study the impacts of immunity on naïve and previously infected cohorts and what these findings may imply for infection and vaccine acquired immunity and vaccine dosing regimens.

Extended dosing intervals and SARS-CoV-2 immune responses

In response to the UK government's announcement in January 2021 to delay the second SARS-CoV-2 vaccine dose, we initiated an investigation into the impact of varying dosing intervals on cellular immune responses. Although our study, published in November 2021, was among the first to provide robust data on this topic, preliminary findings were shared in a preprint during the summer of 2021, and some other reports emerged around the same time from similar studies. Thus, the work highlighted in this chapter contributed to providing key evidence that drove policy decisions in the UK surrounding vaccine dosing regimens. When the UK government announced plans in January 2021 to extend the interval between vaccine doses, we had already collected samples from HCWs who received the BNT162b2 vaccine on a short dosing interval. This positioned us uniquely to investigate the effects of varying dosing intervals on immune responses, especially in the context of T cell responses, where limited data existed at the time. Although subsequent studies have also examined antibody responses and vaccine effectiveness at different dosing intervals, our study was among the first to report on both antibody and T cell responses within this framework. These findings, along with other studies on antibody responses, are discussed further in the discussion section to provide broader context on the implications of extended dosing intervals for vaccine efficacy and effectiveness.

The lack of studies examining dosing intervals highlighted the need for more research, particularly using a large sample size to guide policy decisions. Secondly, there was also a need to understand the dynamics of the cellular response regarding dosing intervals, as the dynamics and kinetics of the T cell responses and antibody responses may differ. Our research which focused on understanding the T cell and antibody responses in a large cohort of healthcare workers who had been vaccinated using a short or extended dosing interval was timely and critical in addressing research gaps.

Long-term impacts of varying dosing intervals and hybrid immunity

By December 2020, when the first SARS-CoV-2 vaccines were administered, the pandemic had been ongoing for approximately a year. By this time, 79 million SARS-CoV-2 infections had been reported globally [176]. By November 2021, the number of people who had been infected with SARS-CoV-2 at least once varied significantly across different countries, ranging from less than 20% in 39 countries to over 70% in 40 countries [177]. Therefore, in addition to understanding the optimal dosing strategy, there was also a need to understand the long-term impact on immune responses of previous SARS-CoV-2 infection on vaccination (hybrid immunity) and if these impacts varied by dosing intervals. Studies have demonstrated that unvaccinated individuals who have had a prior SARS-CoV-2 infection develop SARS-CoV-2-specific neutralizing antibodies and T cell responses [178,179] and these immune responses provide a significant level of protection against reinfection and severe outcomes. Infection induces humoral and cellular immune responses to a broader array of viral antigens, including S, M and N proteins while vaccine mediated immunity is directed against the S protein alone [180]. Immune memory to SARS-CoV-2 can develop through infection, vaccination, or hybrid immunity. Immune memory formed from infection provided significant protection against ancestral strain, with studies indicating 80%–95% protection against symptomatic COVID-19 reinfections for at least eight months [181]. As the SARS-CoV-2 virus continued to evolve, the VOCs posed challenges to vaccine effectiveness. These variants carried mutations, particularly in the S protein, which is the primary target of vaccine-induced immune responses. Studies demonstrated that the neutralizing antibody titres generated by vaccination against the ancestral strain showed reduced potency against these new variants, especially the Omicron variants, leading to decreased VE over time [150,182,183]. For instance, VE against symptomatic infection dropped significantly with Omicron, even among those who had received two doses, raising concerns about the potential for high infection rates. Despite this decline in neutralizing

antibody effectiveness, T cell responses generated by vaccination or prior infection appeared more robust across variants, as they targeted more conserved regions of the virus. This cross-reactive T cell immunity likely contributed to lower severe disease and mortality rates despite high transmission rates of Omicron [184]. Understanding the combined protective effects of hybrid immunity and booster doses has become essential for public health strategies to manage breakthrough infections and sustain protection against severe outcomes. Studying hybrid immunity is crucial for elucidating how the broad immune responses generated by infection can be complemented and enhanced by the targeted protection from vaccination, potentially optimizing immunity across diverse SARS-CoV-2 variants.

Since the emergence of SARS-CoV-2, multiple variants of concern VOCs have risen, each with mutations that impact viral transmission, immune evasion, and vaccine effectiveness (VE). Notably, variants such as Alpha, Beta, Delta, and Omicron have been associated with varying degrees of reduced VE, especially against symptomatic infection, though VE against severe outcomes remains more robust [185]. For example, studies have shown that the Delta variant can partially evade neutralizing antibodies, resulting in a moderate decrease in VE against infection but generally preserved protection against severe disease [186]. The Omicron variant, with numerous mutations in the spike protein, has led to a more pronounced reduction in VE against infection, even in individuals with hybrid immunity [187]. These variants underscore the importance of studying hybrid immunity, as prior infection combined with vaccination may offer a broader immune response that could mitigate the impacts of these mutations. Understanding how hybrid immunity adapts to new variants can inform vaccine strategies to optimize protection in the face of evolving viral challenges.

3.2 Results

Research questions

1. Do T cell responses after two doses of the mRNA vaccine BNT162b2 differ between standard (2-5 weeks) and extended (6-14 weeks) SARS-CoV-2 vaccine dosing intervals in naïve individuals and those with previous infection?
2. What is the impact 6 months later in cohorts receiving different vaccine dosing intervals, with varying infection status (hybrid immunity) on T cell responses?

3.2.1 Cohort

The PITCH HCW study cohort was used to address my research questions and has been described in chapter 2. To address the first research question on the impact of extended dosing intervals on SARS-CoV-2 immune responses, 589 participants were studied who had been vaccinated using the short interval regimen (n=86) or the long interval regimen (n=503) between December 2020 and May 2021. The short and long dosing interval cohorts were further characterized based on infection history, using a combination of PCR, lateral flow tests (LFTs), and serology results. In 2021, cohort members underwent regular PCR screening and had access to PCR testing when symptomatic. Additionally, from late summer 2021, they also had access to LFTs. Infection was defined as a previous positive PCR or LFT result and/or anti-nucleocapsid (anti-N) seropositivity above a specified threshold 3,874 AU/ml on the MSD assay, indicating prior exposure to SARS-CoV-2. This approach allowed for clear differentiation between naïve and previously infected individuals in the cohort, facilitating more accurate comparisons of immune responses across different dosing intervals (Figure 3.1A). Demographic information about study participants is shown in Table 3.1. Whole blood was taken from each donor at several timepoints (Figure 3.1B) with PBMCs, serum and plasma being collected.

	All participants	BNT162b2 Short 2 - 5 Weeks	BNT162b2 Long 6 - 14 Weeks
Dosing Interval			
Median Days	70	23.5	71
Median Weeks	10.00	3.36	10.14
IQR (Days)	63-77	21-27	64-77
Range (Days)	14-105	14-35	45-105
N	589	86	503
Female, N (%)	431 (73%)	45 (52%)	386 (77%)
Male, N (%)	151 (26%)	36 (42%)	115 (23%)
Unreported, N	7	5	2
Age			
Mean Age	42.30	44.96	41.87
Age in years, Median (IQR)	43 (32-52)	45 (37-54)	43 (31-51)
Age Range	21-71	22-64	21-71
Infection Status			
Naïve, N (%)	334 (57%)	57 (66%)	277 (55%)
Previous SARS-CoV-2, N (%)	255 (43%)	29 (34%)	226 (45%)
Ethnicity			
White, N (%)	410 (85%)	58 (84%)	352 (85%)
Asian, N (%)	45 (9%)	7 (10%)	38 (9%)
Black, N (%)	7 (1%)	0 (0%)	7 (2%)
Other, N (%)	20 (4%)	4 (6%)	16 (4%)
Unreported, N	107	17	90

Table 3.1. Characteristics of the PITCH study cohort for extended dosing intervals. Characteristics of the PITCH for studying the impact of extended dosing intervals. Participants had received two doses of BNT162b2 vaccine.

To address the second research question aimed at understanding the long-term impacts of varying dose intervals and hybrid immunity on SARS-CoV-2 immune responses, 684 participants who had received three vaccine doses were studied from 6 months after the second dose (June to December 2021) until 1 month after the third dose (December 2021 to January 2022) [143]. Of the 684 participants, 342 were infection-naïve and 342 had been previously infected with SARS-CoV-2 at the V2+6 months timepoint (Figure 3.1A). Within the naïve and previously infected cohorts, participants were further characterized within vaccine regimen type; AZD1222 (n=92), BNT162b2 short dosing interval (n=84) and BNT162b2 long dosing interval (n=514). For the third dose, all participants received an mRNA vaccine. Demographic information about study participants is shown in Table 3.2.

	All participants	Short 2 - 5 Weeks	Long 6 - 14 Weeks	AZD1222
Dosing Interval				
Median Days	71	24	71	74
Median Weeks	10	3	10	11
IQR (Days)	63-77	21-27	66-78	64.75-78
Range (Days)	14-158	0:38	0:120	53:158
N	684	84	508	92
Female, N (%)	505 (73.8%)	50 (59.5%)	387 (76.2%)	387 (76.2%)
Male, N (%)	179 (26.2%)	34 (40.5%)	121 (23.8%)	121 (23.8%)
Age				
Age in years, Median (IQR)	33-52.3	37-55	33-51.25	27-56
Age Range	22-77	22-71	21-71	22-77
Infection Status				
Naïve, N (%)	342 (50.0%)	49 (41.7%)	387 (76.2%)	45 (51.1%)
Previous SARS-CoV-2, N (%)	342 (50.0%)	35 (58.3%)	121 (23.8%)	47 (48.9%)
Ethnicity				
White, N (%)	464 (83.8%)	56 (84.8%)	337 (84.5%)	71 (79.8%)
Asian, N (%)	56 (10.1%)	5 (7.6%)	39 (9.8%)	12 (13.5%
Black, N (%)	7 (1.3%)	1 (1.5%)	6 (1.5%)	0 (0.0%)
Other, N (%)	27 (4.9%)	4 (6.1%)	17 (4.3%)	6 (6.7%)
Unreported, N	130	18	109	3

Table 3.2. Characteristics of the PITCH study cohort for the long-term impacts of varying dose intervals and hybrid immunity.

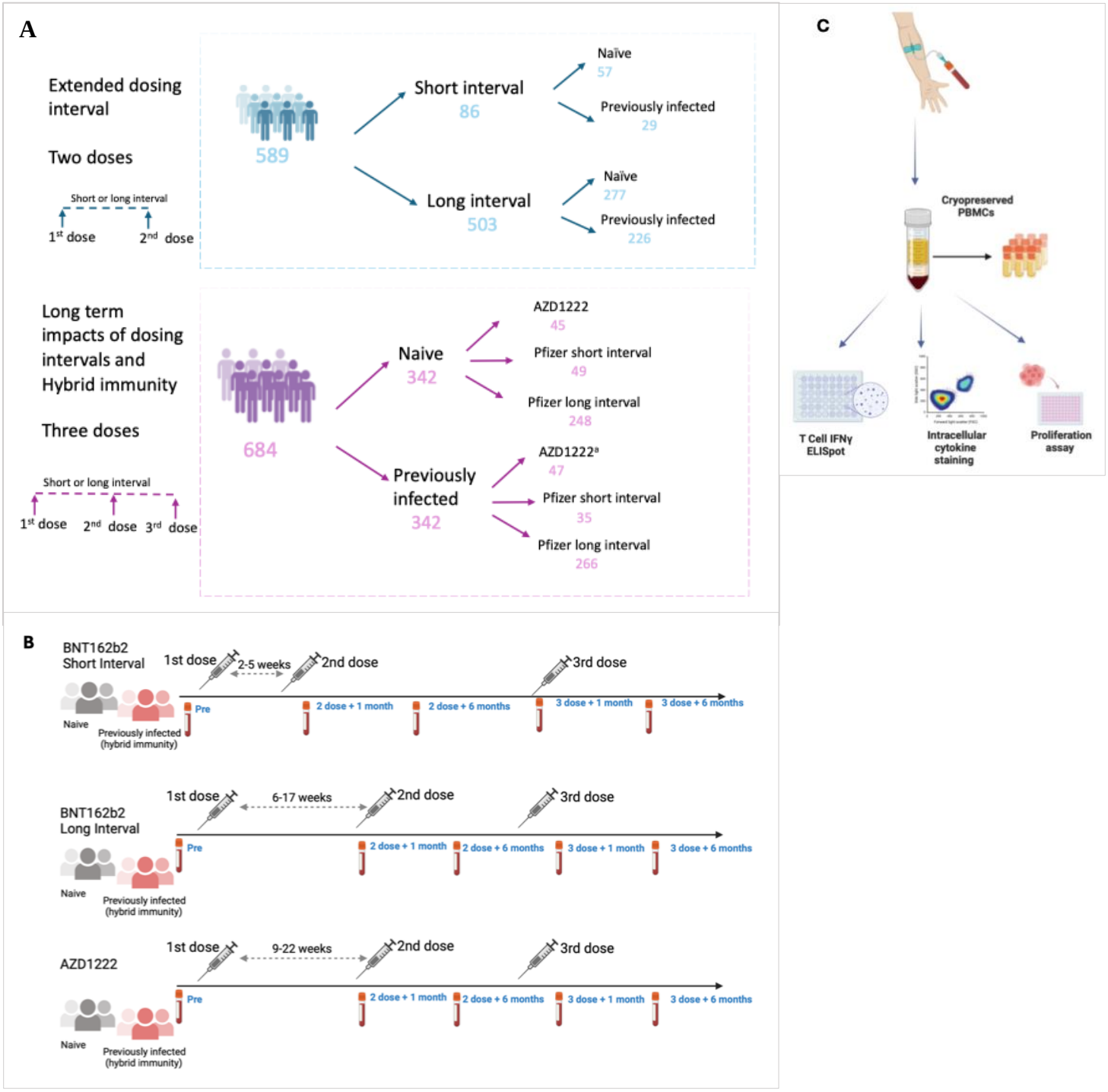


Figure 3.1. Schematic representation of the study cohort. (A) Breakdown of various groups in the study cohort, (B) sampling timeline of various vaccine regimens and (C) T cell experiment flow. [a]six participants seroconverted during the study.

3.2.2 T cell responses to SARS-CoV-2 peptides in naive and previously infected individuals receiving two doses of BNT162b2 with a long dosing interval

To characterize T cell responses in this cohort, T cell responses to SARS-CoV-2 whole spike (178 peptides) and M +N (86 peptides) were measured using an IFNγ ELISpot assay. In naïve individuals, T cell responses specific to SARS-CoV-2 were lower prior to vaccination compared to individuals with prior infection, (Figure 3.2 A and B) indicating no pre-existing immunity against the virus. Four weeks after the first vaccine dose, T cell responses increased in magnitude in naïve individuals, demonstrating that the vaccine effectively primes the immune system in those without prior infection (Figure 3.2 A and B). A further increase in T cell response magnitude was observed following the second dose, highlighting the benefit of an additional vaccine dose in enhancing immune responses in naïve individuals (Figure 3.2A).

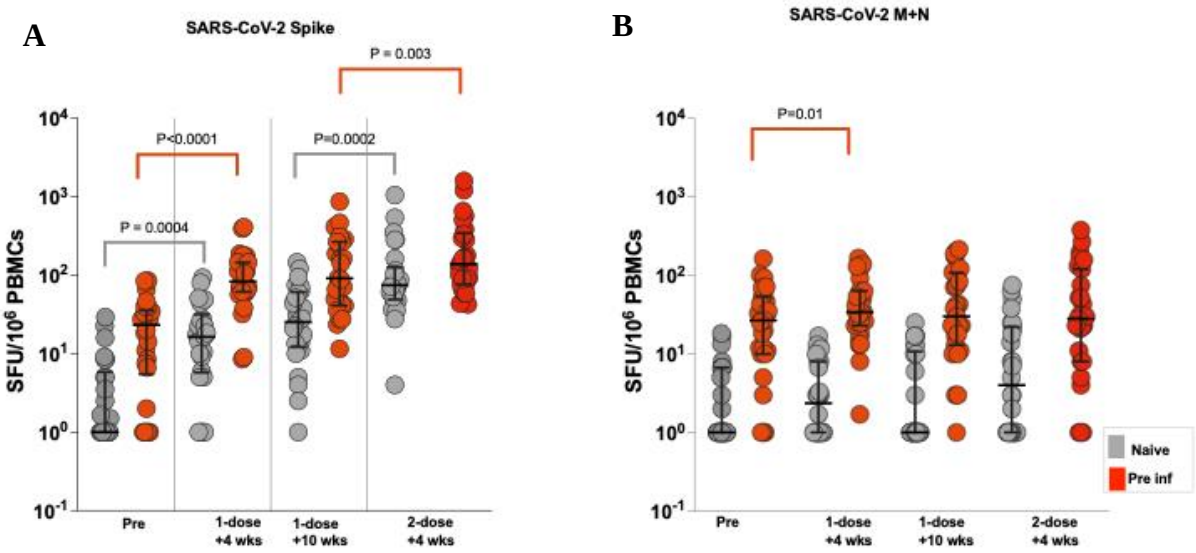


Figure 3.2. T cell responses following long interval dosing regimen. Comparison of IFNγ ELISpot responses to peptides representing SARS-CoV-2 Spike protein (A) and SARS-CoV-2 Membrane and Nucleocapsid proteins (M and N) (B) proteins in 26 naive and 26 previously infected individuals, following a long interval between vaccine doses. Data are presented as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs). Grey circles represent responses in naive individuals, while red circles represent responses in previously infected individuals. Time points include pre-vaccination (Pre), 4 weeks after the first dose (1-dose + 4 weeks), 8–12 weeks after the first dose (1-dose + 10 weeks), and 4 weeks after the second dose (2-dose + 4 weeks). Median values are shown with interquartile ranges as bars. Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test.

In contrast, individuals who had prior exposure to SARS-CoV-2 (Pre inf) start with a higher baseline of T cell responses compared to naïve individuals before vaccination. This was expected, as several studies including those by my research group had already highlighted the T cell responses in convalescent, unvaccinated individuals[131,133,137]. Following vaccination, these individuals also experienced an increase in T cell responses, particularly after the first dose, with a significant rise observed four weeks post-vaccination. The rise in T cell responses observed, indicates that vaccination boosts the existing immune response in those who had prior exposure (Figure 3.2 A and B).

Assessing the SARS-CoV-2 M and N responses among naive individuals, as expected, the baseline T cell response to M+N proteins is low, similar to that observed for the S protein. In contrast, previously infected individuals had detectable M+N responses, indicating this measurement confirms infection status (Figure 3.2B). In individuals with prior infection, there is a detectable T cell response to M+N peptides even before vaccination, which further increases following the first vaccine dose. The presence of an M+N immune response prior to vaccination suggests prior exposure to endemic coronaviruses, such as seasonal coronaviruses. Also, the presence of an M+N immune response prior to vaccination in naïve individuals could suggest prior undetected SARS-CoV-2 exposure, where infection went unnoticed or did not lead to a positive LFT/PCR result. Additionally, these individuals may not have seroconverted for anti-N antibodies, or their antibody levels may have waned below detectable levels over time, as demonstrated by Lumley et al., who observed that anti-N antibody levels tend to decline significantly within a few months post-infection [188].

3.2.3 Comparison of T Cell responses to SARS-CoV-2 peptides in short and long interval dosing cohorts

To study the T cell responses in short and long dosing intervals post-vaccination in naïve and previously infected individuals, T cell responses after two vaccine doses were determined using T cell IFNγ ELISpot. In both the short and long dosing interval cohorts, there is a significant increase in T cell responses to SARS-CoV-2 Spike protein in individuals with previous infection compared to naïve individuals, P = 0.006 and P<0.0001 respectively (Figure 3.3). These findings demonstrate that SARS-CoV-2 infection can produce an immune response similar to a vaccine dose.

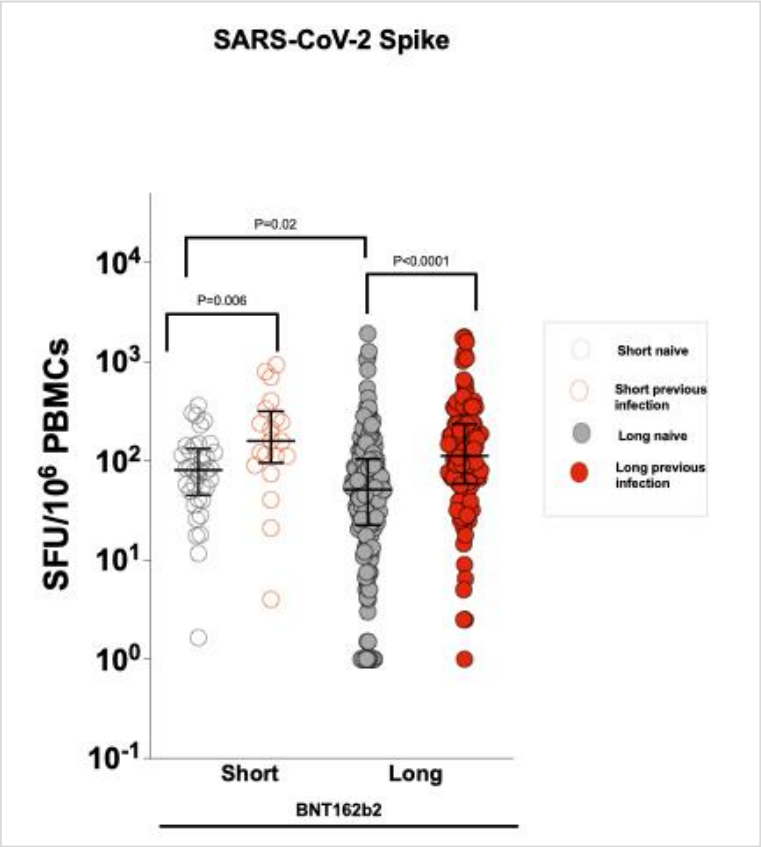


Figure 3.3. T cell responses in short and long dosing interval 4 weeks after 2nd vaccine dose. T cell IFNγ ELISpot responses in four groups of vaccinees: naive short (n = 37), naive long (n = 188), pre-infection short (n = 20), and pre-infection long (n = 124) individuals. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs) and represent responses to peptide pools derived from the SARS-CoV-2 whole spike protein. Naïve (grey) and previously infected (red) donors highlighted. Data are presented as median values with interquartile ranges. Comparisons between groups were made using two-tailed Mann-Whitney tests, with significant p-values indicated.

In the naïve cohort, a comparison of the two dosing intervals shows an increased T cell response in the short interval group compared to the long interval group, P=0.02. Specifically, the median T cell response in the naïve short interval dosing group was 81 SFU/10^6 PBMCs compared to 51 SFU/10^6 PBMCs. In contrast, in the group with prior infection, while the long interval group demonstrates a trend towards a higher median T cell response (159 SFU/10^6 PBMCs in the short dosing interval group compared to 113 SFU/10^6 PBMCs in the long dosing interval group), there is no observed statistically significant difference between the dosing interval groups, suggesting that prior infection might diminish the impact of dosing interval length on subsequent vaccine-induced T cell responses.

3.2.4 Spike-specific CD4+ and CD8+ T Cell responses in short and long dosing interval cohorts

I next demonstrated that analysis of SARS-CoV-2 Spike antigen-specific T cells revealed distinct differences in immune responses based on the interval between vaccine doses, prior infection status and the specific T cell subsets; CD4+ and CD8+. Among the naive group, there was a notable enhancement in the percentage of Spike-specific CD4+ T cells with longer dosing intervals. This was particularly evident in the IL-2 and IFNγ cytokine responses, where their production were significantly higher in the long interval group (P<0.0001 and P=0.02, respectively) (Figure 3.4A) compared to the short interval dosing group. Specifically, median CD4+ IL2 responses were 0.05% in the long naïve group compared to 0.02% in the short naïve group. Likewise, median CD4+ IFNγ responses were 0.05% in the long naïve group compared to 0.02% in the short naïve group.

Conversely, in the group with prior infection, no significant differences were observed between short and long intervals in the CD4+ T cell responses, further suggesting that the impact of varying dosing intervals is more pronounced in naïve compared to previously infected cohorts.

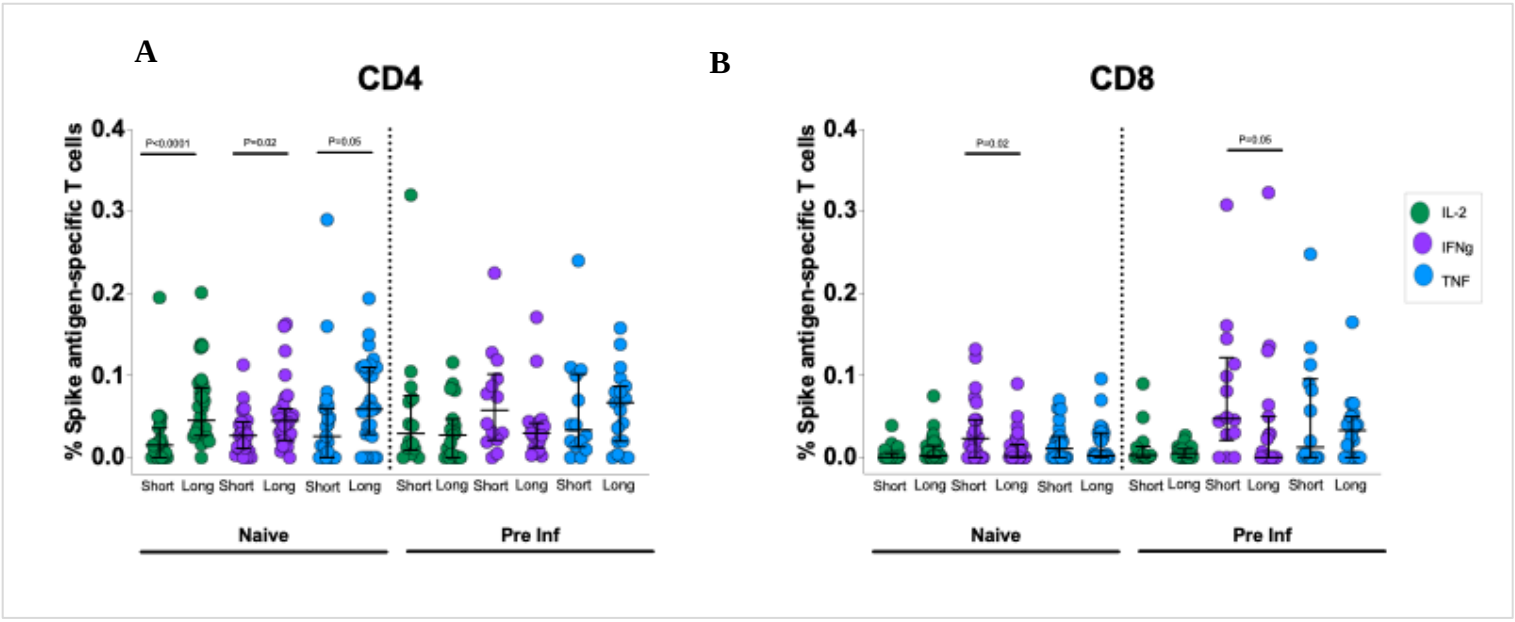


Figure 3.4. CD4+ (A) and CD8+ (B) T cell responses in short and long dosing interval cohort 4 weeks after 2nd vaccine dose. Flow cytometry was used to assess the T cell responses in PBMCs from 53 naïve and 33 previously infected participants (n=86 total) who received either a short or long vaccine dosing interval. The individual cytokine expression levels for IFNγ (purple), IL-2 (green), and TNF (blue) are displayed as a percentage of the CD4+ T and CD8+ T cell population. Bars represent the median with interquartile range. Statistical significance between groups was determined using the Mann-Whitney test with p-values indicated.

Within the CD8+ subset, in the naïve cohort, the short dosing interval group had a higher proportion of Spike-specific CD8+ T cells with IFNγ production (P=0.02) (Figure 3.4B). Median CD8+ IFNγ T cell responses were 0.02% in the short dosing interval group compared to 0.001% in the long dosing interval group.

Similarly, there was an increase in IFNγ production in the group with prior infection (P=0.05) with median CD8+ IFNγ T cell responses in the short dosing interval group 0.05% compared to 0.001% in the long dosing interval group. For other cytokines, IL-2 and TNF, there was no statistically significant difference between the interval dosing groups within the CD8+ subset. These findings suggest that longer dosing intervals enhance both the magnitude and polyfunctionality of the CD4+ T cell response, particularly in individuals without prior infection, underscoring the importance of dose timing in optimising a robust adaptive immune response.

3.2.5 Impact of varying dosing intervals: T cell responses 6 months after 2nd vaccine dose and 1 month after 3rd vaccine dose

Following up from this work that showed the T cell immune responses in short and long dosing intervals at 4 weeks after the 2nd vaccine dose, I carried out additional studies to understand the immune trajectory in the dosing interval cohorts 6 months after the 2nd dose (V2 + 6 months) and 1 month after the 3rd dose (V3+ 1 month). Additionally, participants receiving the AZD1222 vaccine (9-22 weeks between 1st and 3rd dose), were also studied. In participants who received the short and long dosing intervals at V2+6 months, I observed strong immune responses across participants, with participants with previous infection maintaining a higher immune response compared to naïve participants ($P<0.0001$ short dosing interval, $P=0.0194$ long dosing interval) (Figure 3.5A). In the long and short dosing interval groups, median T cell responses were higher in the group with prior infection; short BNT162b2 263 SFU/10^6 PBMCs compared to 161 SFU/10^6 PBMCs in the naïve group and long BNT162b2 148 SFU/10^6 PBMCs in the group with prior infection compared to 83 SFU/10^6 PBMCs in the naïve group. The AZD1222 group also showed high immune responses, although within the AZD1222 group, there was no significant difference in immune responses between the naïve and previously infected groups. Median T cell responses were 78 SFU/10^6 PBMCs in the group with prior infection compared to 62 SFU/10^6 PBMCs in the naïve group. The observed trends at V2+6 months continued after participants received a 3rd vaccine dose, with similar trends observed across the dosing interval groups and the naïve and previously infected cohorts ($P=0.00036$ short dosing interval, $P<0.0001$ long dosing interval) (Figure 3.5B).

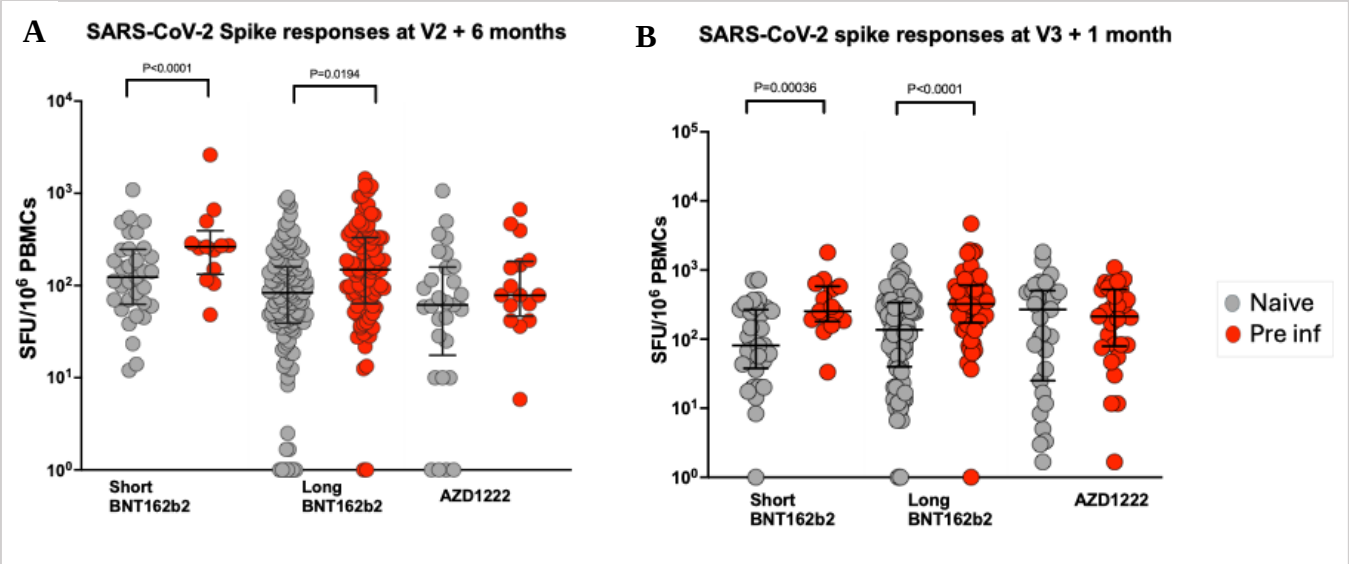


Figure 3.5. Comparison of IFNγ ELISpot responses to SARS-CoV-2 S protein at (A) V2+6 months and (B) V3+1 month. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs). Naïve participants are represented in grey squares and previously infected (pre inf) in red squares. The V2+6 months participants received either two doses BNT162b2 delivered with a short dosing interval (naive = 33, pre inf = 13), or a long dosing interval (naive = 119, pre inf = 93), or 6 months after two doses of AZD1222 vaccine (n = 29, pre inf = 16). The V3+1-month participants received a third vaccine dose across similar dosing intervals. Short dosing interval (naïve = 33, pre inf = 16), long dosing interval (naïve = 82, pre inf = 59), AZD1222 (naïve = 32, pre inf = 30). Data are presented as median values with interquartile ranges. Comparisons between groups were made using two-tailed Mann-Whitney tests, with p-values indicated.

Overall, the data collectively highlight the differential impact of varying dosing intervals and prior infection on the immune response to SARS-CoV-2 vaccines at 2 timepoints, V2+6 months and V3+1 month. The Pfizer BNT162b2 vaccine, regardless of dosing interval, consistently elicited stronger immune responses in individuals with prior SARS-CoV-2 infection, emphasizing the role of hybrid immunity in enhancing vaccine effectiveness. In contrast, the AZD1222 vaccine induced a more uniform but comparatively lower magnitude of response, with minimal distinction between naïve and previously infected individuals.

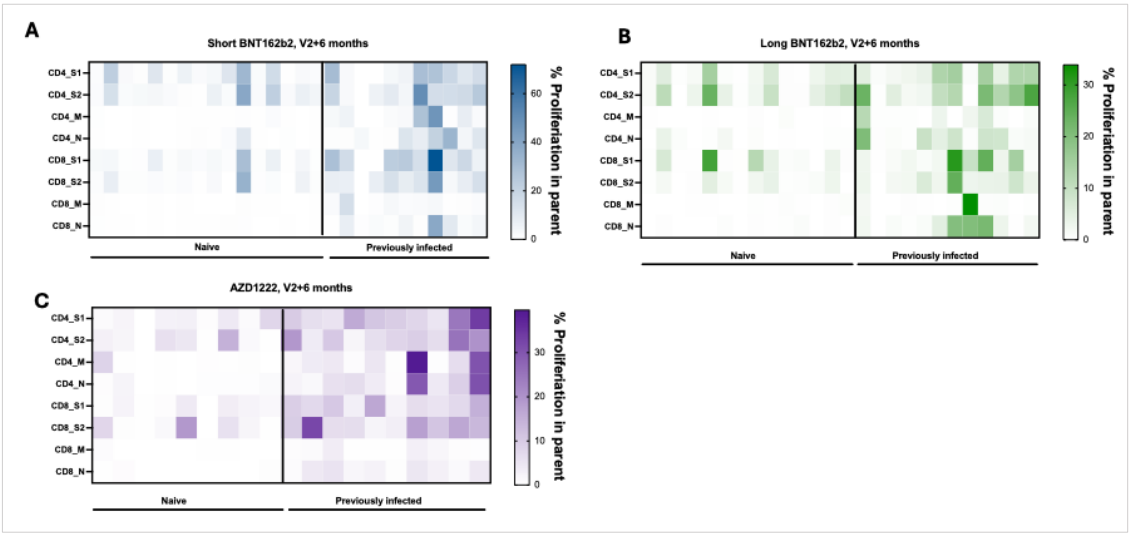
3.2.6 Proliferation responses to SARS-CoV-2 peptides at V2+6 months

To further understand the immune responses across the different cohorts, I performed a T cell CTV proliferation assay at the V2+6-month timepoint. CD4+ and CD8+ responses were assessed across SARS-CoV-2 S1, S2, M and N proteins in the short dosing interval, long dosing interval and AZD1222 groups. SARS-CoV-2 S1 and S2 peptides are derived from whole SARS-CoV-2 spike (178 peptides). S1 is made of 92 peptides and S2 is made up of 86 peptides. All are 15-18 amino acids in length, overlapping by 10 amino acids. Generally, across all groups, individuals with prior SARS-CoV-2 infection exhibited higher and more widespread T cell responses across different viral antigens, as expected especially with higher T cell responses to the M and N proteins compared to naïve individuals in all vaccine regimens (Figure 3.6, A-C).

The M and N antigens are not included in any of the vaccine formulations; thus, it is expected that individuals with prior SARS-CoV-2 infection show significantly higher T cell responses to these antigens compared to naïve individuals, as these responses result from infection. In contrast, responses to the S1 and S2 spike protein regions, which are included in the vaccine, are observed in both naïve and previously infected individuals, with previously infected participants generally exhibiting broader and more robust responses, likely due to the combined effects of infection-induced and vaccine-induced immunity. These findings suggest that prior infection not only enhances the breadth of T cell responses to multiple SARS-CoV-2 proteins but also highlights the role of non-spike antigens such as M and N in shaping a more robust and durable immune response post-vaccination. Specifically, within the CD4 subset, there was an increase in T cell proliferation among all antigens in the previously infected short dosing interval compared to the naïve short dosing interval. Similarly, within the AZD1222 group, there was an increase in T cell proliferation among S1, S2, M and N in the group with prior infection compared to the naïve group (Figure 3.6, D-G).

Among the long dosing interval group, there was a significant increase in T cell proliferation in the previously infected group compared to the naïve in M and N proteins only. Interestingly, within the CD8 subset (Figure 3.6, H-K), there was a marked difference between vaccine regimens, with short dosing interval with prior infection having a significant increase in T cell proliferation to S1, S2 and M proteins compared to the long dosing interval with prior infection. Overall, as expected S1 and S2 responses were higher in the groups with prior infection due to

the established combined effect of infection and vaccination on immune responses, while the responses to M and N were low or absent in the naïve group, further confirming absence of infection with SARS-CoV-2.



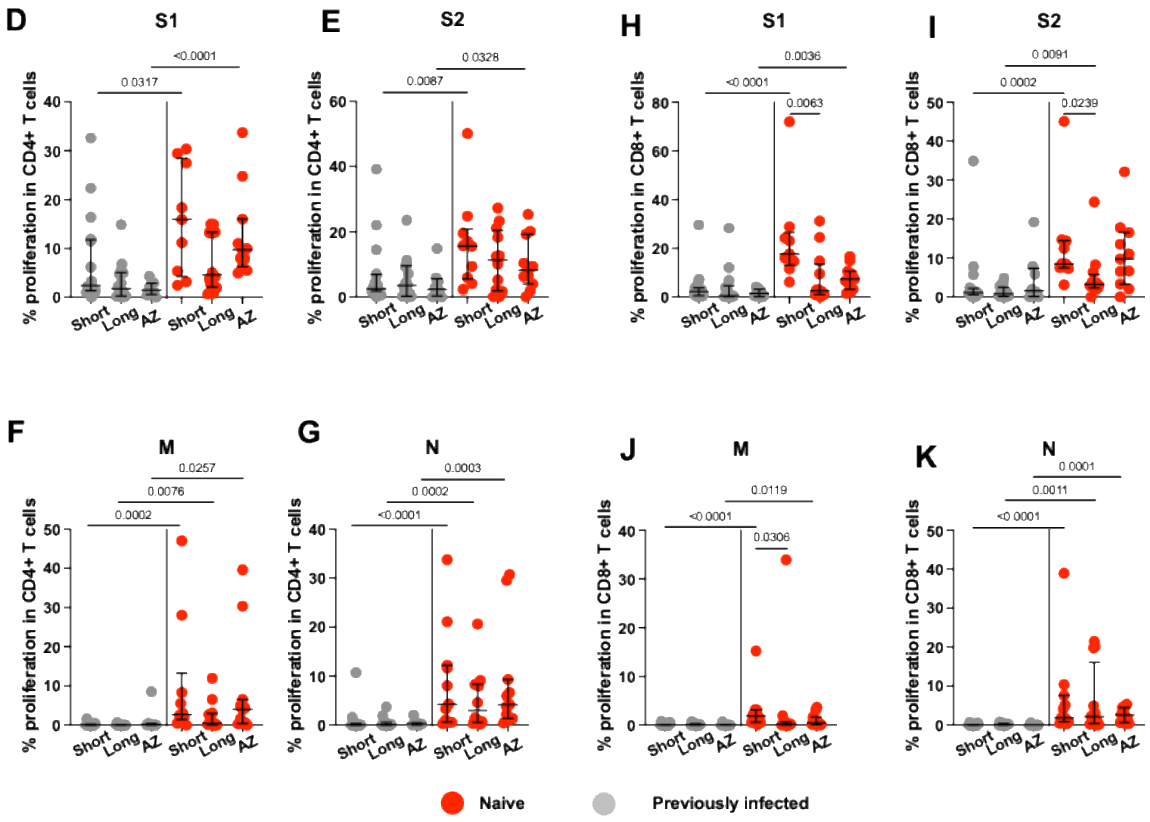


Figure 3.6. T cell proliferation responses to SARS-CoV-2 peptides at V2+6 months. Heatmap showing proliferation responses among CD4 and CD8 subsets to SARS-CoV-2 peptides S1, S2, M and N (A-C), n=73. Comparison of CD4 (D-G) and CD8 (H-K) T cell proliferation responses across vaccine regimens in naïve (grey) and previously infected (red) cohorts. Bars represent the median with interquartile range. Statistical significance between two groups was determined using the Mann-Whitney test and among three groups using the Kruskal-Wallis test with p-values indicated and only statistically significant values shown.

3.2.7 Characterization of CD4+ and CD8+ T cell responses at V2+6 months

To further characterize the immune responses and access T cell functionality I performed an ICS assay on the V2+6 months cohort, measuring IFNγ, IL-2 and TNF responses. Firstly, there were both CD4+ and CD8+ responses to all cytokines in all vaccine regimen groups, regardless of prior infection status. Although both CD4+ and CD8+ T cells responded across all vaccine regimens, it is notable that the overall cytokine responses were higher in the CD4+ T cell population (Figure 3.7, A-C) compared to CD8+ T cells (Figure 3.7, D-F). This suggests that the vaccine-induced immune response might be skewed toward CD4+ helper T cell activity rather than CD8+ T cells at this time point. It is important to note that the 15-mer peptides used in this assay are optimized for CD4+ T cell stimulation, which may contribute to the higher observed cytokine responses in CD4+ T cells compared to CD8+ T cells. However, our group has consistently demonstrated the capability to measure robust CD8+ T cell responses to SARS-CoV-2 and other pathogens using this peptide approach, indicating that while there may be a slight bias, the results still provide a reliable measure of CD8+ T cell activity. There was a significant increase within the CD4+ subset across cytokine expression for IL-2 and TNF in the naïve short group compared to the AZD1222 group (IL-2, P=0.0080 and TNF, P=0.024, Figure 3.7, A-C). Median CD4+ IL-2 and TNF responses in the naïve short dosing interval group were 0.060% and 0.18% respectively compared to 0.011% and 0.039% in the AZD1222 group. A similar trend was observed between the naïve long dosing interval and AZD1222 group across IFNγ, IL-2 and TNF (P=0.0121, P=0.0039 and P=0.0388 respectively). Likewise, median CD4+ IFNγ, IL-2 and TNF responses in the naïve short dosing interval group were 0.071%, 0.058% and 0.22% respectively compared to 0.024%,0.011% and 0.039% in the AZD1222 cohort.

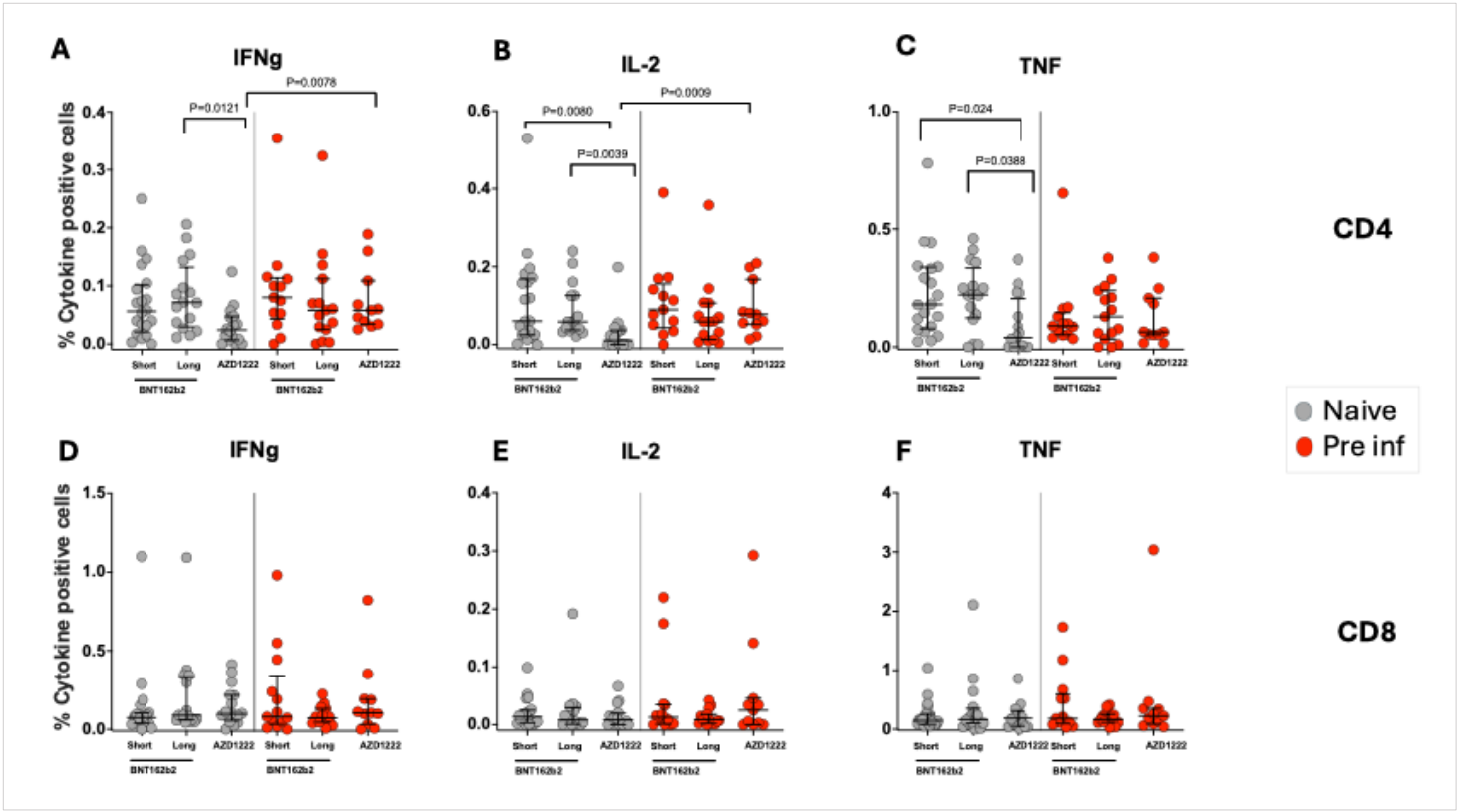


Figure 3.7. T cell ICS responses in BNT162b2 short, BNT162b2 long dosing interval and AZD1222 cohorts 6 months after 2nd vaccine dose. Flow cytometry was used to assess the T cell responses in cryopreserved PBMCs from 49 naïve (grey) and 46 previously infected participants (red). Individual cytokine expression levels for IFNγ, IL-2 and TNF are displayed as a percentage of the CD4+ T and CD8+ T cell population. Bars represent the median with interquartile range. Statistical significance between groups was determined using the Mann-Whitney test with p-values indicated and only statistically significant values shown.

In contrast, comparisons across all vaccine regimens in the group with prior infection show no significant difference. However, within the AZD1222 group, there is a marked increase IFNγ and IL-2 production in the group with prior infection compared to the naïve group. Additionally, in the group with prior infection, there appears to be higher variability (IQR range) in responses, particularly within the CD8+ subset, across all cytokines in all vaccine groups. This variability could reflect a heterogeneous memory response in individuals previously exposed to SARS-CoV-2, potentially influenced by differences in prior viral load, the severity of infection, timing between infection and vaccination or genetic differences including HLA type. Overall, previously infected individuals generally show a higher median

percentage of cytokine-positive cells than the naïve cohort across cytokines. This could suggest enhanced T cell priming or memory in individuals with prior exposure to SARS-CoV-2, which may lead to more robust vaccine-induced T cell responses. Alternatively, the higher cytokine responses in previously infected individuals may simply reflect the additive effect of an additional exposure to the spike protein through infection. This cumulative exposure could enhance both the breadth and magnitude of the immune response, as each encounter with the spike antigen contributes to an incremental boost in T cell priming and memory.

3.3 Discussion

In this chapter I provide work on examining the impact of varying dose intervals on T cell immune responses and their long-term impact, taking into consideration hybrid immunity. I showed that after a single vaccine dose, SARS-CoV-2 vaccines boost the magnitude of T cell responses in both naïve and previously infected individuals. Regardless of dosing interval, subsequent SARS-CoV-2 vaccines doses boost T cell responses in both groups, with a more pronounced boost in naïve individuals. In both dosing intervals, there is minimal waning of T cell responses, with responses being maintained up to 6 months post vaccination. Finally, while there are differences in the T cell responses observed in the naïve short and long dosing interval groups, these differences even out in groups with prior infection.

Since our work, several studies have investigated the optimal dosing intervals for SARS-CoV-2 vaccines, highlighting potential vaccination strategies for maximizing vaccine efficacy and effectiveness. A study in 750 participants aged 50-89 years looking at serological responses and vaccine effectiveness (VE) in varying dosing intervals found that extending the interval between the first and second doses of the BNT162b2 and AZD1222 vaccines resulted in higher antibody titres [189]. Specifically, participants who received their second dose of BNT162b2 65-84 days after the first dose had six-fold higher geometric mean titres (GMTs) compared to those who received it 19-29 days apart. The extended dosing interval also led to higher vaccine effectiveness. The VE after two doses was higher for both vaccines across all age groups compared to a single dose, however, the magnitude depended on the interval between doses. Notably, for the BNT162b2 vaccine, VE was higher when doses were administered more than six weeks apart compared to the authorized three-week schedule, including among those aged 80 and older [189]. These conclusions are in line with findings from a study by Skowronski et al. that showed VE was significantly higher against both infection and hospitalization using an

extended dosing interval (7 to 8 weeks) compared to 3 to 4 weeks intervals between doses [190]. The Moderna mRNA-1273 vaccine has also been evaluated for varying dosing intervals. A study by Grunau et al. in 186 participants found that extending the interval between doses to six weeks, compared to the standard four weeks, resulted in higher SARS-CoV-2 S, N and RBD antibody titres [191].

These findings are consistent with other research suggesting that longer intervals between doses can enhance the durability and strength of the antibody immune response, concluding on an increased vaccine effectiveness using these findings. Based on findings from these studies, significant policy decisions were made globally regarding SARS-CoV-2 vaccination strategies. For example, national governments recommended extending the dosing intervals for mRNA vaccines up to 16 weeks to ensure broader population coverage with the first dose. This recommendation aimed to maximize vaccine distribution and enhance public health outcomes, particularly in the context of limited vaccine supplies [192]. Similarly, WHO also recommended countries extend the interval between SARS-CoV-2 vaccine doses up to 8 to 12 weeks [193].

My T cell IFNγ ELISpot data, combined with the national PITCH data, indicates that short dosing intervals lead to a more elevated T cell response to SARS-CoV-2 spike as measured by IFNγ ELISpot following the second dose in naive individuals compared to those who received a longer interval. The magnitude of the T cell response in the short dosing interval and long dosing interval cohort with previous infection were similar, suggesting that prior infection may induce a baseline level of T cell immunity that is robust enough to mitigate the effects of varying vaccine regimens on overall T cell activation. This implies that the immune memory from infection priming plays a dominant role in shaping the T cell response, regardless of the dosing regimen. However, ICS assays revealed that long dosing intervals are associated with higher proportions of polyfunctional CD4+ T cells producing IL-2 and IFNγ, particularly in naive individuals. These polyfunctional T cells are considered more effective at orchestrating immune responses [194], suggesting that extended dosing intervals may enhance the quality of CD4+ T cell-mediated immunity. This trend is not observed in the previously infected group, as both short and long intervals show similar levels of cytokine production, which aligns with the previous evidence that prior infection may level out these differences. A similar pattern is observed at V2+6 months, with the naïve BNT162b2 short and long dosing interval groups having an increased T cell response compared to the AZD1222 group but with differences

evening out in the groups with prior infection. The CD8+ T cell responses, although detectable in the ICS assay, were comparatively less robust, suggesting that the dosing schedule may differentially regulate these two T cell subsets, with a stronger influence on CD4+ T cell polyfunctionality. Equally, the CTV assay, which measures T cell proliferation, demonstrated a strong proliferative response in the CD4+ subset, particularly in those receiving a longer dosing interval at V2+6 months. This was more evident in naive individuals, aligning with the hypothesis that extended intervals may provide the immune system with more time to establish a robust memory T cell pool, which could be crucial for long-term protection.

There is evidence suggesting a complex interplay between the humoral and cellular responses to SARS-CoV-2, as while T cell responses remain largely stable across dosing intervals, there is a notable enhancement in antibody responses with extended dosing intervals. Our research using the same cohort included in this chapter found that extended the dosing interval resulted in higher peak levels of neutralizing antibodies and B cells compared to the short dosing interval [143,195]. After the first SARS-CoV-2 vaccine dose, there was an initial peak in antibody levels which gradually declined over the next several weeks, with a marked reduction in the naïve cohort. Neutralizing antibody levels were 2 to 4 times higher in the long dosing interval group, and this effect was most pronounced in the naïve group compared to the group with prior infection. These trends were observed in SARS-CoV-2 S protein and RBD, suggesting that an extended dosing interval allows the immune system more time to mature and develop a stronger antibody response, particularly against the key regions of the virus critical for facilitating infection. Our study by Payne et al. demonstrated that an extended dosing interval allowed for a more robust B cell memory response, particularly at the time of the second dose [196]. This was reflected in a greater magnitude of SARS-CoV-2-specific B cells in the longer interval group, supporting the notion that B cells require sufficient time to mature and develop optimal memory. This maturation likely contributes to the observed enhancement in antibody responses upon subsequent antigen exposure, underlining the advantage of extended intervals in achieving stronger and more durable immune memory.

Overall, the findings from cellular and humoral responses highlight the complementary roles of antibodies and T cells in mediating vaccine-induced immunity. The rapid decline of antibody levels after the primary vaccination series emphasises the importance of booster doses to maintain adequate protection, particularly amidst the transition from a pandemic to endemic phase of the virus [197]. Booster doses become essential in sustaining antibody levels,

especially in individuals with no prior infection. Unlike antibodies, T cell responses are more durable and sustained over time, providing a more stable layer of protection even as antibody levels decline. T cells play a crucial role in mitigating severe disease by coordinating immune responses and directly eliminating infected cells. Their persistence following SARS-CoV-2 vaccination indicates a contribution to long-term immunity, particularly through the formation of memory T cells, which can quickly respond to reinfection.

In vaccinated individuals, studies on humoral and cellular responses have shown the impact of hybrid immunity on vaccine effectiveness and immunological responses. A cohort study in over 1.5 million participants found that prior infection with SARS-CoV-2 was associated with a lower risk of breakthrough infection following mRNA vaccination, suggesting that natural immunity may provide an additional layer of protection [198]. Studies examining the impact of a single vaccine dose in naïve and previously infected individuals showed that antibody levels were 7 to 20 times higher after a single dose in previously infected compared to naïve donors [139,199]. Additionally, the T cell responses in previously infected individuals were approximately five times greater than in those without prior infection, with CD4+ T cell responses dominant in previously infected individuals, whereas infection naive participants exhibited balanced CD4+ and CD8+ responses [139]. Another study demonstrated that after two vaccine doses, individuals with hybrid immunity exhibit significantly higher neutralizing antibody titres compared to those who were only vaccinated, with an 8.5 to 15.7 fold increase in neutralization observed in the hybrid immunity cohort [200]. Similarly, a study by Guibert et al. showed that previously infected vaccinated individuals exhibited significantly higher levels of memory T cell frequencies compared to naïve vaccinated individuals [201]. Taken together, these studies highlight the need for studies evaluating the potential long-term impacts of hybrid immunity, particularly on how dosing interval variations may also play a role. These studies will provide insights into optimizing vaccine strategies, particularly concerning the timing and vaccination prioritization strategies. Therefore, our research also examined the long-term trajectory of varying dose intervals with considerations for the landscape of hybrid immunity.

The findings from this work imply that booster doses are crucial for maintaining both antibody and T cell responses regardless of vaccine regimen and prior infection status. The third vaccine dose not only restores antibody levels but also enhances T cell activity, reinforcing protection

against the virus over time. This sustained immunity is particularly important for high-risk groups such as healthcare workers, who are more frequently exposed to the virus. Given the waning of antibodies after the primary vaccination series, periodic booster doses may be necessary to ensure continued protection, especially for those most vulnerable to severe outcomes. The durability of T cell responses also suggests that long-term protection against severe disease does not rely solely on antibody levels. This finding has been key in informing policy decisions, which transitioned from aims of minimizing infections to preventing severe disease. Finally, the findings on the role of hybrid immunity among the short dosing interval, long dosing interval and AZD1222 group where prior infection enhances vaccine-induced immune responses emerges as a significant advantage. Individuals with prior exposure to the virus tend to exhibit stronger and more durable immunity, both in terms of antibodies and T cells. This has important implications for future vaccine strategies, as it suggests that a tailored approach to booster dosing may be beneficial. Specifically, naive individuals may require more frequent boosters to sustain immunity, while those with hybrid immunity may have longer-lasting protection and need less frequent doses [202]. By the V2+6 month timepoint, however, differences in immune responses due to the dosing interval (short BNT162b2, long BNT162b2, or AZD1222) become less pronounced, with comparable T cell responses across regimens. Although individuals who initially received the AZD1222 vaccine showed lower antibody levels, these differences were effectively balanced by the third dose, suggesting that booster doses play a critical role in equalizing antibody levels across regimens and reinforcing immunity.

Our findings are consistent with other research; Parry et al examined how varying dose intervals in cohorts receiving two doses of the Pfizer-BioNTech BNT162b2 vaccine can impact cellular and humoral responses. The extended dosing interval (11–12 weeks) led to a 3.5-fold higher peak antibody response compared to the standard (3 weeks) interval. Median antibody titres in the extended interval group reached 4030 U/ml, compared to 1138 U/ml in the standard interval group [203]. Compared to the extending dosing interval group, T cell responses were higher in the standard interval group, with peak cellular response at 72 SFU/10^6 PBMCs in the standard interval group compared to 20 SFU/10^6 PBMCs in the extended interval group. Interestingly, T cell responses in both cohorts showed a decline from V2+2 months, with a faster decline in the standard interval cohort. This observed decline was not seen in our cohort, perhaps due to the age group studied. We studied healthcare workers, with more than 40% below 36 years, while Parry et al. primarily studied older populations who were 80 years and

older, thus immunosenescence playing a role in trends observed. Also, this contrast with our findings could be due to several factors, including differences in the assays used. Parry et al. employed the Oxford Immunotec T-SPOT assay, which, though ELISpot-based, involved longer sample transit times and distinct peptide pools that may affect sensitivity and detection levels.

In a different Canadian study, Hall et al., studied a cohort of healthcare workers with a median age of 40 and evaluated the impact of varying dose intervals on T cell functionality. Participants had received two doses of the Pfizer-BioNTech BNT162b2 vaccine in a short (21-38 days interval between doses) or long (8-16 weeks) dosing regimen. They observed that polyfunctional (IFNγ and IL-2 producing) CD4+ and CD8+ T cells were induced in both groups, with no significant difference between the short and extended dosing interval groups [204]. This is in contrast with our findings, and the differences in T cell polyfunctionality in varying dose intervals we observed may be due to further studying how hybrid immunity may affect varying dosing intervals, which was not included in the Hall et al. study. The Hall et al. study recruited participants without documented prior COVID-19 infection, though two participants in the delayed-interval group had detectable anti-RBD antibodies before vaccination but no recorded infection history, suggesting possible asymptomatic exposure. Lastly, as previous work had shown, humoral responses from the Hall et al. study showed that the extended dosing interval significantly increased RBD antibody titres compared to the standard dosing interval [204].

Vaccine effectiveness was explored using the UK SIREN study (which includes PITCH participants from the Liverpool, Sheffield and Newcastle sites) analysed the vaccine effectiveness of the BNT162b2 mRNA COVID-19 vaccine in HCWs. Results showed that 21 days after the first dose, VE against SARS-CoV-2 infection was 70% (95% CI 55–85), increasing to 85% (74–96) after the second dose. This study underscores the vaccine's ability to reduce both symptomatic and asymptomatic infections among healthcare workers, potentially lowering transmission risks.

In conclusion, this chapter provides evidence that extending the dosing interval of SARS-CoV-2 vaccines significantly enhances the functional quality of the immune response, particularly in SARS-CoV-2 naïve individuals. The increase in polyfunctional CD4+ T cells co-expressing IL-2 and IFNγ observed with the longer dosing interval suggests improved T cell differentiation

and potential for sustained immune memory, which is critical for long-term protection. Polyfunctional T cells are known to coordinate multiple immune processes, such as promoting B cell help, maintaining immune memory, and orchestrating effector responses, thus contributing to a more robust and durable immunity. However, the impact of dosing interval on T cell polyfunctionality was less pronounced in previously infected individuals, indicating that hybrid immunity may induce a baseline level of T cell activation that minimizes the influence of varying dosing regimens. These findings suggest that while extended dosing intervals enhance the quality of T cell responses in naïve individuals, prior infection leads to a more uniform T cell response regardless of the interval between doses. From a policy perspective, these findings support the use of extended dosing intervals, especially in naïve individuals, to enhance both antibody and T cell responses, while recognizing that those with hybrid immunity may not require as frequent booster doses. Further research is needed to explore the long-term durability of these immune responses across different populations and to understand the specific mechanisms driving the enhanced polyfunctionality of T cells in extended intervals. Additionally, studies investigating the interplay between humoral and cellular responses in varying age groups and risk populations could further inform vaccine strategies.

Chapter 4: T cell Responses to SARS-CoV-2 Variants of Concern

Aim: To understand the impact of SARS-CoV-2 variants on T cell responses in healthcare workers vaccinated against COVID-19

Hypothesis:

- T cell responses elicited by SARS-CoV-2 vaccination are maintained against SARS-CoV-2 variants of concern

Abstract

This chapter investigates the impact of SARS-CoV-2 variants of concern (VOCs) on T cell responses in healthcare workers (HCWs) vaccinated against COVID-19. The objective was to evaluate whether vaccine-induced T cell responses are maintained against mutations in Alpha, Beta, Gamma, Delta, and Omicron variants.

Using PBMC samples from HCWs, T cell IFNγ ELISpot assays revealed that while there were some reductions in T cell responses to mutated peptides in VOCs, the overall magnitude of T cell responses remained robust. Responses to mutated peptides varied across donors but were predominantly maintained due to the recognition of conserved regions within the Spike (S) protein. Importantly, whole S peptide analyses demonstrated minimal loss in T cell recognition for Alpha, Beta, Gamma, and Delta variants compared to the ancestral strain. For Omicron, responses to conserved regions accounted for a significant portion of the overall response, although mutations in BA.1 led to modest declines in recognition.

These findings highlight that while mutations in VOCs can impact individual peptide responses, their overall effect on T cell immunity is limited, highlighting the robustness of T cell responses in recognizing VOCs, despite the frequency and location of mutations. While current vaccines primarily target the S protein, further emphasis on broadening T cell immunity by including additional epitopes, such as those outside of S, may further enhance protection against emerging SARS-CoV-2 variants. The work presented in this chapter highlights the need to continually refine vaccine strategies to address the evolving viral landscape.

Contributions

Work presented in this chapter has previously been published in the following papers;

Skelly, Donal T., Adam C. Harding, Javier Gilbert-Jaramillo, Michael L. Knight, Stephanie Longet, Anthony Brown, **Sandra Adele**, Emily Adland et al. "Two doses of SARS-CoV-2 vaccination induce robust immune responses to emerging SARS-CoV-2 variants of concern." *Nature communications* 12, no. 1 (2021): 5061.

In this study, I contributed to the T cell work: I designed the peptides with senior support and I was responsible for conducting the T cell ELISpot assays. Additionally, I performed all the T cell data analysis and generated all the T cell figures (Figure 4.2.2) for inclusion in the manuscript.

Payne, Rebecca P., Stephanie Longet, James A. Austin, Donal T. Skelly, Wanwisa Dejnirattisai, **Sandra Adele**, Naomi Meardon et al. "Immunogenicity of standard and extended dosing intervals of BNT162b2 mRNA vaccine." *Cell* 184, no. 23 (2021): 5699-5714.

In this study, I contributed significantly to the T cell analysis as part of our role as the lead site for T cell work. I was responsible for conducting ELISpot assays within the Oxford PITCH site on samples from our cohort to assess T cell responses to varying dosing intervals. I prepared Figure 4.2.3 based on data from our PITCH sites, including data I generated.

Moore, Shona C., Barbara Kronsteiner, Stephanie Longet, **Sandra Adele***, et al. "Evolution of long-term vaccine-induced and hybrid immunity in healthcare workers after different COVID-19 vaccine regimens." *Med* 4, no. 3 (2023): 191-215.

**Joint first author*

In this publication, I played a central role in the T cell response component of the research, with our site designated as the lead for T cell analysis. My specific contributions included conducting ELISpot and CTV assays, performing peptide design, and leading both site-wide and site-specific analyses within the PITCH cohort. I independently analysed resulting T cell data with Figures 4.2.7, 4.2.9 and 4.2.10 included in the publication.

All other figures are unpublished data I generated

4.1 Introduction

As COVID-19 vaccination efforts began in December 2020, the emergence of viral variants threatened the effectiveness of existing vaccines and raised critical questions about the durability and breadth of immune protection. It was important to carry out studies on how mutations in VOCs could affect immune responses. These studies are essential for understanding the potential changes in viral behaviour such as increased transmissibility, immune evasion and the implications for vaccine efficacy and long-term immunity. Early during the pandemic, research efforts were heavily focused on neutralising antibodies as the primary immune correlate of protection, as antibodies, particularly those that bind to the S protein of SARS-CoV-2, were shown to be effective in neutralizing the virus and preventing infection. Several key studies demonstrated that the levels of neutralizing antibodies correlated with protection against symptomatic and severe disease during the pre-Alpha variant era [205,206]. This understanding drove the development of vaccines designed to elicit strong humoral responses, primarily targeting the S protein.

The early focus on neutralizing antibodies as the primary mechanism of vaccine-induced immunity, while important, quickly proved insufficient to fully explain the dynamics of SARS-CoV-2 immunity, particularly in the context of VOCs as several studies highlighted the rapid waning of antibody levels [143,195] yet the protection against severe disease and death provided by prior infection and vaccination remained robust and long-lasting. In early 2021, research primarily focused on neutralizing antibodies as the main correlate of protection against SARS-CoV-2. However, studies such as Tomic et al. demonstrated that, after infection, antibody responses waned significantly over time (at 28, 90, and 180 days), whereas T cell responses showed much greater durability, indicating a longer-lasting cellular immune component [207]. Given the distinct kinetics between cellular and humoral immunity, it was important to study how T cell responses could provide broad and durable protection across different SARS-CoV-2 variants within the context of waning antibody responses.

Unlike antibodies, which primarily target specific regions of the S protein, T cells can recognise a broader range of viral proteins, and their epitopes are often more conserved, even in variants undergoing significant antigenic drift [129,208]. A study by Grifoni et al. showed that SARS-CoV-2-specific T cells are robustly activated in individuals who recover from COVID-19. The study found that while the S protein is a key target of T cell immunity, significant T cell

responses are also directed at other viral proteins, including NSPs, M and N proteins and accessory proteins such as ORF3 and ORF8 [132]. This observation is crucial because it highlights that T cell responses to SARS-CoV-2 infection are not restricted to the spike protein; instead, T cells recognise a wide range of epitopes across the entire viral proteome. Another study by Peng et al. which our laboratory contributed to provides additional evidence that SARS-CoV-2-specific T cell responses are not restricted to the S protein. In this study, robust memory CD4+ and CD8+ T cell responses were observed in convalescent individuals, with strong reactivity to the M and N proteins, in addition to the S protein [209]. Interestingly, these T cell responses were more polyfunctional than those typically observed in acute infection, particularly when stimulated by non-spike proteins. These responses were found to be durable, indicating that memory T cells could provide long-lasting protection even in the absence of high levels of circulating antibodies [145]. Lastly, Ogbe et al showed that while S-reactive T cells were detectable by 7-day proliferation assay in many seronegative individuals due to cross-reactivity with seasonal coronaviruses, T cell responses to the M, N, and ORF proteins were specific to SARS-CoV-2-infected individuals [133]. This distinction is critical because it shows that while cross-reactive immunity to S protein may exist, the broader T cell responses to non-spike proteins are specific more markers of SARS-CoV-2 infection. These studies highlight the breadth of the T cell epitope repertoire and emphasize the importance of studying variants of concern, particularly considering how T cells, unlike antibodies, target a wider array of viral proteins beyond the spike. The ability of T cells to target more conserved regions of the virus is particularly important for variants of concern, which often have mutations in the spike protein that can reduce the efficacy of antibody-mediated neutralization. Since T cells target more stable and conserved regions across the virus, they are likely to remain effective even against variants with significant changes in the spike protein. Angyal et al. demonstrated that even after a single vaccine dose, T cell responses in previously infected individuals were broad and directed at conserved viral regions, reinforcing the potential of T cells to provide cross-variant immunity despite spike protein mutations [210]. Swadling et al. also demonstrated that robust T cell responses directed against conserved non-spike proteins of SARS-CoV-2 contribute to protective immunity, reinforcing the importance of T cells in providing cross-variant protection even when spike protein mutations occur [211].

The emerged VOCs, Alpha, Beta, Gamma, Delta and Omicron carried mutations in key regions of the spike protein, which led to reduced neutralization efficacy of antibodies elicited by infection with the ancestral strain or by vaccines based on the original viral sequence

[57,60,62,212,213]. This loss of neutralization was particularly striking with Omicron, which contained over 30 mutations in the spike protein [214]. Early data showed that neutralizing antibody titres against Omicron were significantly lower, raising global concerns that Omicron might trigger a surge in COVID-19 mortality due to immune escape [215,216]. Despite these concerns, the global mortality rate during the Omicron wave was relatively low compared to earlier waves, even though infection rates reached unprecedented levels, leading the highest number of cases (Figure 4.1). While the global mortality rate during the Omicron wave was relatively low compared to earlier waves, regions with low vaccination coverage experienced significant mortality. In Hong Kong, for instance, a study reported that 96% of COVID-19–associated deaths during the Omicron surge occurred in individuals aged 60 and above, with 70% of these fatalities among the unvaccinated. This underscores the continued virulence of the Omicron variant, particularly among unvaccinated populations[217]. This discrepancy was especially evident when comparing the number of cases during the Omicron wave to other VOC waves. This suggested that factors beyond neutralizing antibodies were contributing to protection against severe disease and death.

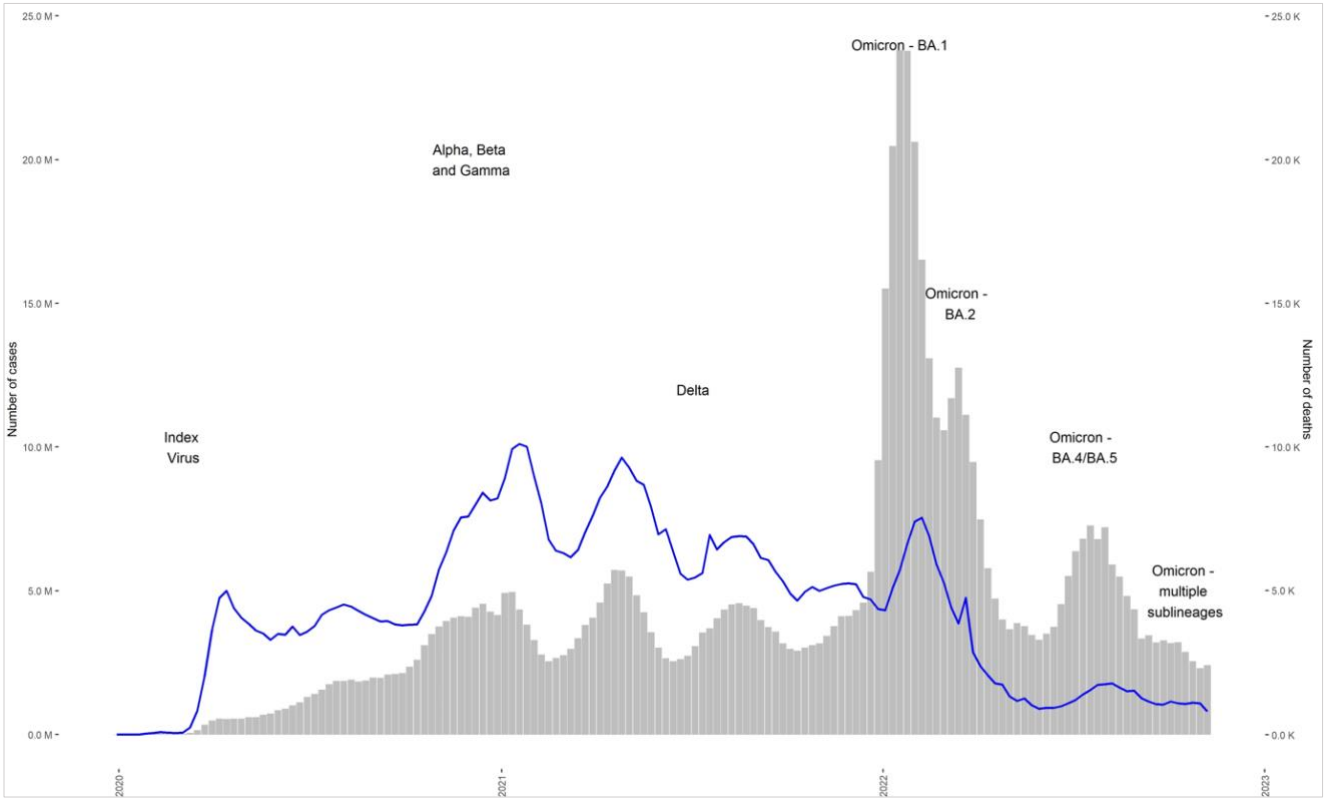


Figure 4.1. COVID-19 cases and deaths during SARS-CoV-2 variant waves. Graph showing SARS-CoV-2 cases (grey) and deaths (blue) from January 2020 to September 2023. Source: World Health Organisation [218].

The reduced mortality during the Omicron wave, despite the diminished neutralization capacity of antibodies, led researchers to hypothesise that T cells might play a critical role in mitigating severe disease. In chapter 1, studies were discussed that highlighted the role of T cells in controlling severe disease outcomes, with robust T cell responses linked to better clinical protection, especially during the initial wave of infections [219,220]. As discussed, T cells are known to recognise a broader range of viral proteins, including conserved regions of SARS-CoV-2. However, at the time, limited direct evidence existed to confirm the extent of cross-reactive T cell responses against these variants, underscoring the need for further research into their role in protective immunity. While T cells were hypothesised to play a significant role in mitigating severe outcomes, it is important to acknowledge that other functions of antibodies, beyond neutralization, might also contribute to protection against death. Antibodies can mediate immune responses such as antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and complement activation. These functions collectively enhance viral clearance by recruiting immune cells to infected sites and promoting the destruction of virus-infected cells, as demonstrated in influenza and other viral infections [221]. These non-neutralizing functions could partially explain the observed protection during the Omicron wave in individuals who had been vaccinated or previously infected.

The reduced efficacy of neutralizing antibodies as VOCs emerged highlighted the urgent need to investigate other immune mechanisms, such as cross-reactive T cell responses. Understanding these responses is critical not only for predicting the effectiveness of vaccines against future variants but also for designing vaccines that elicit broader and more durable immunity. When the first VOC, Alpha, emerged in late 2020, the lack of direct evidence for cross-reactive T cell responses to VOCs underscored a major gap in knowledge, pointing to a crucial area for research as the pandemic continued to evolve. Thus, in this chapter I aimed to investigate the breadth and magnitude of T cell responses to SARS-CoV-2 VOCs, taking into consideration prior immunity from infection or vaccination. In this chapter, I aim to understand the impact of SARS-CoV-2 variants on T cell responses in HCWs vaccinated against COVID-19. The hypothesis driving this work is that T cell responses elicited by SARS-CoV-2 vaccination are maintained against SARS-CoV-2 VOCs, potentially providing broad and durable immunity despite viral mutations.

4.2 Results

Research questions

3. Are T cell responses generated to SARS-CoV-2 spike regions with mutations?
4. Is there evidence of loss of T cell response in SARS-CoV-2 variants compared to ancestral SARS-CoV-2?

4.2.1 Cohort and design

To study T cell responses to VOCs, the PITCH HCW cohort was used. PBMCs were collected between January 2021 and August 2023 from donors who had received one, two and three doses of either the BNT162b2 or the AZD1222 vaccine. Both infection-naïve and individuals with prior SARS-CoV-2 infection were included in the study. For Alpha, Beta and Gamma, PBMCs were collected after the first vaccine dose and 7 days after the second dose. For Delta, PBMCS were collected 28 days after the 2nd dose and for Omicron, 28 days after the second dose as well as 180 days after the second and third dose (Figure 4.2.1A).

At the onset of these studies in January 2021, with the emergence of the Alpha variant, I was tasked with systematically evaluating the impact of SARS-CoV-2 VOCs on T cell responses. This process began with identifying mutations in the emerging consensus sequences of each VOC as they appeared, comparing these sequences to the ancestral strain. Once I identified key mutations, I designed and ordered overlapping peptide pools that covered the regions surrounding these mutations. These peptides were specifically chosen to encompass significant alterations in the viral structure, allowing us to assess whether these mutations influenced T cell recognition. By testing these peptides in our vaccinated HCW cohort, I aimed to determine the extent to which T cell responses elicited by the vaccine could still target these mutated epitopes, ultimately providing insight into the breadth and durability of T cell immunity across SARS-CoV-2 variants. Chapter 2 further describes the peptide design methodology in detail and Figure 4.2.1B shows a graphical representation of the different layers of peptide interactions studied in this chapter and Table 4.2.1 further shows the peptide interactions studied and their corresponding figures.

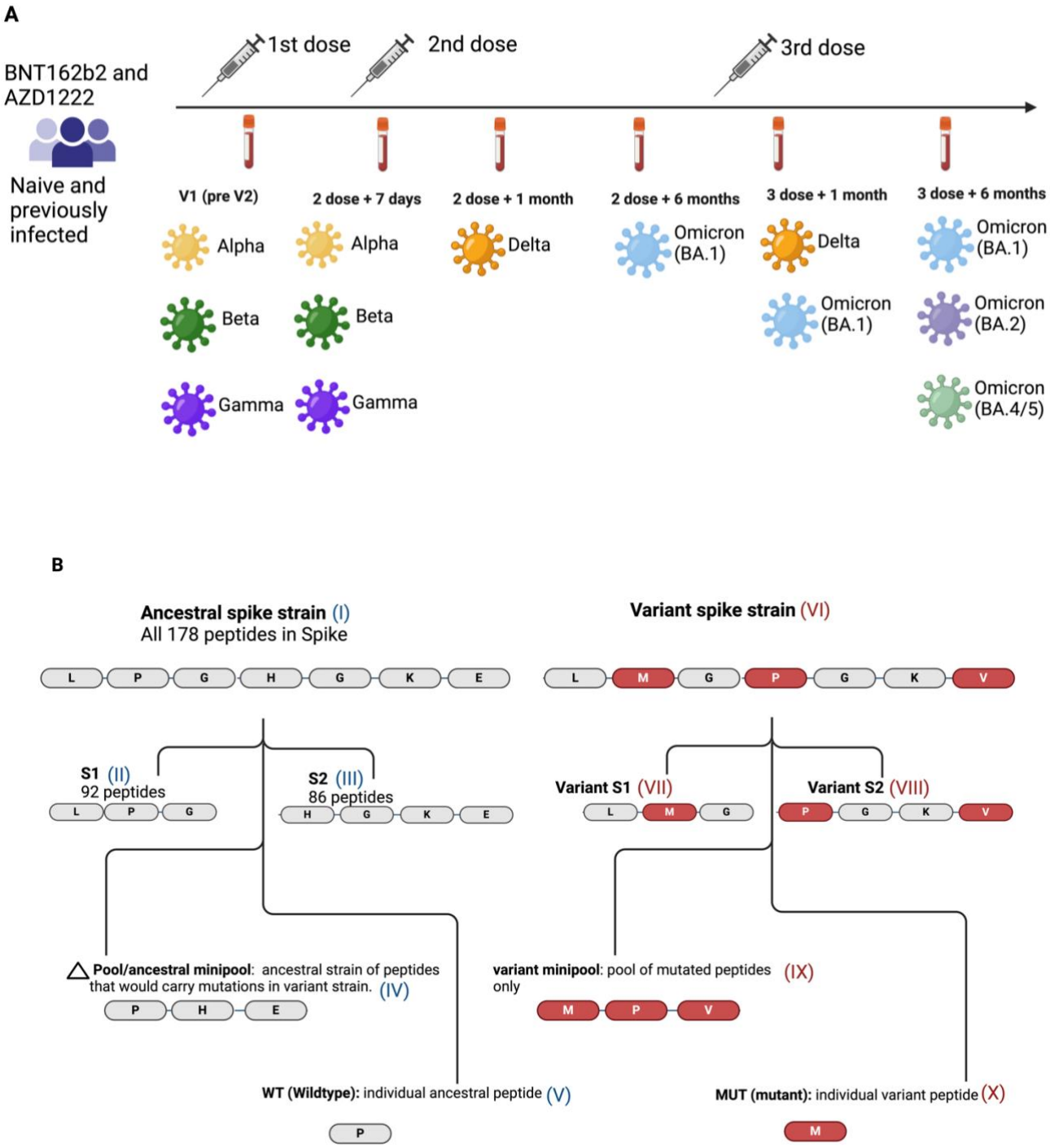


Figure 4.2.1. PITCH study cohort and peptide design strategy. (A) Timepoints of PBMCs collected to study each VOC and (B) peptide design and pooling strategy and nomenclature used. Roman numerals refer to each peptide pool used, with I-V referring to pools with no mutations (blue) and VI-X referring to pools with variant mutations (red).

Figure	Peptide Pool strategy
Figure 4.2.2 A	II,III
Figure 4.2.2 B	IV
Figure 4.2.2 C	V
Figure 4.2.2 D	IV
Figure 4.2.3 A,B	I,VI
Figure 4.2.4	II,III, VII,VIII
Figure 4.2.5	V,X
Figure 4.2.6	I,VI
Figure 4.2.7	See Figure 4.2.7
Figure 4.2.8	IV,IX
Figure 4.2.9	I,VI
Figure 4.2.10 A,C	II,VII
Figure 4.2.11	III,VIII
Figure 4.2.10 B,D	I,VI

Table 4.2.1. Peptide design strategy used and results figures. Table showing each chapter figure and the corresponding peptide design strategy used from Figure 4.2.1 B. Blue refers to peptide pools with unmutated regions while red refers to peptide pools with mutated regions.

4.2.2 T cell responses to Alpha, Beta and Gamma Variants

The first 3 VOCs, Alpha, Beta and Gamma had 9, 8 and 12 mutations respectively, corresponding to 22 peptides in total compared to the ancestral SARS-CoV-2 strain(Wuhan-Hu-1) of 178 peptides. All three variants share the N501Y and D614G mutation in the S protein, while Beta and Gamma share the E484K mutation. These mutations (discussed in depth in Chapter 1) have been shown to enhance viral transmissibility and immune escape by improving ACE2 receptor binding and reducing neutralization by antibodies [222]. I examined how T cell responses to S may be impacted both at the peptide level and at the mutation level in 24 donors who had received 2 doses of BNT162b2 SARS-CoV-2 vaccine using T cell IFNγ ELISpot assays. First, I assessed responses to all 178 peptides in the ancestral S protein subunits, S1 and S2. All donors had T cell responses against S protein, range and median magnitude 15 – 1046 SFU/10^6 PBMCs and 435 SFU/10^6 respectively (Figure 4.2.2 A).

Next, I assessed T cell responses to the mutated regions in Alpha, Beta and Gamma. I designed an ancestral S peptide pool of 17, 21 or 22 peptides corresponding to Alpha, Beta and Gamma respectively. T cell IFNγ ELISpot results showed that 18/24 donors showed a response to the Alpha pool, 21/24 donors to the Beta pool and 20/24 donors made a response to the Gamma peptide pool (Figure 4.2.2 B). These responses to the variant pools contributed 0-67% of

responses to the S protein. Specifically, the range of T cell responses to the Alpha pool contributed 0-67% (mean 13%) of the total S response, the Beta pool contributed 0-44% (mean 14%) of the total S response while the responses to the Gamma pool contributed to 0-29% (mean 10%) of the total S response (Fig 4.2.2 D). Overall, 16/24 individuals had T cell responses to all 3 VOC pools, 4/24 had T cell responses to 2 pools, 3/24 had T cell responses to 1 pool and 1/24 did not have T cell responses against any of the VOC pools. These findings indicated that the majority of individuals who received 2 doses of a SARS-CoV-2 vaccine generated robust T cell responses to the S protein, including responses targeting regions of the protein affected by mutations in Alpha, Beta, and Gamma variants. While T cell responses varied in their magnitude, most donors demonstrated cross-reactive responses to multiple variants, suggesting that vaccination induces T cells capable of recognizing and responding to mutated viral epitopes in VOCs.

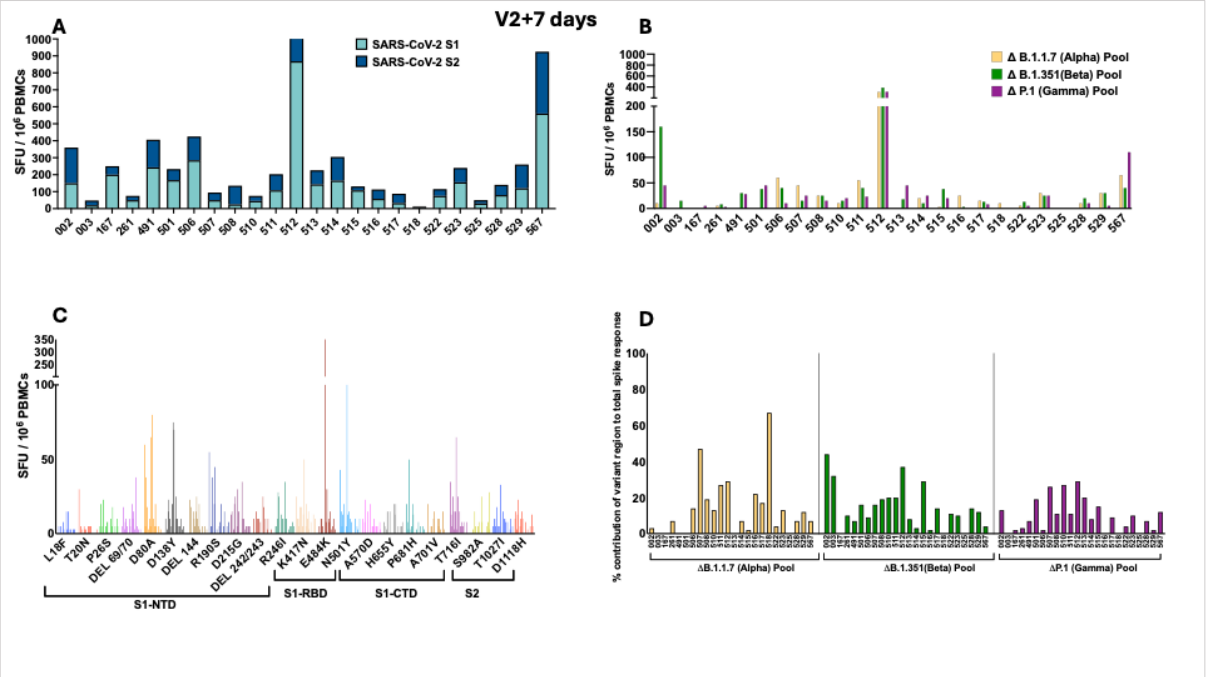


Figure 4.2.2 T cell responses to ancestral, Alpha, Beta and Gamma pools after two doses of SARS-CoV-2 vaccine. T cell responses in 24 donors to (A) ancestral S protein, (B) peptides from the ancestral strain mapped to regions with mutations in Alpha (17 peptides), Beta (21 peptides) and Gamma (22 peptides) pools, (C) individual peptides from the ancestral strain mapped to mutated peptides in Alpha, Beta and Gamma (22 peptides) and (D) percent contribution of each variant to the overall T cell response.

Additionally, I assessed individual peptide responses to the combined 22 ancestral peptides that were mutated in Alpha, Beta and Gamma. These mutations in S spanned across the N-terminal domain (NTD), RBD and the C-terminal domain (CTD). Each donor had T cell responses ranging from 0 to all 22 peptides (mean = 13), further demonstrating the breadth of the T cell response. Responses to peptides in key functional domains such as the RBD are particularly relevant, given the role of these domains in viral entry and immune evasion. Furthermore, this diversity in recognition underscores the potential for T cell-mediated immunity to provide protection across a wide range of SARS-CoV-2 variants, even when specific mutations alter key regions of the S protein. These results highlight the breadth of the T cell responses, showing that most of the T cell responses generated by the vaccine are directed against viral epitopes that are conserved between the ancestral SARS-CoV-2 strain and the VOCs.

Despite the mutations present in the Alpha, Beta, and Gamma variants, T cells retained the ability to recognise and respond to many of the altered peptides. This resilience in recognition likely stems from the nature of T cell epitopes, which often include flanking regions adjacent to the mutation site. Studies have shown that TCRs can tolerate certain amino acid substitutions within epitopes, particularly when these changes occur outside the critical anchor residues that directly interact with MHC molecules or the TCR itself [223,224]. As a result, minor variations in epitope sequence often do not disrupt T cell recognition, unlike antibody responses that rely on precise structural integrity of the target region [225]. This characteristic allows T cell responses generated by vaccination to target conserved regions of the viral proteome, maintaining cross-reactivity across multiple SARS-CoV-2 variants, even when key functional domains like the RBD undergo mutations. These findings underscore the robustness of T cell-mediated immunity against SARS-CoV-2 variants and support its role in broad and durable protection

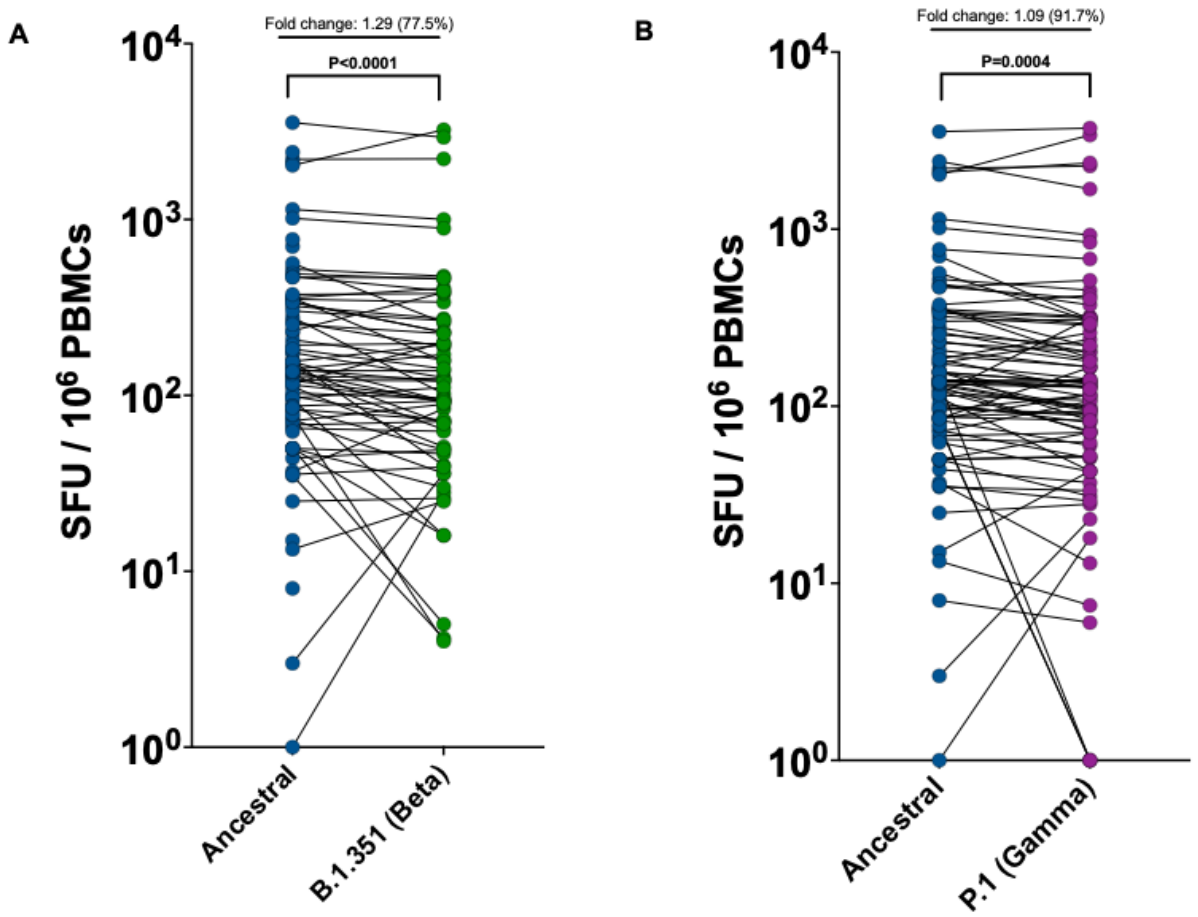


Figure 4.2.3 Paired T cell responses to ancestral, Beta and Gamma 178 whole Spike peptides after two doses of SARS-CoV-2 vaccine. T cell responses in 82 donors to (A) 178 ancestral strain peptides compared to corresponding 178 variant peptides in Beta and (B) 178 ancestral strain peptides compared to corresponding 178 variant peptides in Gamma. Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test. Fold change values refer to p values shown. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

Lastly, using ancestral S and mutated Beta and Gamma Whole S containing all 178 peptides, I studied paired responses in the ancestral strain and respective variant strains in a cohort of 82 donors who had received two doses of SARS-CoV-2 vaccine. The data demonstrates that while there is a statistically significant decline in T cell responses to the Beta and Gamma variant S strain compared to the ancestral strain ($P<0.0001$ and $P=0.0004$, respectively), the overall magnitude of T cell responses remains largely sustained across the VOCs (Figure 4.2.3). Importantly however, the individual-level differences observed where some donors show a marked loss and others a gain in T cell response highlight the variability in immune responses between individuals, even when population-level trends suggest a general preservation of immunity, so some individuals are vulnerable to a drop in T cell immunity with a given

mutation. The dynamics of these trends are explored in depth in chapter 5. These findings suggest that, despite mutations in the S protein in Beta and Gamma variants, vaccine-induced T cell immunity is broadly maintained, potentially contributing to continued protection against these VOCs at the population level.

4.2.3 T cell responses in Delta

The Delta VOC contains 7 mutations in the S protein, corresponding to 17 peptides. The mutations include the D614G mutation seen in Alpha, Beta and Gamma. Additionally, the P681R mutation in Delta is an analogous change to the P681H mutation observed in the Alpha variant, where histidine (H) is replaced by arginine (R) in Delta. Using T cell IFNγ ELISpot, I studied T cell responses to ancestral strain containing in S1(92/178) and S2 (86/178) subunits and the corresponding Delta variant S1 and S2 subunits. Responses were studied in 33 donors after two doses of BNT162b2 (n=21) or two doses of AZD1222 (n=13) (Figure 4.2.4). The results showed no significant difference in T cell responses between the ancestral and Delta S peptides in both S1 and S2, indicating that vaccine-induced T cell immunity remains robust and cross-reactive, effectively recognizing and responding to the Delta variant despite its mutations.

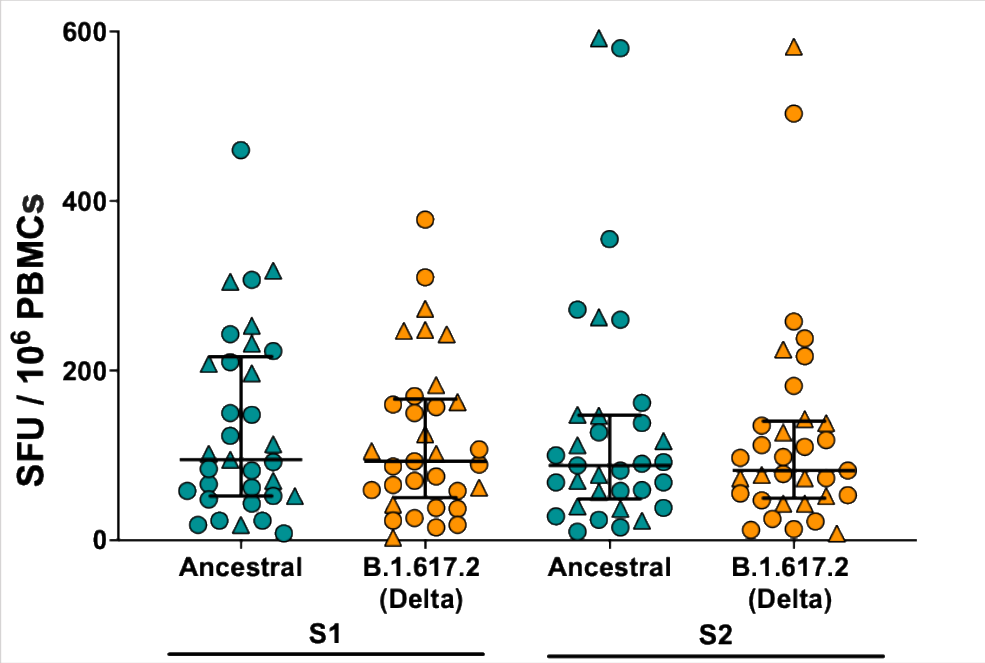
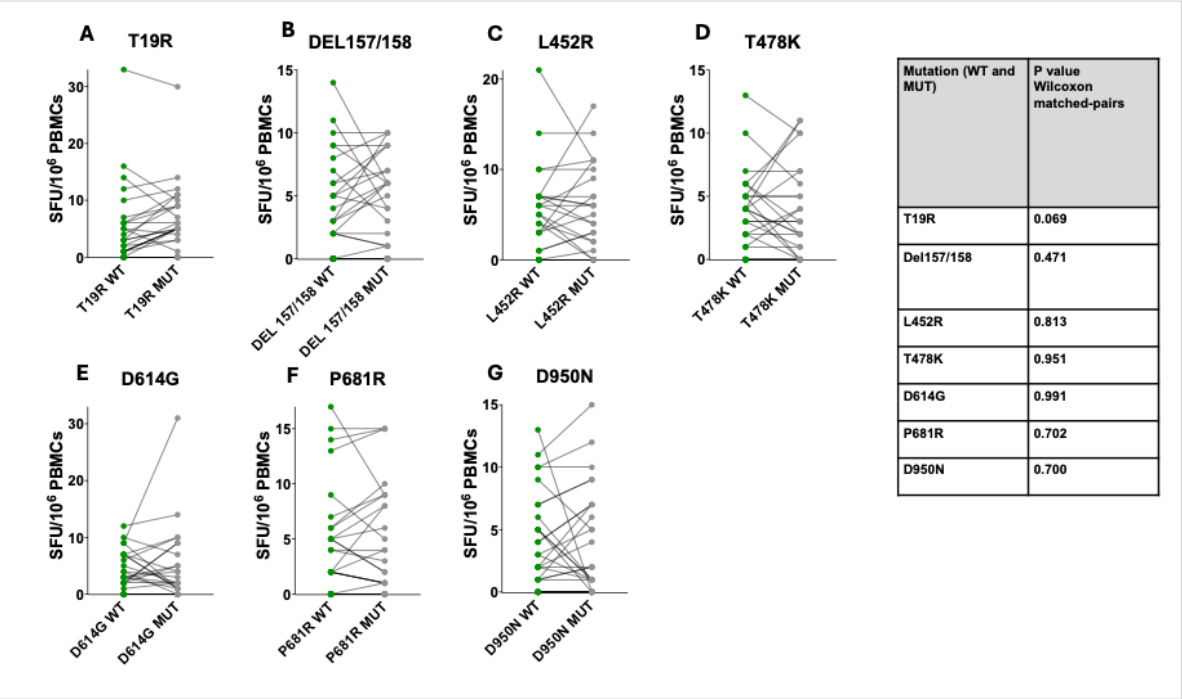


Figure 4.2.4 T cell IFN**γ** ELISpot responses to ancestral S1 and S2 peptides and corresponding Delta variants. T cell responses are measured to ancestral S1 (92 peptides) and S2 (86 peptides) and their corresponding mutated peptides in Delta variant. T cell responses in 33 donors who two doses of the BNT162b2 vaccine (circles, n=21) or two doses of the AZD1222 vaccine (triangles, n=13). Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

I further assessed T cell responses to individual mutations in a subset of 22 donors who had received two BNT162b2 SARS-CoV-2 vaccine doses using ancestral and Delta peptides individual peptides. Individual peptides were either wildtype (WT) peptide, containing no mutations, or the corresponding mutant (MT) peptide, containing mutations in Delta. In total, 7 individual peptides were assessed. Although there is a general trend toward reduced T cell recognition of the mutated peptides compared to the ancestral peptides across all mutations, the responses remain largely sustained, suggesting that despite some reduction in recognition, the overall magnitude of T cell reactivity is preserved. Statistical analysis showed no significant difference between the ancestral or Delta T cell responses for any of the individual peptides tested, indicating that the T cell responses to the Delta variant mutations are comparable to those elicited by the ancestral strain. To ensure that our experiment was adequately powered, I conducted a post hoc power calculation. This analysis confirmed that with n=22 donors and an alpha level of 0.05, the study was sufficiently powered to detect a decline of at least 71.9% in T cell responses between the ancestral and Delta individual peptides

tested. While this indicates our sample size was powered to detect relatively large differences and thus miss out on small differences that could be biologically relevant. While no published studies exist exploring individual mutations in Delta, studies looking at the full S proteome in larger sample sizes have also shown no significant decline in T cell responses when comparing ancestral and Delta variants [226,227]. This consistency across studies suggests that T cell responses to Delta mutations remain robust, supporting the idea that T cell-mediated immunity is broadly effective across these SARS-CoV-2 variants.



Mutation (WT and MUT)	P value Wilcoxon matched-pairs
T19R	0.069
Del157/158	0.471
L452R	0.813
T478K	0.951
D614G	0.991
P681R	0.702
D950N	0.700

Figure 4.2.5 Paired T cell responses to ancestral and Delta individual mutations in 22 donors after two doses of SARS-CoV-2 vaccine. Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test. WT=ancestral, MUT=variant. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

4.2.4 T cell responses in Omicron

The Omicron variant, the fourth VOC to emerge, contains 32 mutations in its S protein, compared to only 7 mutations in the Delta variant. Omicron shares the T478K, D614G, and P681H mutations with Delta. Notably, in Omicron, the amino acid at position 681 is replaced by histidine (H), similar to the Alpha variant, instead of arginine (R) as seen in Delta. Omicron, which emerged in November 2021 and was designated as B.1.1.529, quickly evolved into several descendent lineages while retaining similarities to the original parental lineage.

Consequently, BA.1 and BA.2 were classified in February 2022, followed by BA.3, BA.4, and BA.5 in May 2022. One of the challenges with designing peptides for Omicron was the high frequency of mutations within the S protein. Unlike previous variants, where we could design peptides targeting individual mutations, the extensive mutation profile of Omicron required a more comprehensive approach. To accurately cover BA.1, I created a consensus sequence that incorporated all significant mutations, dividing it into overlapping peptides rather than targeting each mutation individually. This strategy allowed for a more accurate representation of the Omicron variant in T cell assays and underscored the complexity of addressing highly mutated variants.

This research initially focused on BA.1, the first omicron variant, and then went on to include BA.2, BA.4 and BA.5. Firstly, due to the large increase in mutations in S, particularly compared the most recent VOC Delta, I wanted to assess T cell responses to the ancestral, Delta and BA.1 strains in the same donors 28 days after a third dose of SARS-CoV-2. Using PBMCs, I performed T cell IFNγ ELISpot assays using ancestral whole S peptide and variant peptides, Delta and BA.1. Responses were assessed in 12 donors who had received three doses of the BNT162b2 vaccine. This study was conducted in March 2022, (Omicron emerged in November 2021). Thus, it is worth noting the challenges in distinguishing immune responses attributable to vaccination from those influenced by prior exposure to specific SARS-CoV-2 variants. This complexity is further compounded by the fact that many people did not undergo regular testing, particularly if they were asymptomatic, making it difficult to confirm variant-specific infections and accurately attribute immune responses to specific exposures. There was a significant decline in T cell responses to Delta variant compared to the ancestral strain ($P=0.0024$), however, there were no differences between the ancestral strain and BA.1 S peptide (Figure 4.2.6). This contrasts with results highlighted in Figure 4.2.5, where there was no difference observed between ancestral and Delta S. These findings are likely explained by heterogeneity between individuals, driven by HLA type and other genetic factors as well as by past exposures to SARS-CoV-2 and possibly other pathogens. Whilst there may be individual T cell decline in responses, at a population level overall, T cell responses to VOCs are well maintained.

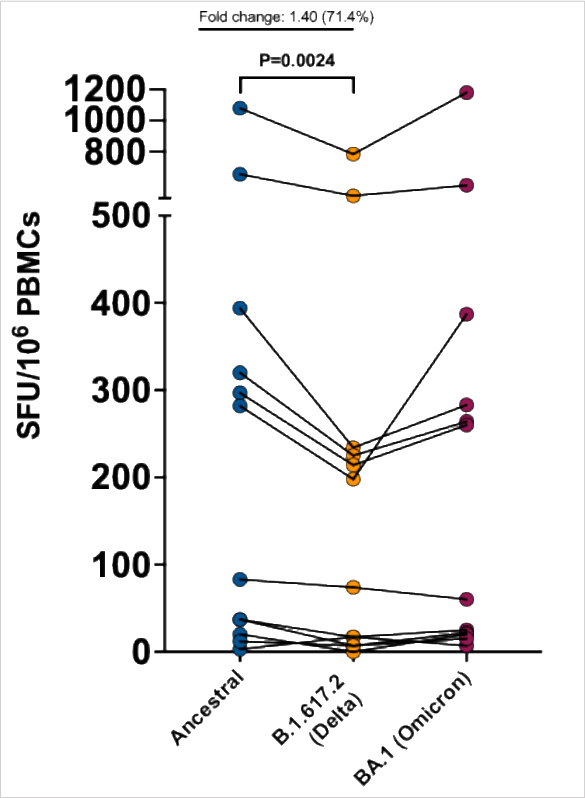


Figure 4.2.6. Paired T cell responses to ancestral strain, Delta and BA.1 variant peptides. T cell responses in 12 donors at V3+28 to ancestral strain (all 178 S peptides) and the corresponding variant strains in Delta and BA.1. Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test with only significant comparisons highlighted. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

Secondly, I assessed the contributions of T cell responses to ancestral and BA.1 S protein using T cell IFNγ ELISpot in 8 donors at the V2+6-month timepoint. I used two peptide pools; a conserved peptide pool consisting of S1/S2 peptides conserved in both ancestral and BA.1 and an Omicron BA.1 peptide pool consisting of ancestral peptides that have been mutated in BA.1. The S1 conserved region consisted of 56 peptides, the S2 conserved region consisted of 71 peptides, the Omicron BA.1 S1 region consisted of 36 peptides and the Omicron BA.1 S2 region consisted of 15 peptides. The peptide pooling design is shown in Figure 4.2.7 A. Our results show that in S1, the mean T cell responses directed towards the conserved region was 42% of the total S1 response, range 7-89% (Figure 4.2.7 B). In the Omicron BA.1 region, a mean of 58% of the total S1 response was directed towards peptides that carry mutations in BA.1, range 11-93%. In S2, 46% of the total S2 response were directed towards conserved peptides, range 24-92% and a mean of 54% of the total S2 response was directed towards

peptides that carry mutations in BA.2, range 8-67% (Figure 4.2.7 C). These findings suggest that while T cells still recognise conserved regions, in some donors, a significant proportion of the response is directed towards the mutated regions, and this can lead to significant loss of T cell responses.

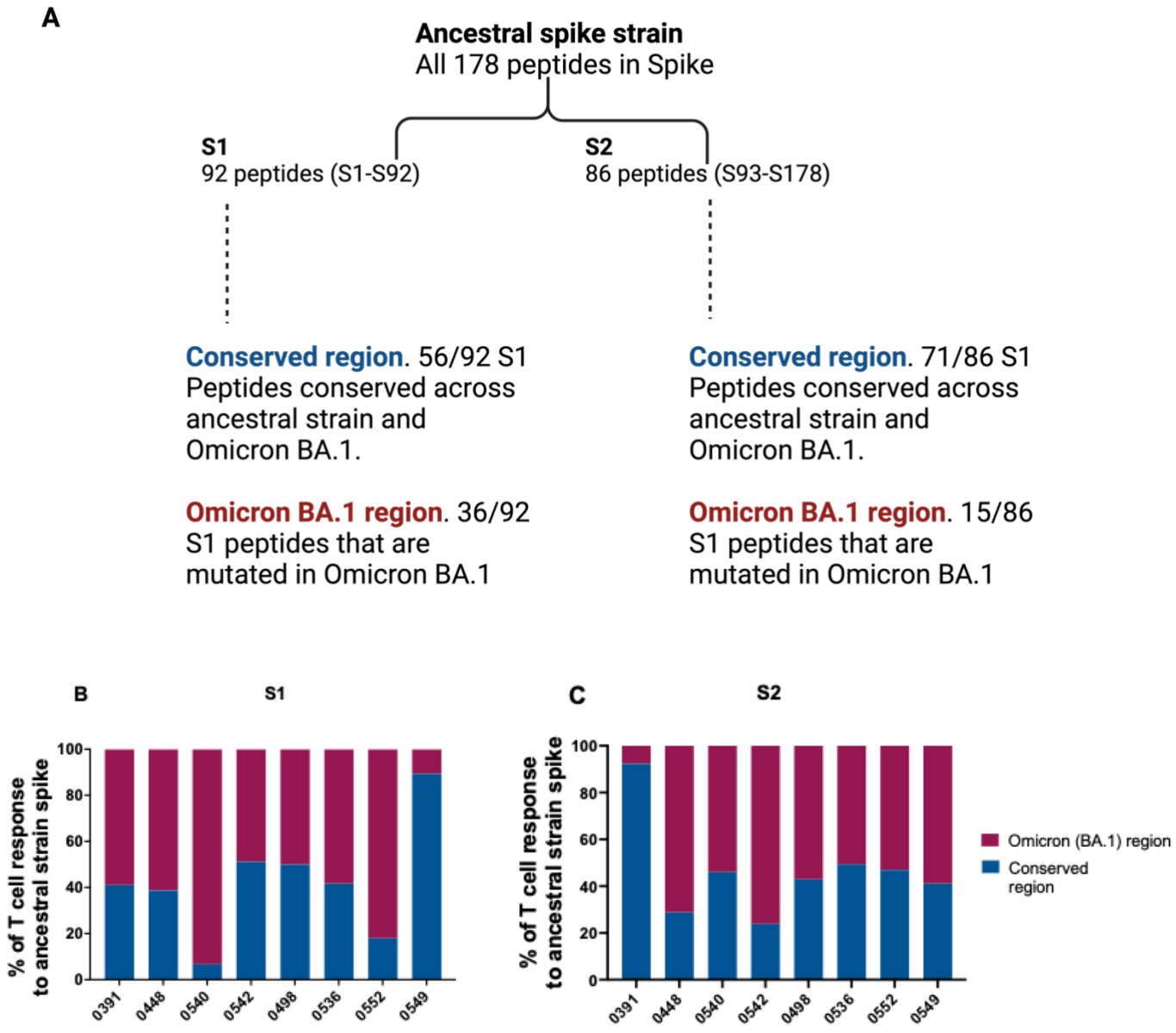


Figure 4.2.7. Percent contributions of T cell responses. (A) Peptide pooling strategy to determine percent contributions to conserved S1 and S2 regions, 56/92 and 71/86 peptides respectively and Omicron BA.1 S1 and S2 regions, 36/92 and 15/86 respectively. T cell responses to (A) S1 and (B) S2 in 8 donors at V2+6 months to conserved regions (blue) and regions with mutations in Omicron BA.1 (red).

Next, I looked at T cell responses to ancestral and BA.1 S minipools which consisted of comparing the 51/178 BA.1 mutated peptides in S to their corresponding ancestral strain peptides in 23 donors at V2+6 months. There was a significant decline observed in the T cell responses to the BA.1 minipool compared to the ancestral pool, P=0.0006 (Figure 4.2.8). This indicates that mutations present in the BA.1 variant lead to a reduced T cell recognition compared to the ancestral, unmutated sequences. The decrease in T cell response suggests that the mutations in the Omicron BA.1 variant may partially escape pre-existing T cell immunity. These findings underscore the potential challenges in maintaining immune protection as new variants with substantial mutations emerge, emphasizing the need for updated vaccines that incorporate these variant-specific changes to sustain robust T cell-mediated immunity.

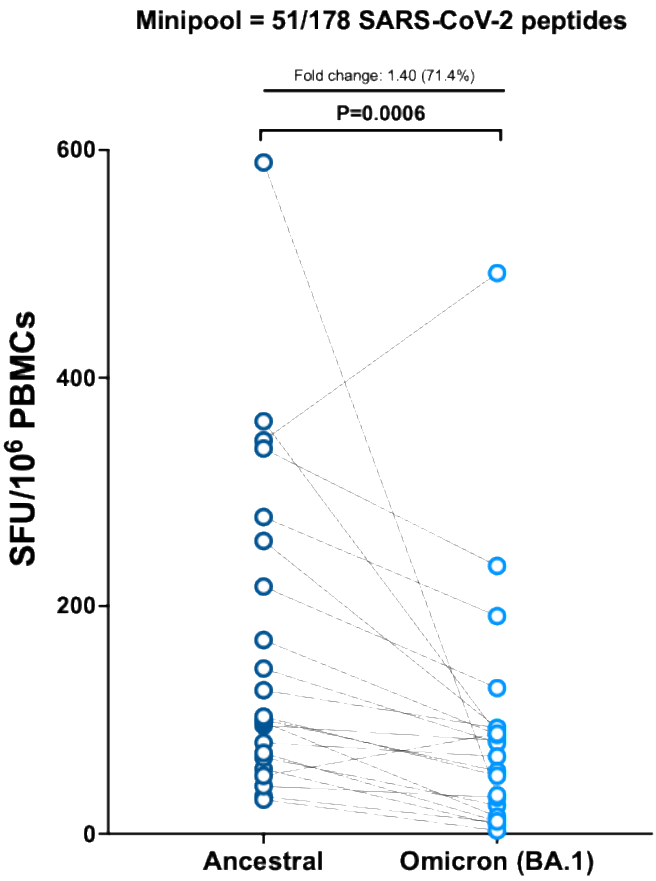


Figure 4.2.8. T cell responses to ancestral and BA.1 mini peptide pools. T cell responses to 51/178 peptides in S with mutations in BA.1. in 19 donors at V2+6 months. Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

The observations from the peptide minipools were consistent when using the whole S peptide pools (all 178 peptides) for both the ancestral and BA.1 variants in a cohort of 36 donors at the V2+6 months and V3+1-month timepoints. As shown in Figure 4.2.9 A, at V2+6 months, there was a significant decrease in T cell responses to the BA.1 variant S peptide pool (178 peptides) compared to the ancestral strain S pool (178 peptides) ($P = 0.0046$). Median T cell responses were 113 SFU/10^6 PBMCs in the ancestral strain compared to 82 SFU/10^6 PBMCs in the variant BA.1 strain. Similarly, at the V3+1month timepoint (Figure 4.2.9 B), the decline in T cell response to the BA.1 variant peptide pool was also present ($P = 0.0157$). At the V3+1-month timepoint, median T cell responses were 248 SFU/10^6 PBMCs in the ancestral strain compared to 197 SFU/10^6 PBMCs in the variant BA.1 strain. These results suggest that the mutations in the BA.1 variant lead to a considerable reduction in T cell recognition over time, indicating potential immune escape and highlighting the importance of variant-specific considerations in vaccine design and booster strategies.

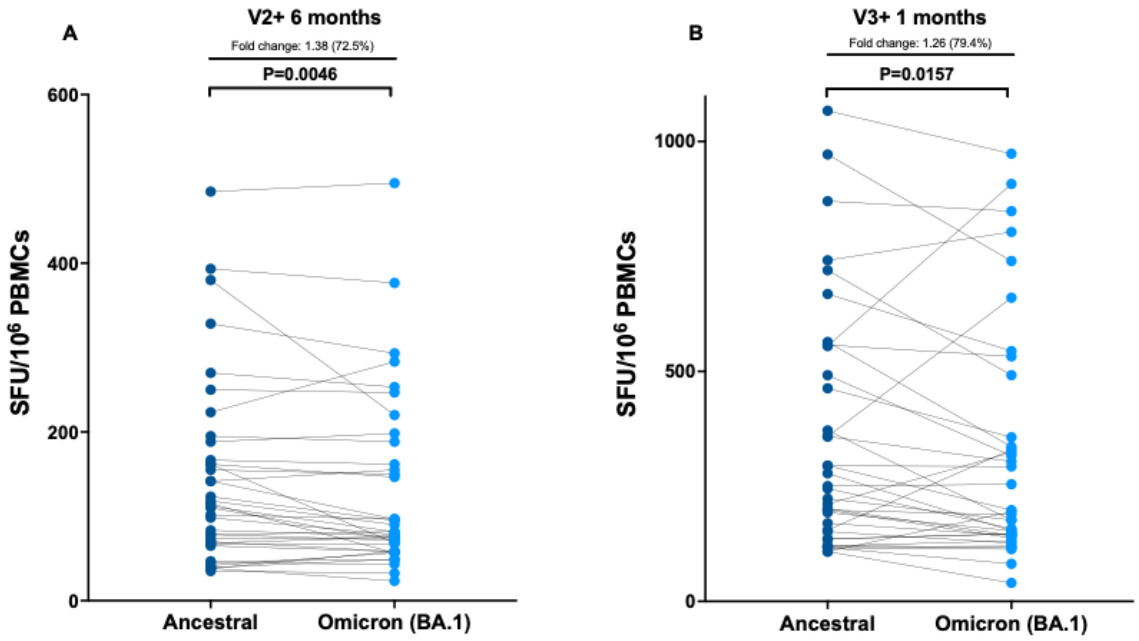


Figure 4.2.9. T cell responses to ancestral strain and variant BA.1 peptides. T cell responses to ancestral S strain (178 peptides) and corresponding variant BA.1 strain at (A) V2+6 months (n=36) and (B) V3+1 month (n=36). Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs). Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test.

I also assessed T cell proliferation responses to SARS-CoV-2 S1 and S2 in 25 donors at V2+6 months. This 7-day assay gives more opportunity to measure memory responses and gives an indication of CD4+ and CD8+ T cell subset contributions. Among the CD4+ T cells, there was no significant decline in proliferation between ancestral and BA.1 S1 peptides. However, there was a significant difference for S2 peptides (P=0.0115) (Figure 4.2.10 A-B). Median CD4+ responses in ancestral S1 and S2 were 11% and 12.5% respectively, while median CD4+ responses in Omicron BA.1 S1 and S2 were 6.4% and 11.8% respectively. Similarly, among the CD8+ T cells, there was no significant decline in proliferation between ancestral and BA.1 S2 peptides, however among S1 there was a significant decline (P=0.0342) (Figure 4.2.10 C-D). Median CD8+ responses in ancestral S1 and S2 were 9.5% and 8.4% respectively, while median CD8+ responses in Omicron BA.1 S1 and S2 were 4.1% and 5.2% respectively.

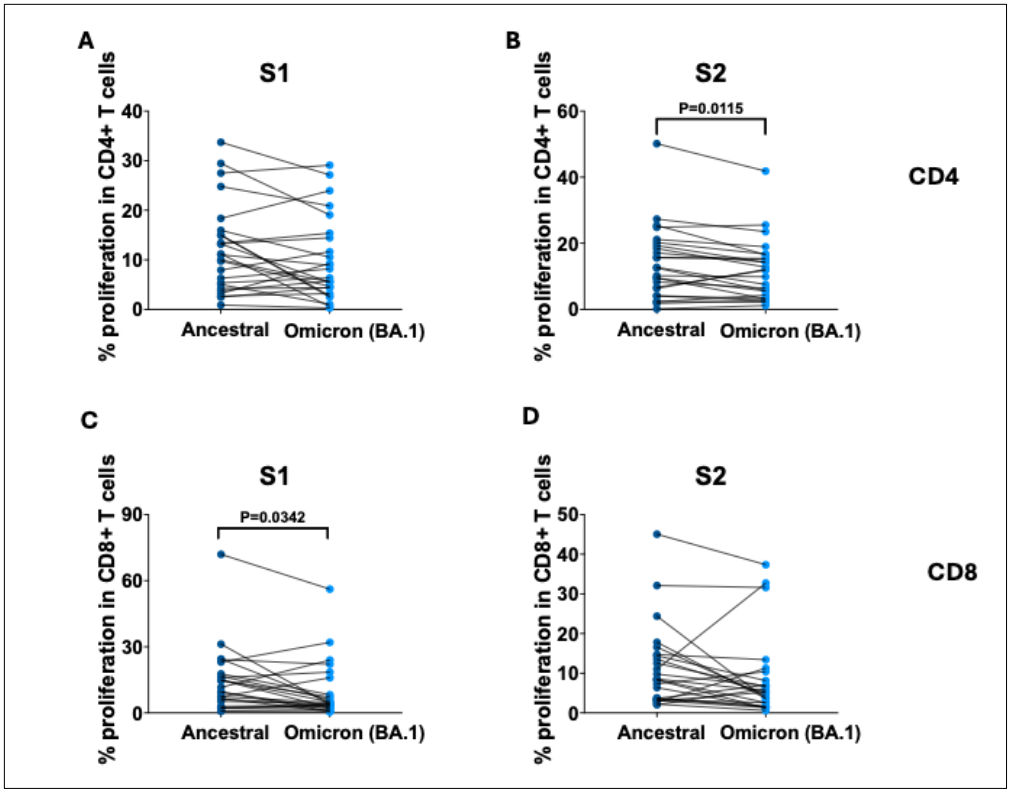


Figure 4.2.10. T cell proliferation responses after 7 days stimulation. T cell proliferation responses to ancestral strain S1(92/178) and S2 (86/178) peptides and the corresponding variant peptides, BA.1 S1and S2. Paired T cell CD4+ proliferation responses in 25 donors at V2+6 months to (A) S1 and (B) S2 peptides. Paired T cell CD8+ proliferation responses to (C) S1 and (D) S2 peptides. Statistical significance of paired comparisons was assessed using the Wilcoxon matched-pairs signed-rank test.

Lastly, we expanded the study of T cell responses to include BA.2, BA.4 and BA.5. BA.4 and BA.5 share similar mutations in S, with differences in non-S regions of SARS-CoV-2. BA.2 has 9 additional mutations in the S protein compared to BA.1, and BA.4/5 have 6 additional mutations in the S protein compared to BA.1. Using PBMCs from 64 donors at V3+6 months, I measured T cell responses to the S protein using an IFNγ ELISpot assay. There was no significant decline in T cell responses across the three descendent Omicron lineages assessed (Figure 4.2.11). The results suggest that T cell-mediated immunity provides a level of cross-protection, which is crucial for long-term immunity and may reduce the severity of infections.

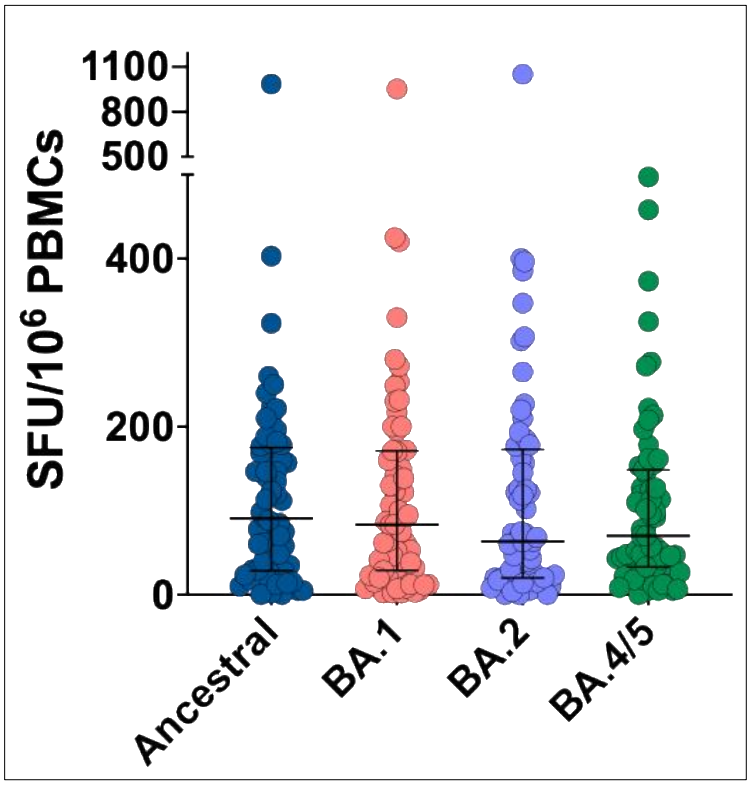


Figure 4.2.11. T cell responses to ancestral strain, BA.1, BA.2 and BA.4/5 peptides using T cell IFN**γ** ELISpot assay. T cell responses to whole S ancestral strain (178 peptides) were compared to corresponding variant strains in BA.1, BA.2 and BA.4/5. Responses to ancestral and variant peptides are measured in 64 donors at V3+6 months. Lines with median and interquartile range are shown. Responses are measured as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs). Statistical significance was assessed using the Mann-Whitney test.

4.3 Discussion

In this chapter, I investigated T cell responses at multiple levels to provide a comprehensive view of immune recognition across SARS-CoV-2 variants. I examined responses to individual ancestral peptides compared with their mutated counterparts, offering a granular view of how specific mutations affect immune recognition. Additionally, I analysed responses to minipools encompassing all peptides from mutated regions, providing insights into broader segment-based reactivity. Also, I assessed T cell responses directed at conserved regions, which are likely to be recognised across different variants and contribute to 'bystander immunity'—where conserved epitope recognition can stimulate other immune cells like MAIT and NK cells. Finally, I looked at the overall response to all 178 peptides in the ancestral sequence versus those in VOCs. These layers of analysis allow us to understand the balance between mutation-specific responses and the broader, variant-spanning immunity provided by conserved epitopes, which may help sustain T cell-mediated protection despite VOC-associated mutations.

The results in this chapter show that across the VOCs studied, T cell responses are durable and maintained, although at an individual level, some people may experience significant decline in T cell responses. In individuals, several factors may influence the magnitude and quality of T cell responses to VOCs such as HLA type, prior infection history for SARS-CoV-2 and other pathogens, sex and comorbidities. HLA molecules are responsible for presenting viral peptides to T cells, and different HLA alleles present distinct sets of epitopes. This variability can lead to individual differences in the breadth and specificity of T cell responses to SARS-CoV-2 variants. Some HLA types may be more efficient at binding and presenting conserved epitopes that remain unchanged in different variants, facilitating cross-reactive T cell responses. Conversely, certain HLA types may not bind these conserved epitopes as effectively, potentially resulting in reduced immune protection or variability in immune responses across the population. This variability can contribute to differences in susceptibility to infection, disease severity, and vaccine responses among individuals.

Age is another critical factor; older adults often exhibit a decline in T cell function and a reduction in the diversity of their TCR repertoire, which can impair their ability to mount effective immune responses against new infections or variants [228]. Similarly, immune-compromised individuals, including those with conditions such as HIV, cancer, or organ

transplantation, may have diminished T cell responses due to underlying disease or immunosuppressive treatments [229,230]. Studies have reported that these populations may have a reduced breadth and strength of T cell responses, which could impact their ability to recognise and respond to emerging variants.
Sex differences have also been noted in immune responses, with females generally exhibiting stronger T cell responses than males [231–233]. These factors underscore the need for tailored vaccination and booster strategies to address the diverse immune landscapes across different population groups. By understanding the factors influencing T cell responses, more effective and inclusive public health interventions can be developed.

The results presented in this chapter are in line with other studies which show that among the VOCs studied (Alpha, Beta, Gamma, Delta, Omicron and their descendent lineages), while there are minor differences observed, these changes are generally small in magnitude and do not significantly compromise immune recognition [165,171,208,222,234–238]. This contrasts with data from antibody studies which shows significant decline over time across VOCs. Overall, these findings suggest that compared to their impact on antibody responses, VOCs have a relatively limited impact on T cell-mediated immunity, highlighting the stability and robustness of T cell responses in sustaining protective immunity despite viral evolution. While antibody responses are crucial for initial viral neutralization, the enduring nature of T cell immunity underscores its pivotal role in long-term protection against SARS-CoV-2 and its variants. For Alpha and Beta, the neutralization potency of antibodies induced by the original SARS-CoV-2 lineage is significantly reduced against the Beta variant and to a lesser extent against the Alpha variant, while T cell responses are not majorly affected by the mutations found in these variants with most of the response directed against epitopes that are conserved between the original lineage and the VOCs [239]. Additionally, CD4+ and CD8+ T cells from individuals exposed to the ancestral SARS-CoV-2 strain through infection or vaccination effectively recognise the Alpha, Beta and Gamma variants [240], with 93% and 97% of CD4+ and CD8+ T cell epitopes respectively remain unaffected by the mutations in these variants.

Similarly, studies on T cell responses to Delta also showed that T cell responses are maintained when compared to the ancestral strain. Using a whole blood assay, Stanley et al. demonstrated that both CD4+ and CD8+ T cells in infected and vaccinated individuals exhibit robust cross-reactive immune responses against Delta, with memory T cells induced by either infection or vaccination generating similar immune responses against both the ancestral and Delta strains

[241]. Another study found no significant differences in cytokine production (IL-2 and IFN-γ) when stimulating blood samples with peptides from the ancestral, Delta, or Omicron variants. This suggests that the T cell responses elicited by vaccination are robust and maintain efficacy against multiple SARS-CoV-2 variants [242]. Tarke et al. found that T cell responses induced by different SARS-CoV-2 vaccine platforms are capable of cross-recognizing various viral variants, including the Alpha, Beta, Gamma, Delta and Omicron [226]. They also observed that about 90% of CD4+ and 87% of CD8+ memory T cell responses were preserved against the variants six months post-vaccination. On average, vaccinees recognised a median of 11 CD4+ and 10 CD8+ Spike protein epitopes. The study found that more than 80% of these epitopes were conserved in Omicron, further supporting the potential robustness of T cell immunity across different variants [226]. In contrast to the T cell responses, antibody responses show significant waning over time. A study in participants who had received two doses of the BNT162b2 or the AZD1222 vaccine showed neutralization of Delta by vaccine sera was reduced by 2.5- to 4.3-fold compared to the ancestral strain [243]. The study also highlighted significant antigenic divergence across the variants. Delta exhibited unique mutations that caused it to diverge antigenically from other variants, with Beta and Gamma showing markedly reduced neutralization against Delta, suggesting individuals previously infected with these variants might be more susceptible to reinfection by Delta. These findings underscore the durability and broad recognition of T cell responses against SARS-CoV-2 VOCs, Alpha, Beta, Gamma, Delta, and Omicron. While antibody responses may show significant reduction in neutralization potency and wane over time, T cell immunity remains robust, recognizing conserved epitopes across these variants. This highlights the crucial role of T cells in long-term protection and the potential resilience of vaccine-induced immunity in the face of emerging SARS-CoV-2 variants.

Some studies have also assessed the impact of breakthrough infections with Delta or Omicron on subsequent T cell responses. Neale et al. conducted a nested case-control study within the PITCH cohort, focusing on HCWs who received two doses of the COVID-19 vaccine during the Delta variant wave. They demonstrated that individuals who later experienced Delta breakthrough infections had lower T cell responses to Delta peptides at V2+28 compared to controls, specifically showing a drop in both CD4+ and CD8+ IFNγ and TNF responses to Delta peptides compared to ancestral peptides [142]. This drop in T cell responses among cases, as opposed to controls, suggests that a reduction in T cell reactivity to Delta-specific mutations may correlate with an increased susceptibility to breakthrough infections. This finding

underscores that for some individuals, even minor reductions in T cell responses can be associated with a higher risk of breakthrough, emphasizing the role of T cells in protective immunity against SARS-CoV-2. Tarke et al. showed that following Delta or Omicron breakthrough infections, individuals show a significant boost in CD4+ T cell responses to both Spike (S) and non-Spike antigens, such as the membrane and nucleocapsid proteins (M and N) [140]. These enhanced T cell responses target both conserved epitopes and newly arising variant-specific epitopes, suggesting that breakthrough infections may improve the immune system's ability to recognise and respond to new viral variants effectively [140]. Reynolds et al. demonstrated that following Omicron infection, T cell responses, particularly to the S protein, showed variability based on prior infection history. Their study revealed that triple-vaccinated individuals without prior infection experienced a more pronounced increase in S-specific T cell responses post-Omicron infection compared to previously infected individuals, who showed muted responses due to immune imprinting from earlier infections [244]. This suggests that prior SARS-CoV-2 exposure can attenuate the magnitude of T cell responses upon subsequent Omicron infection, potentially limiting the immune system's adaptability to emerging variants. Additionally, Hailey et al. further explored the effects of Omicron infection in previously infected and naïve populations. They found that Omicron breakthrough infections in previously vaccinated individuals generally enhanced immunity against a broad array of SARS-CoV-2 antigens, both S and non-S [245]. This boost, however, was more pronounced in previously infection-naïve individuals, who displayed robust increases in mucosal and systemic immunity. Importantly, T cell responses to conserved and non-S antigens remained strong across both groups, highlighting the capacity for Omicron infections to reinforce immunity to a range of viral targets, regardless of prior infection history.

While it has been shown that repeated S exposures do not enhance the frequency of S-specific T cell responses [141], data from the PITCH study revealed that each initial exposure to the S protein—whether from infection or vaccination—approximately doubled the magnitude of the T cell ELISpot response up to the third exposure [143]. After this third dose, T cell responses reached a plateau, highlighting that while additional doses may not further increase response magnitude, they may still enhance T cell immunity by diversifying the response across different S epitopes, potentially providing broader protection against emerging variants.

These findings are interesting, particularly within the context of providing evidence for updating vaccines to include the S protein of mutated peptides. In August 2022, bivalent

vaccines containing the spike protein components of both the ancestral strain and the Omicron variant (BA.1 or BA.4/BA.5) were approved for use as booster doses. A study analysing T cell responses in healthy donors and people living with HIV after a bivalent vaccine containing mRNA for both ancestral and Omicron BA.5 S proteins primarily induce cross-reactive CD4+ T cell responses, with most of the T cells activated by the bivalent vaccine being able to recognise both the ancestral and BA.5 spike proteins [246]. However, they found that BA.5-Specific T Cell responses were limited, with fewer than 10% of the spike-specific CD4+ T cell receptors in all donors reactive to BA.5, suggesting that the bivalent vaccine does not significantly increase BA.5-specific T cell responses compared to the original monovalent vaccine. Additionally, the majority of T cell epitopes targeted by the T cells were not affected by mutations present in the BA.5 spike protein, indicating that the cross-reactive T cell responses target conserved regions of the S protein [246]. The predominance of cross-reactive T cells suggests that the current bivalent vaccines may not induce a significantly stronger variant-specific T cell response compared to monovalent vaccines. However, the cross-reactive nature of these T cells may still provide broad protection against multiple variants. Another study on bivalent vaccines in individuals with and without prior infection found that CD4+ and CD8+ T cell responses to the S protein of both the ancestral strain and Omicron subvariants were similar regardless of prior infection status [247]. The study also found that vaccine-induced CD4+ and CD8+ T cells targeted conserved regions of the S protein across different BA.1, BA.2 and BA.4/5, demonstrating the robustness of T cell immunity in recognizing various SARS-CoV-2 variants.

The durable T cell responses observed in the presence of VOCs can provide an explanation for declining hospitalization rates in successive variant waves, even as antibody levels decline. T cell responses have been associated with better clinical outcomes in SARS-CoV-2 infection and several studies indicate that robust T cell immunity, particularly the presence of strong CD4+ and CD8+ T cell responses, correlates with milder disease and a reduced risk of severe COVID-19 [219,223,248]. Additionally, CD4+ and CD8+ T cell responses target conserved epitopes across VOCs, contributing to their cross-reactive nature. This is especially important for breakthrough infections, where the rapid activation of memory T cells can help limit viral replication and disease progression [249]. Breakthrough infections with Delta or Omicron appear to further enhance T cell responses, especially against novel variant-specific epitopes, suggesting that such infections may broaden the immune system's recognition of emerging viral variants. Importantly, bivalent vaccines, which include components of both the ancestral

strain and Omicron variants, predominantly induce cross-reactive T cell responses. Although they do not significantly enhance variant-specific T cell responses, the cross-reactive T cells still provide broad protection against multiple variants.

Overall, these findings underscore the importance of T cell immunity, especially as antibody responses wane over time. It suggests that monitoring T cell responses in addition to antibody levels could provide a more comprehensive understanding of immune protection and guide vaccine booster strategies. Maintaining a strong T cell response may be key to mitigating the impact of future SARS-CoV-2 variants and achieving long-term pandemic control. Moving forward, vaccine strategies may benefit from focusing on the induction of broad, cross-reactive T cell responses to ensure lasting protection in diverse populations. One of the key challenges in designing vaccines for SARS-CoV-2 is the extensive mutational landscape of the virus, particularly in variants like Omicron. Traditional vaccine strategies have primarily targeted the S protein, which undergoes significant mutations in new variants. Swadling et al. highlighted the importance of T cell responses against more conserved viral regions, particularly non-structural proteins like RNA polymerase (NSP12) and other components of the replication-transcription complex (RTC). These regions are expressed early in the viral life cycle and tend to be more conserved across different SARS-CoV-2 variants and even other coronaviruses. Consequently, T cell responses targeting these non-spike regions could offer a more stable, cross-reactive immunity across variants. Incorporating these conserved epitopes into vaccine designs could enhance the breadth and durability of T cell responses, offering a more robust protection against SARS-CoV-2 and future emerging coronaviruses. This further highlights the vital role of T cells in sustaining durable immunity and supports ongoing efforts to refine vaccine formulations and booster strategies to enhance protection against SARS-CoV-2.

Chapter 5: Peptide Matrix Mapping of SARS-CoV-2 and SARS-CoV-2 Variant T cell Responses

Aim: To explore the breadth of SARS-CoV-2 responses in SARS-CoV-2 vaccinated cohorts and identify T cell epitopes

Hypothesis:
I hypothesise that:

- After a third COVID-19 vaccine dose, donors previously infected with SARS-CoV-2 will exhibit a greater breadth of SARS-CoV-2-specific T cell responses compared to naïve donors

- Mutations in the SARS-CoV-2 Omicron variant are predicted to have minimal impact on T cell recognition of viral epitopes.

- The breadth of SARS-CoV-2-specific T cell responses is anticipated to increase between the third monovalent vaccine dose and the fourth bivalent vaccine dose in vaccinated donors.

- Specific T cell epitopes within the HLA-DQB1*06 allele potentially play a key role in the reduced risk of breakthrough infection associated with this allele .

Abstract
This chapter investigates the breadth and durability of SARS-CoV-2 T cell responses in vaccinated individuals, focusing on differences between those with and without prior infection. I assessed the immune response to SARS-CoV-2 spike peptides and explored T cell epitopes associated with protective HLA alleles, particularly HLA-DQB1*06.

Using PBMCs from vaccinated individuals after the third (V3+28 and V3+6 months) and fourth (V4+28) vaccine doses, T cell responses were measured by IFNγ ELISpot and intracellular cytokine staining assays. A peptide matrix was created to identify SARS-CoV-2 spike epitopes, including regions with mutations in Omicron. HLA typing identified allele-specific responses, with HLA-DQB1*06 carriers analysed for differential epitope recognition.

Findings reveal broad and durable T cell responses to multiple SARS-CoV-2 spike epitopes, especially in previously infected individuals, with significant responses in the S1 domain, including NTD and RBD. Despite mutations in Omicron, many T cell epitopes remained conserved, highlighting their potential role in cross-variant protection. Interestingly, T cell breadth and magnitude declined in donors with multiple SARS-CoV-2 infections after the fourth vaccine dose, suggesting that additional booster doses may not significantly enhance T cell responses. In contrast, donors with fewer infections demonstrated an expanded T cell response after the fourth dose. Additionally, HLA-DQB1*06 carriers displayed enhanced reactivity to specific peptides, particularly S125, suggesting a potential role for this allele in modulating SARS-CoV-2 immunity.

These results underscore the critical role of T cell response breadth in providing durable immunity after successive SARS-CoV-2 vaccinations. My findings demonstrate that while the third and fourth vaccine doses maintain broad T cell responses, there is variability in the breadth and magnitude depending on prior infection history. In particular, some individuals showed a narrower T cell response after the fourth dose, suggesting that successive exposures may lead to a more focused but potentially less adaptable immune profile. This observation highlights the importance of considering individual immune histories when designing booster strategies. While exploratory, the observed association of HLA-DQB1*06 with responses to certain SARS-CoV-2 peptides suggests allele-specific effects that merit further investigation. Together, these findings emphasise the potential benefits of vaccination strategies that enhance T cell diversity to sustain protection over time.

Contributions

In this chapter, I was responsible for each stage of the study: cohort recruitment, sample processing, all experiments and data analysis. I independently developed the peptide matrix and optimised the protocol for T cell line cultures. Additionally, I performed all bioinformatic analyses for epitope mapping and HLA typing, ensuring a comprehensive approach to examining T cell responses.

5.1 Introduction

Studying the breadth of T cell responses to SARS-CoV-2 is crucial for understanding long-term immunity and the effectiveness of vaccines against different viral variants. The breadth of T cell responses, defined as the number of distinct viral epitopes targeted by the immune system, is critical in determining the durability and effectiveness of the immune response. A broader response suggests a more diverse T cell repertoire capable of recognizing multiple epitopes, potentially improving cross-protection against viral variants. This breadth is particularly important in the context of SARS-CoV-2, as mutations in key regions of the spike protein can result in immune evasion by some viral variants. Unlike antibodies, which typically target specific regions of the virus, T cells can recognise and respond to a broader range of viral peptides, including those that may remain conserved across different variants. This broad recognition makes T cells a critical component of the immune system's defence against emerging variants that may partially escape neutralizing antibodies. Additionally, the breadth of the T cell response helps explain variations in immunity among individuals with different exposure histories, such as those with prior infections or varying numbers of vaccine doses. By understanding the diversity of T cell responses, there can be better insights into why some individuals maintain protection against severe disease despite waning antibody levels or encountering immune-evasive variants. This knowledge can also inform the design of next-generation vaccines that elicit stronger and more durable cellular immune responses, offering better protection against both current and future strains of the virus.

Prior to the work highlighted in this chapter, studies had not comprehensively defined the breadth (number of antigenic regions/epitopes targeted by T cells) of T cell responses in vaccinees across the S protein or any other protein. Stimulating PBMCs with peptides from the entire S protein requires a high number of cells and is very time intensive, thus studies have focused on creating peptide pools based on the RBD or NTD regions alone. Tarke et al. mapped T cell responses in SARS-CoV-2 infection, identifying immunodominant and subdominant epitopes across the viral genome, but did not address the breadth of responses in vaccinated individuals [275]. Additionally, Saini et al. similarly characterized SARS-CoV-2-specific CD8+ T cell responses, emphasizing immunodominance and activation profiles [276]. However, their study focused exclusively on CD8+ T cells and did not evaluate CD4+ T cell epitopes, nor did it assess the breadth of responses across the entire S protein in vaccinated individuals.

Grifoni et al. developed peptide megapools spanning the RBD, NTD, S and M proteins[223]. These megapools allowed for an assessment of the breadth of T cell responses, enabling the identification of both CD4+ and CD8+ T cell targets beyond the commonly studied regions. By using bioinformatic predictions and epitope pools, they were able to overcome the challenges of cell availability and time constraints, providing a broader understanding of T cell immunity in convalescent COVID-19 patients and revealing that T cell responses were not limited to the S protein alone but also targeted M, N, and other viral proteins [223]. However, a key limitation of the Grifoni et al. study is that it primarily focused on convalescent COVID-19 patients and unexposed individuals, without including data from vaccinated individuals. This limits the applicability of their findings, given that the current landscape of SARS-CoV-2 immunity is shaped by a combination of infections and multiple rounds of vaccination, which together influence the breadth and specificity of T cell responses. Moreover, the study did not explore the durability of T cell responses over time, so it remains unclear how long the observed immune responses persist, especially in the absence of a vaccine-induced boost. Finally, while the use of epitope megapools provided broad coverage of T cell targets, the approach is dependent on bioinformatic predictions, which may not capture all potential epitopes or account for differences in T cell responses across diverse populations with varying HLA types. Ferretti et al. also assessed the breadth of T cell responses using a genome-wide screening technology called T-scan to map the CD8+ T cell responses in convalescent COVID-19 patients [250]. This method allowed them to screen patient T cells against every possible SARS-CoV-2 epitope, without relying on predicted epitopes. Interestingly, they found that 90% of the shared epitopes recognised by CD8+ T cells in COVID-19 patients were located outside the S protein and instead the epitopes were predominantly located in regions such as ORF1ab and the N protein. Another important finding from this study was that CD8+ T cells specific to SARS-CoV-2 showed almost no cross-reactivity with epitopes from seasonal coronaviruses, suggesting that prior infections with those coronaviruses are unlikely to provide CD8+ T-cell-mediated protection against SARS-CoV-2 [251]. The Ferretti et al. study, while comprehensive in its mapping of the breadth of CD8+ T cell responses to SARS-CoV-2, has several limitations: the study focuses exclusively on CD8+ T cell responses, without examining CD4+ T cell responses, thus limiting the understanding of the full spectrum of immune responses to SARS-CoV-2. The study focused exclusively on convalescent patients who had recovered from SARS-CoV-2 infection and did not include individuals who had received COVID-19 vaccines. As a result, the findings may not reflect the CD8+ T cell responses induced by vaccination, especially since most vaccines target the spike protein,

whereas the study found most natural CD8+ responses were directed elsewhere. Secondly, the study used polyclonal expansion of memory CD8+ T cells ex vivo to increase the number of cells available for screening. While this allowed for broader testing, it may have altered the relative abundance of some T cell clonotypes, potentially skewing the results by amplifying some clonotypes more than others [251,252]. Therefore, the work in this chapter studies the breadth of T cell responses in vaccinated individuals, providing new insights into how SARS-CoV-2 vaccines, particularly those targeting the S protein, influence the breadth and specificity of both CD4+ and CD8+ T cell responses. By overcoming previous limitations in cell availability and assay complexity, this research expands upon earlier studies by examining T cell responses across the entire S protein and comparing these to natural infection responses, thereby offering a more comprehensive understanding of T cell-mediated immunity in the context of both vaccination and natural infection.

To date, there are no studies directly comparing the breadth of T cell responses in individuals after a third SARS-CoV-2 vaccine dose to those after a fourth bivalent dose. Individuals with different SARS-CoV-2 infection histories (e.g. never infected, previously infected once or multiple times) have varying levels of pre-existing immunity, which can affect their response to vaccination. Prior infection likely broadens T cell responses by exposing individuals to a more diverse array of viral epitopes beyond those included in vaccines, which typically focus on the S protein. Therefore, understanding how infection history shapes T cell responses to subsequent vaccine doses is crucial for tailoring immunization strategies.

Additionally, a broad T cell response is critical for cross-variant protection, as SARS-CoV-2 continues to evolve. Comparing the breadth of T cell responses after multiple doses in individuals with different infection histories helps to assess whether vaccination strategies generate T cells that can recognise diverse viral variants, including those that may evade antibody responses. This is particularly important given that some variants may escape neutralizing antibodies but still be controlled by a robust and diverse T cell response [253–255]. comparing the breadth of T cell responses between the third and fourth doses, also provides insights into the durability of the immune response. If the breadth narrows over time after the third dose but is restored or even expanded after the fourth, this suggests that booster doses are necessary to maintain long-term T cell immunity, especially in individuals who may have experienced immune waning due to prior infection or time elapsed since vaccination [256].

This chapter provides critical insights into how successive vaccinations and prior infections shape the breadth and durability of T cell responses, which is essential for optimizing booster strategies and ensuring long-term protection against both current and future variants of SARS-CoV-2. Studying these responses also informs public health strategies for optimizing vaccine schedules. Understanding whether additional doses meaningfully expand T cell breadth, especially in individuals with prior infections, can guide decisions on booster policies for different populations, such as immunocompromised individuals or those at higher risk of severe disease.

T cell epitopes in a protective HLA allele

As the COVID-19 pandemic evolved, several studies showed diversity in immune responses, for example T cell and antibody responses, across donors with similar infection and/or vaccination profiles [196,257,258]. One of the questions this prompted was a need to understand gene associations with immune responses in SARS-CoV-2, with the goal of identifying T cell epitopes that could be protective. Also, identification of these epitopes could be useful in designing updated vaccines against SARS-CoV-2.

Several studies have explored the associations between HLA types and SARS-CoV-2. For example, Langton et al. explored how specific HLA genotypes influence the severity of COVID-19. They found a significant association between the HLA-DRB1*04 allele and milder COVID-19 outcomes, as this allele was more frequent in asymptomatic individuals compared to those with severe symptoms [259]. Conversely, the HLA-DRB1*01 allele was less common in asymptomatic individuals, suggesting a potential role in increased disease susceptibility. Another study by Augusto et al. found an association between HLA-B*15:01 and asymptomatic SARS-CoV-2 infection, with individuals carrying this allele being over twice as likely to remain asymptomatic upon infection compared to non-carriers [260]. The study further showed that T cells from pre-pandemic samples of HLA-B*15:01carriers were reactive to a SARS-CoV-2 peptide, NQKLIANQF, also present in seasonal coronaviruses, OC43 and HKU1, indicating potential cross-reactive immunity.

An Oxford-based study by Mentzer et al. in 1,076 participants found that individuals carrying the HLA-DQB1*06 allele demonstrated higher antibody responses to the SARS-CoV-2 S protein following vaccination and were less likely to experience breakthrough infections

compared to non HLA-DQB1*06 cohorts, with HLA-DQB1*06 associated with a 37% reduction in breakthrough infection risk, and carriers showed increased memory B cell responses [261]. Specifically, Individuals with HLA-DQB1*06 alleles had a lower likelihood ($P<0.02$) of experiencing PCR-confirmed breakthrough infections during the waves of the ancestral SARS-CoV-2 virus and the subsequent Alpha variant, compared to those without these alleles[261]. Additionally, a study by Xie et al. in 175,000 participants found HLA-DQB1*06 to be associated with stronger antibody responses following SARS-CoV-2 vaccination, and a 30% lower risk of breakthrough infection [262]. Interestingly, in a cohort of 80 transplant patients, HLA-DQB1*06 was associated with increased likelihood of SARS-CoV-2 infection ($P<0.012$) [263]. The immune systems of transplant patients are often compromised due to immunosuppressive therapies, which can affect how their immune systems respond to infections such as SARS-CoV-2 [264]. As a result, findings in this population may not be generalizable to the general population or other specific groups. For instance, while HLA-DQB1*06 may be associated with enhanced immune response in the general population, transplant patients' immunosuppressed status might interfere with this potential protective effect. Also, HLA-DQB1*06 is likely to influence immune responses in complex ways, not solely through antibody production but also by affecting T cell responses and other immune mechanisms. The effectiveness of HLA alleles can be context-dependent, varying with factors like infection severity, previous exposures, or the immune status of individuals.

Building on these findings, which underscore the context-dependent nature of HLA impact on immune responses, Chapter 1 provided a detailed review of SARS-CoV-2 epitopes and their HLA associations. This chapter aims to advance our understanding by leveraging the peptide targets identified in this chapter, further identifying specific T cell epitopes associated with SARS-CoV-2 and validating these using in vitro assays.

5.2 Results

Research questions

5. What is the breadth (number of SARS-CoV-2 peptide responses) of SARS-CoV-2 responses in naïve and previously infected donors after a third COVID-19 vaccine dose?
6. Is there loss of T cell response to Omicron (BA.2,BA.4 and BA.5) compared to ancestral SARS-CoV-2?
7. In vaccinated donors after a 4th vaccine dose, is there a difference in breadth of SARS-CoV-2 responses compared to their 3rd vaccine dose?
8. Can HLA-DQB1:*06 restricted T cell epitopes be identified?

5.2.1 Cohort and experiment design

To study the breadth of T cell responses, PBMCs from the PITCH study cohort of donors after a third vaccine dose (V3+28 days and V3+6 months) and fourth dose of SARS-CoV-2 vaccine, V4+28 days were used. Firstly, a SARS-CoV-2 peptide matrix was developed which included all 178 peptides in SARS-CoV-2 spike (15-18 monomer in lengths, overlapping by 10 amino acids). From the 178 peptides, a pool of 38 peptides were constructed, containing 8-10 peptides per pool. Using a T cell IFNγ ELISpot assay, PBMCs from donors were used to determine peptide responses, with a positive response cut off as the background + 2 standard deviations for each donor. Once positive peptide responses to the pools were confirmed, identified antigenic targets were clonally expanded using a 14-day T cell line culture for a subset of single peptides, prioritizing peptides with mutations in Omicron BA2, and BA.4/5. Figure 5.2.1 shows the flow of experiments. Following clonal expansion, cytokine responses, IFNγ, IL-2 and TNF were measured following restimulation with peptides in ICS using flow cytometry. Additionally, to study T cell epitopes, the HLA type of each donor was determined.

To design the peptide matrix, each of the 178 peptides in SARS-CoV-2 S were included twice, in two pools. Thus, to get a positive peptide response, a donor had to show a positive response to the two pools containing a particular peptide. Figure 5.2.2 shows the peptide matrix, with peptides with mutations in BA.2, BA.4 and BA.5 S protein highlighted.

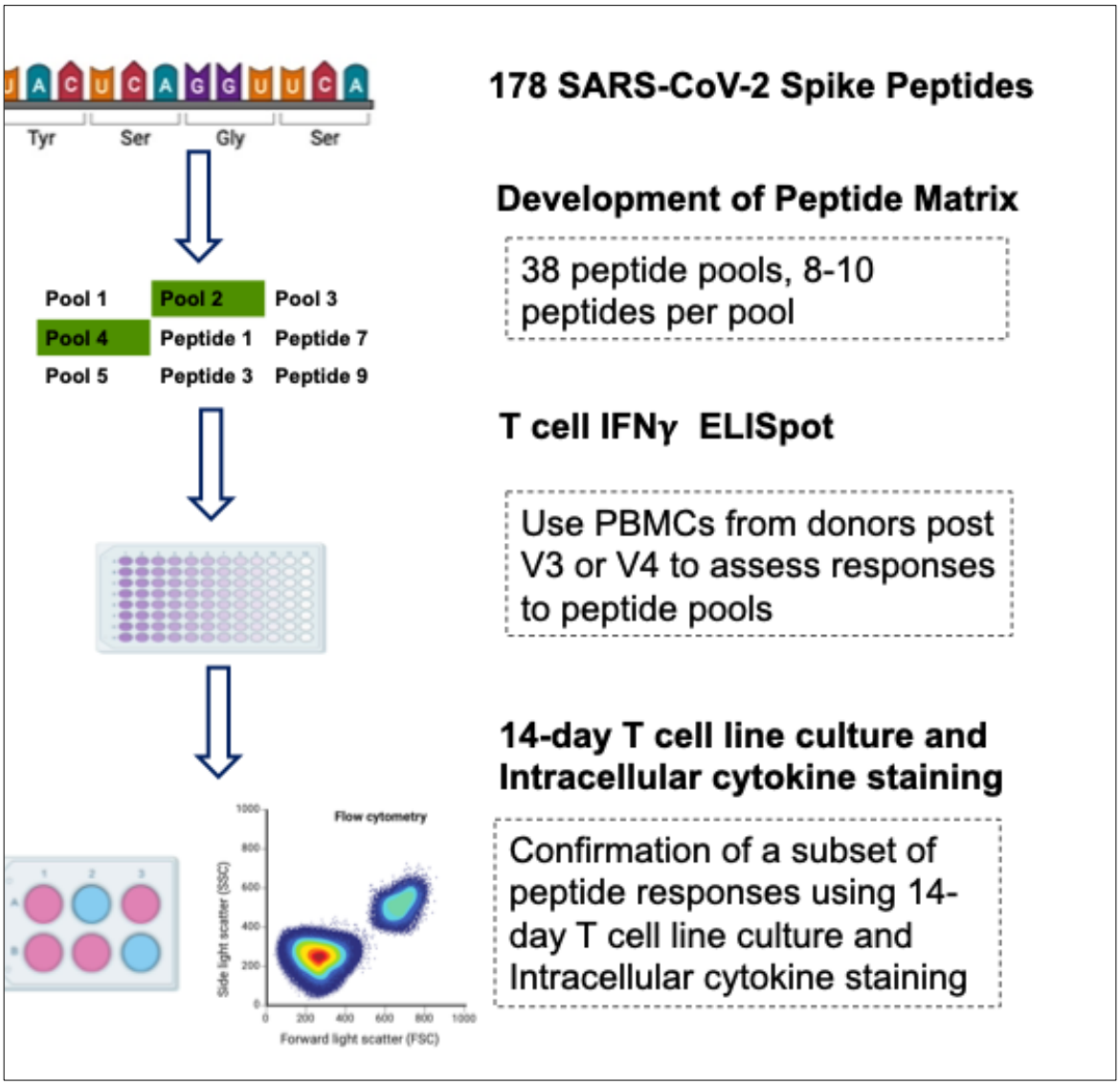


Figure 5.2.1. Experimental design. Experimental design used in mapping T cell peptide responses highlighting development of peptide matrix, T cell IFN**γ** ELISpot assay and confirmation of peptide responses using a 14-day T cell line culture.

Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9	Pool 10	
S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_10	Pool 11
S_11	S_12	S_13	S_14	S_15	S_16	S_17	S_18	S_19	S_20	Pool 12
S_21	S_22	S_23	S_24	S_25	S_26	S_27	S_28	S_29	S_30	Pool 13
S_31	S_32	S_33	S_34	S_35	S_36	S_37	S_38	S_39	S_40	Pool 14
S_41	S_42	S_43	S_44	S_45	S_46	S_47	S_48	S_49	S_50	Pool 15
S_51	S_52	S_53	S_54	S_55	S_56	S_57	S_58	S_59	S_60	Pool 16
S_61	S_62	S_63	S_64	S_65	S_66	S_67	S_68	S_69	S_70	Pool 17
S_71	S_72	S_73	S_74	S_75	S_76	S_77	S_78	S_79	S_80	Pool 18
S_81	S_82	S_83	S_84	S_85	S_86	S_87	S_88	S_89	S_90	Pool 19

Pool 20	Pool 21	Pool 22	Pool 23	Pool 24	Pool 25	Pool 26	Pool 27	Pool 28	Pool 29	
S_91	S_92	S_93	S_94	S_95	S_96	S_97	S_98	S_99	S_100	Pool 30
S_101	S_102	S_103	S_104	S_105	S_106	S_107	S_108	S_109	S_110	Pool 31
S_111	S_112	S_113	S_114	S_115	S_116	S_117	S_118	S_119	S_120	Pool 32
S_121	S_122	S_123	S_124	S_125	S_126	S_127	S_128	S_129	S_130	Pool 33
S_131	S_132	S_133	S_134	S_135	S_136	S_137	S_138	S_139	S_140	Pool 34
S_141	S_142	S_143	S_144	S_145	S_146	S_147	S_148	S_149	S_150	Pool 35
S_151	S_152	S_153	S_154	S_155	S_156	S_157	S_158	S_159	S_160	Pool 36
S_161	S_162	S_163	S_164	S_165	S_166	S_167	S_168	S_169	S_170	Pool 37
S_171	S_172	S_173	S_174	S_175	S_176	S_177	S_178			Pool 38

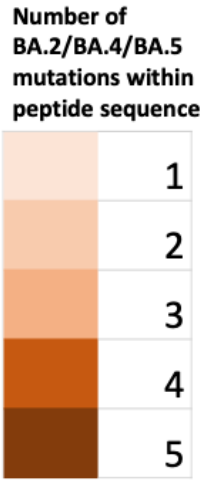
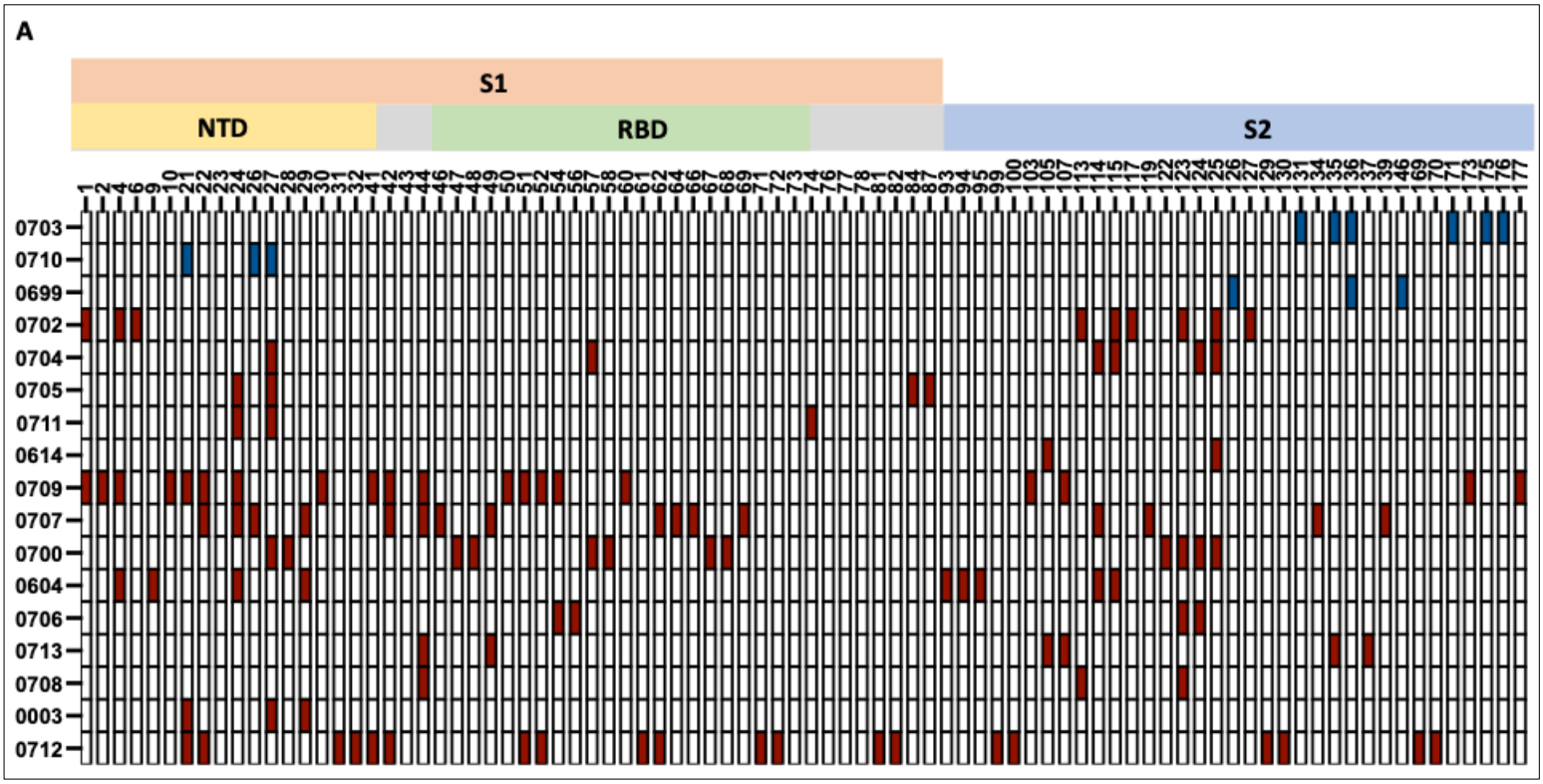


Figure 5.2.2. SARS-CoV-2 spike matrix showing 38 peptide pools. Each pool contains 8-10 individual SARS-CoV-2 spike peptides of 15-18 amino acids in length. The shades of brown highlight the number of peptides within the sequence that are mutated in BA.2, BA.4 and BA.5.

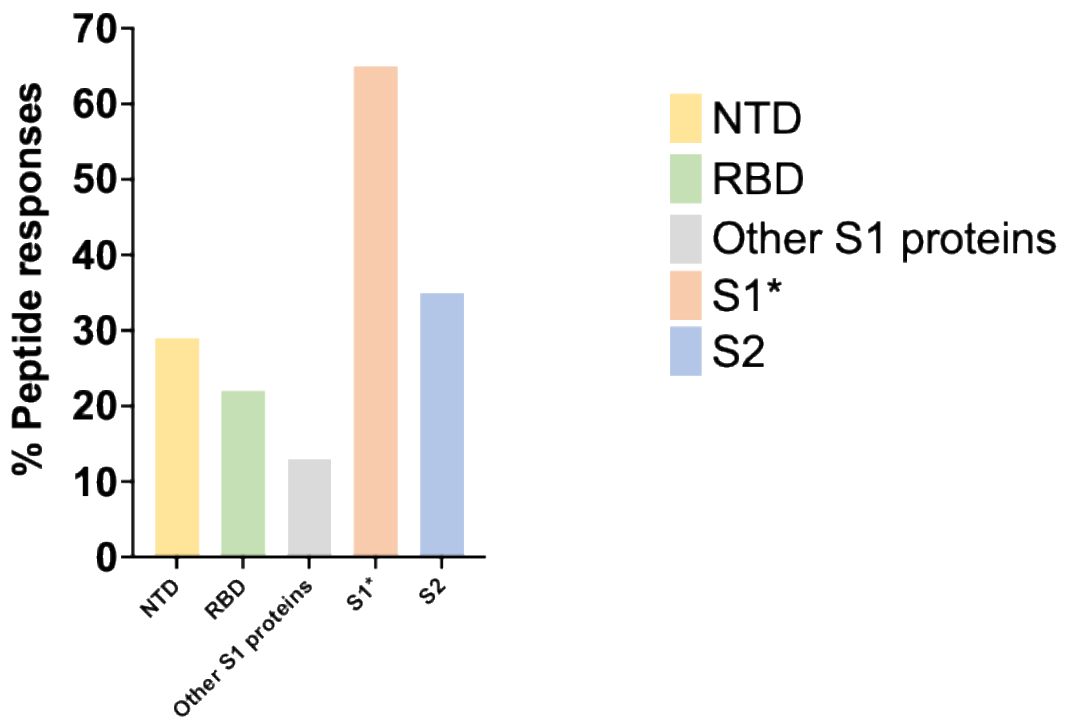
5.2.2 Breadth of T cell responses to SARS-CoV-2 in vaccinated donors

The breadth of `T cell responses to SARS-CoV-2 peptides was assessed in 17 donors 6 months after a third vaccine dose using a T cell IFNγ ELISpot assay. Of the 17 donors, 14 had known previous infection with SARS-CoV-2 (determined by positive PCR, LFT and/or anti-N antibodies), while 3 were infection-naïve. All volunteers generated T-cells targeting >1 ancestral (wildtype) peptide (median 6, range 2-21). In all, 86 distinct antigenic targets were identified, predominantly located in SARS-CoV-2 S1 (65%) (Figure 5.2.3 A). Detailed responses within each donor to total S and the peptide pools are shown in Figure 5.2.3 C. Within S1, T cell responses identified in the NTD were 29%, RBD 22% and other S1 proteins 14%. S2 responses made up 35% of the total S response (Figure 5.2.3 B). Of the 86 identified targets, 56/86 were conserved between ancestral and BA.2/BA.4/BA.5; however, 6/18 NTD and 13/23 RBD targets contained mutations in BA.2/BA.4/BA.5 relative to the ancestral strain. These results highlight the broad and diverse nature of T cell responses following vaccination, particularly in previously infected individuals, with significant targeting of peptides within the

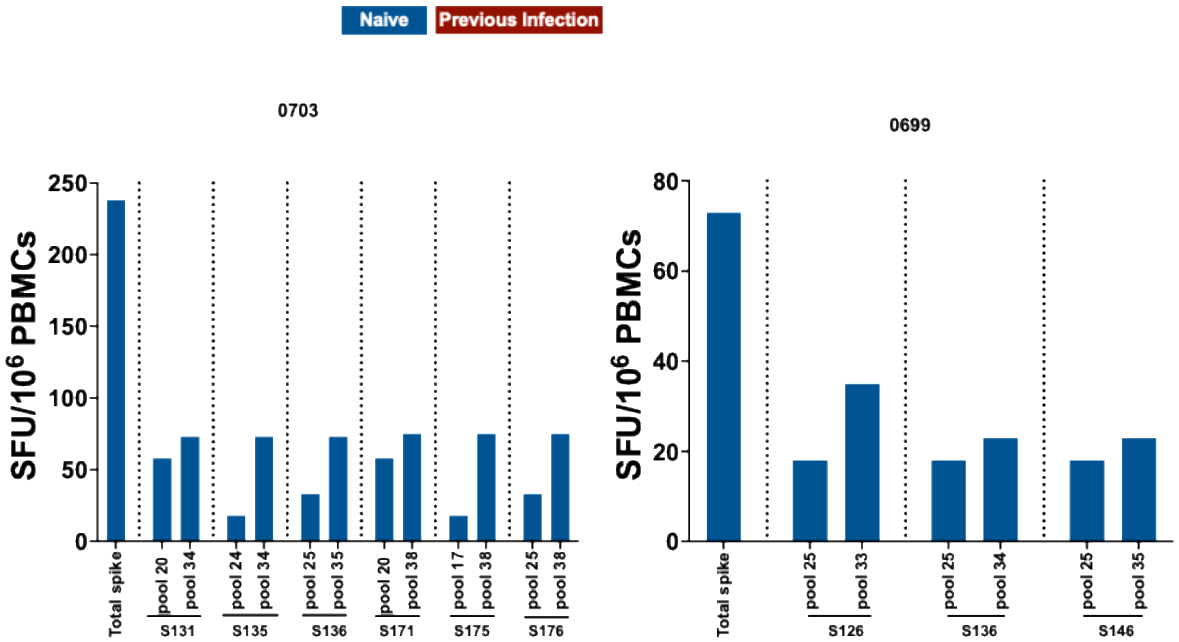
S1 domain, including NTD and RBD regions. Despite mutations in BA.2/BA.4/BA.5, a substantial proportion of T cell epitopes remain conserved between the ancestral strain and these variants. However, mutations in the NTD and RBD of the variants reduce recognition of specific epitopes in these regions, which may have implications for immune escape. This suggests that while T cell immunity remains largely robust, particularly in conserved regions of the S protein, mutations in key functional domains like the RBD could potentially diminish the breadth of immune protection in some individuals.

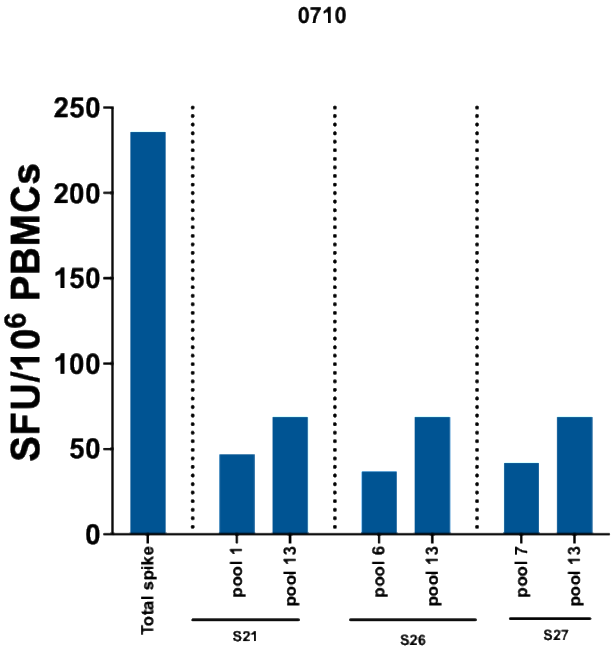


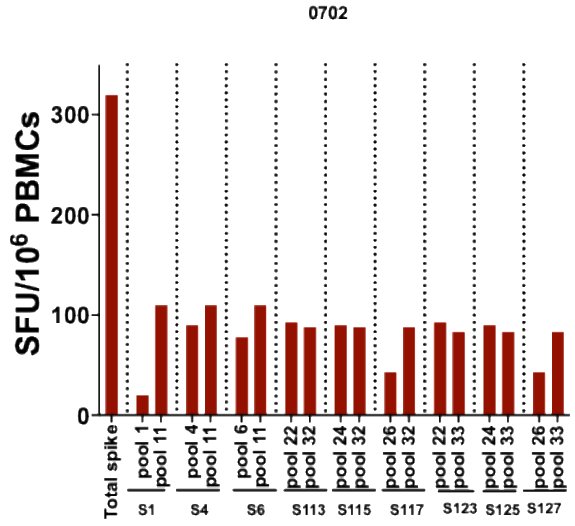
B

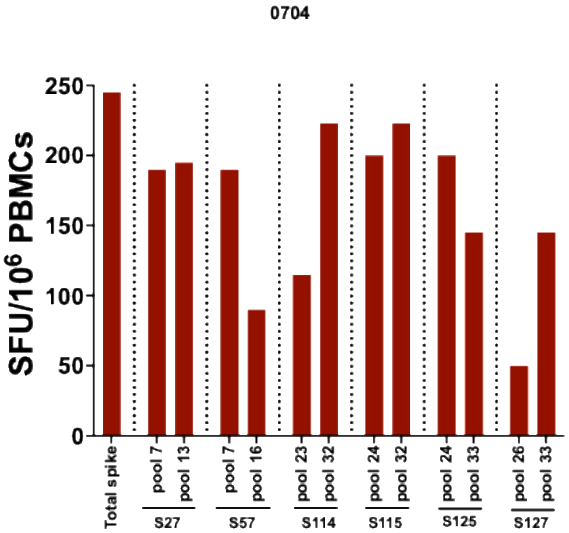

% Peptide responses
70
60
50
40
30
20
10
0
NTD
RBD
Other S1 proteins
S1*
S2
NTD
RBD
Other S1 proteins
S1*
S2

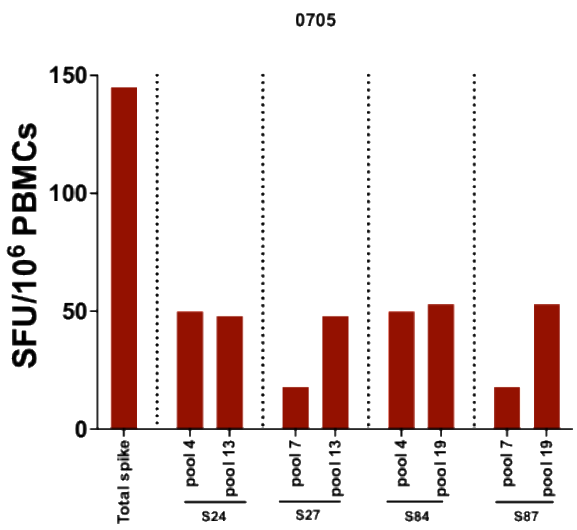
C

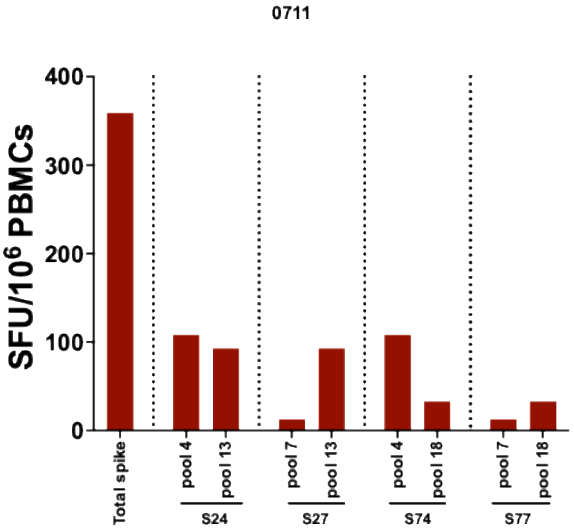

Naive
Previous Infection
0703
SFU/10^6 PBMCs
250
200
150
100
50
0
Total spike
pool 20
pool 34
S131
pool 24
pool 34
S135
pool 25
pool 35
S136
pool 20
pool 38
S171
pool 17
pool 38
S175
pool 25
pool 38
S176
0699
SFU/10^6 PBMCs
80
60
40
20
0
Total spike
pool 25
pool 33
S126
pool 25
pool 34
S136
pool 25
pool 35
S146

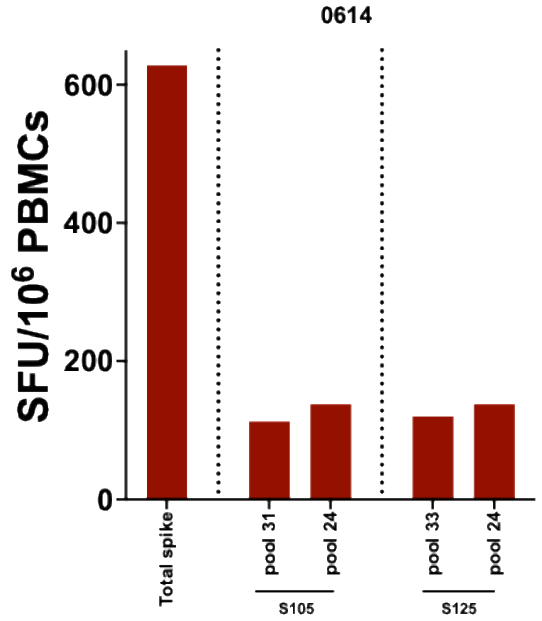

0710
SFU/10^6 PBMCs
250
200
150
100
50
0
Total spike
pool 1
pool 13
pool 6
pool 13
pool 7
pool 13
S21
S26
S27

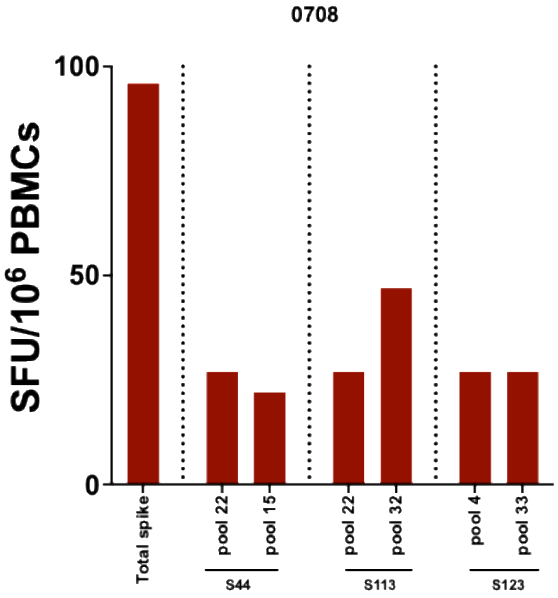

0702
SFU/10^6 PBMCs
300
200
100
0
Total spike
pool 1
pool 11
pool 4
pool 11
pool 6
pool 11
pool 22
pool 32
pool 24
pool 32
pool 26
pool 32
pool 22
pool 33
pool 24
pool 33
pool 26
pool 33
S1
S4
S6
S113
S115
S117
S123
S125
S127

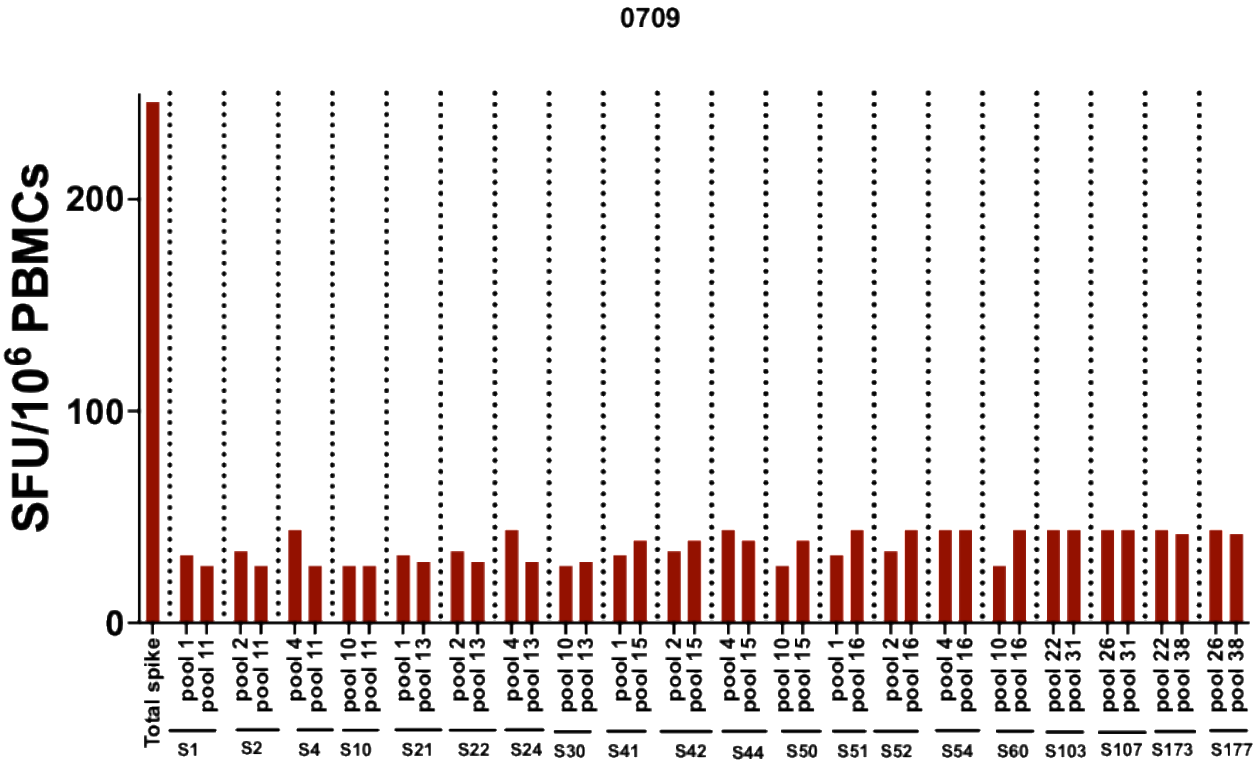

0704
SFU/10^6 PBMCs
250
200
150
100
50
0
Total spike
pool 7
pool 13
pool 7
pool 16
pool 23
pool 32
pool 24
pool 32
pool 24
pool 33
pool 26
pool 33
S27
S57
S114
S115
S125
S127

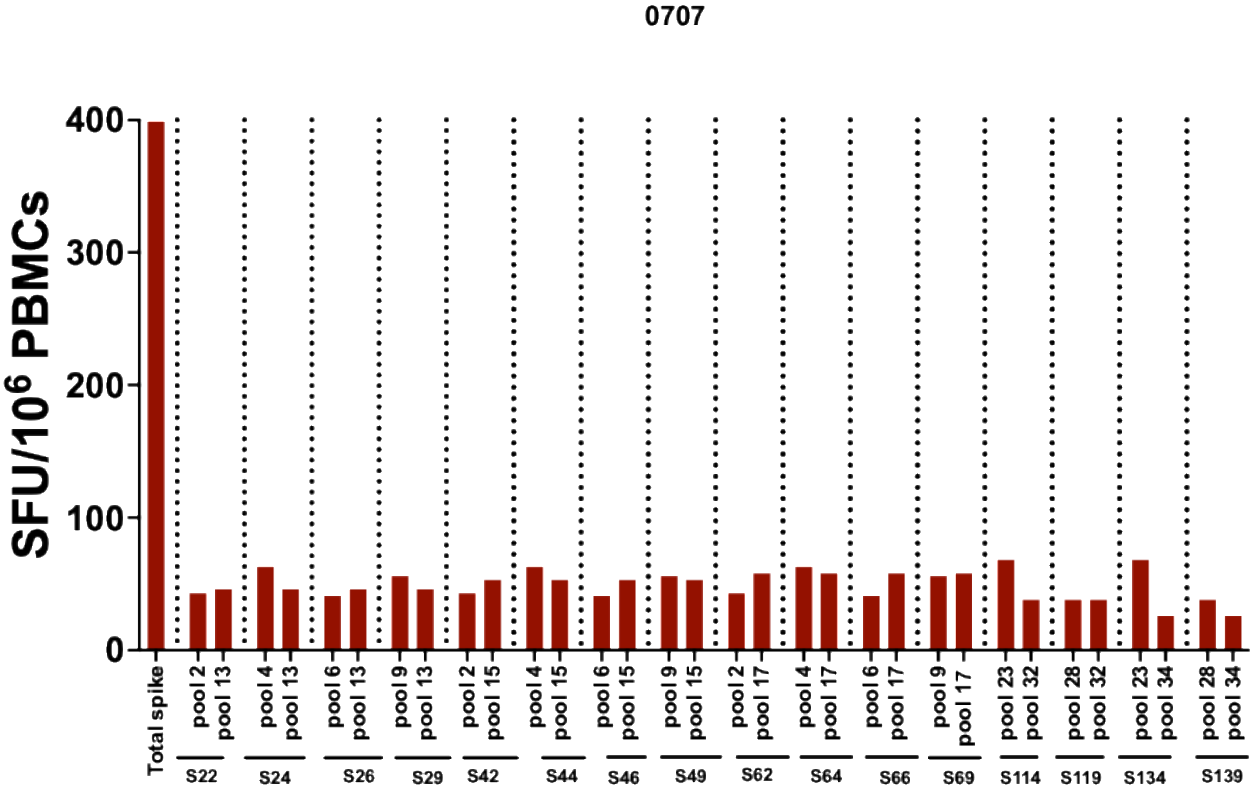

0705
SFU/10^6 PBMCs
150
100
50
0
Total spike
pool 4
pool 13
pool 7
pool 13
pool 4
pool 19
pool 7
pool 19
S24
S27
S84
S87

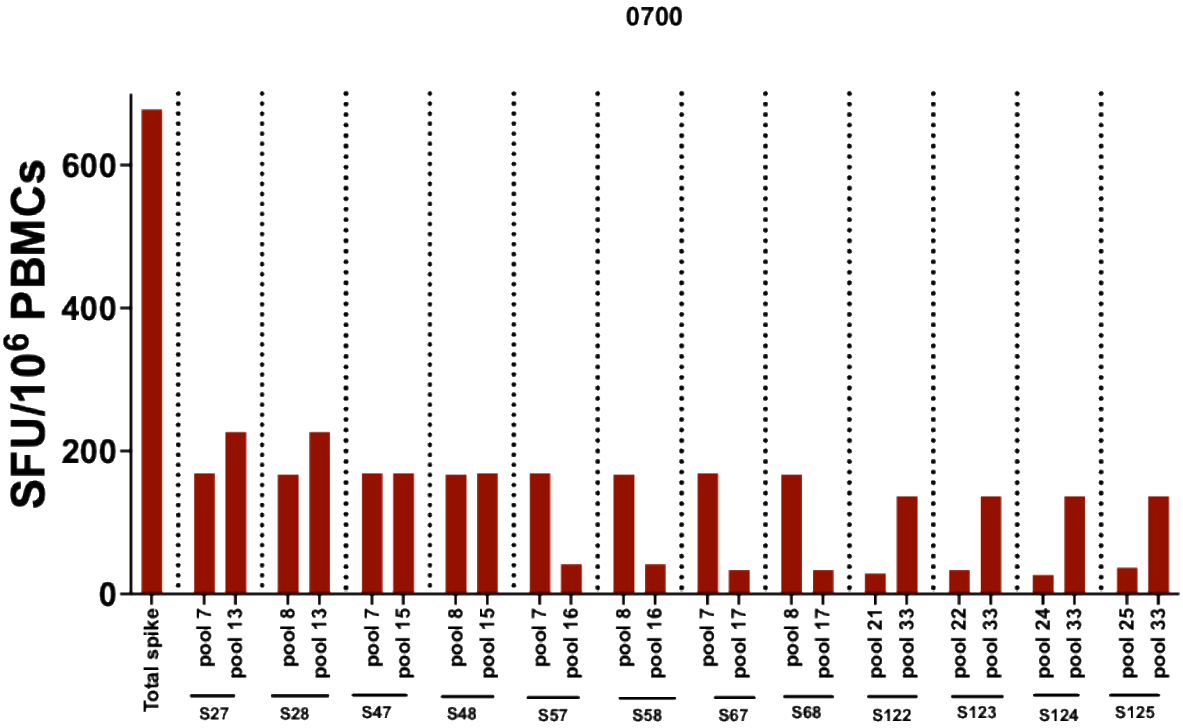

0711
SFU/10^6 PBMCs
400
300
200
100
0
Total spike
pool 4
pool 13
pool 7
pool 13
pool 4
pool 18
pool 7
pool 18
S24
S27
S74
S77

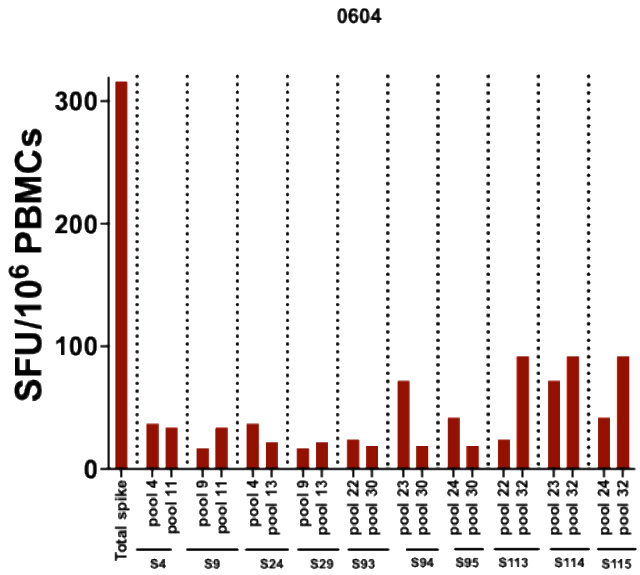

0614
SFU/10^6 PBMCs
600
400
200
0
Total spike
pool 31
pool 24
pool 33
pool 24
S105
S125

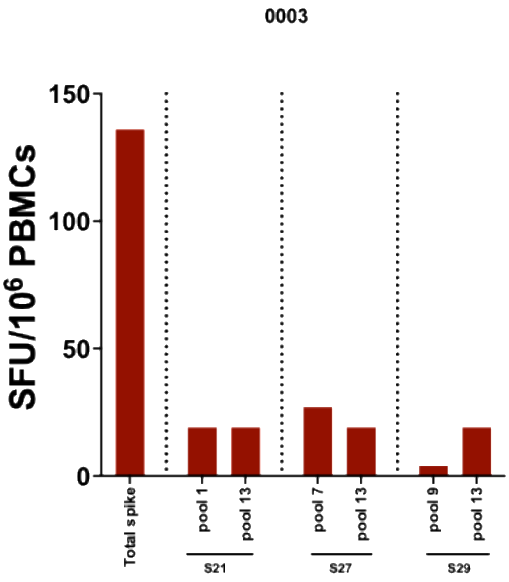

0708
SFU/10^6 PBMCs
100
50
0
Total spike
pool 22
pool 15
pool 22
pool 32
pool 4
pool 33
S44
S113
S123


0709
SFU/10^6 PBMCs
0
100
200
Total spike
pool 1
pool 11
pool 2
pool 11
pool 4
pool 11
pool 10
pool 11
pool 1
pool 13
pool 2
pool 13
pool 4
pool 13
pool 10
pool 13
pool 1
pool 15
pool 2
pool 15
pool 4
pool 15
pool 10
pool 15
pool 1
pool 16
pool 2
pool 16
pool 4
pool 16
pool 10
pool 16
pool 22
pool 31
pool 26
pool 31
pool 22
pool 38
pool 26
pool 38
S1
S2
S4
S10
S21
S22
S24
S30
S41
S42
S44
S50
S51
S52
S54
S60
S103
S107
S173
S177


0707
SFU/10^6 PBMCs
0
100
200
300
400
Total spike
pool 2
pool 13
pool 4
pool 13
pool 6
pool 13
pool 9
pool 13
pool 2
pool 15
pool 4
pool 15
pool 6
pool 15
pool 9
pool 15
pool 2
pool 17
pool 4
pool 17
pool 6
pool 17
pool 9
pool 17
pool 23
pool 32
pool 28
pool 32
pool 23
pool 34
pool 28
pool 34
S22
S24
S26
S29
S42
S44
S46
S49
S62
S64
S66
S69
S114
S119
S134
S139


0700
SFU/10^6 PBMCs
600
400
200
0
Total spike
pool 7
pool 13
pool 8
pool 13
pool 7
pool 15
pool 8
pool 15
pool 7
pool 16
pool 8
pool 16
pool 7
pool 17
pool 8
pool 17
pool 21
pool 33
pool 22
pool 33
pool 24
pool 33
pool 25
pool 33
S27
S28
S47
S48
S57
S58
S67
S68
S122
S123
S124
S125


0604
SFU/10^6 PBMCs
300
200
100
0
Total spike
pool 4
pool 11
pool 9
pool 11
pool 4
pool 13
pool 9
pool 13
pool 22
pool 30
pool 23
pool 30
pool 24
pool 30
pool 22
pool 32
pool 23
pool 32
pool 24
pool 32
S4
S9
S24
S29
S93
S94
S95
S113
S114
S115


0003
SFU/10^6 PBMCs
150
100
50
0
Total spike
pool 1
pool 13
pool 7
pool 13
pool 9
pool 13
S21
S27
S29

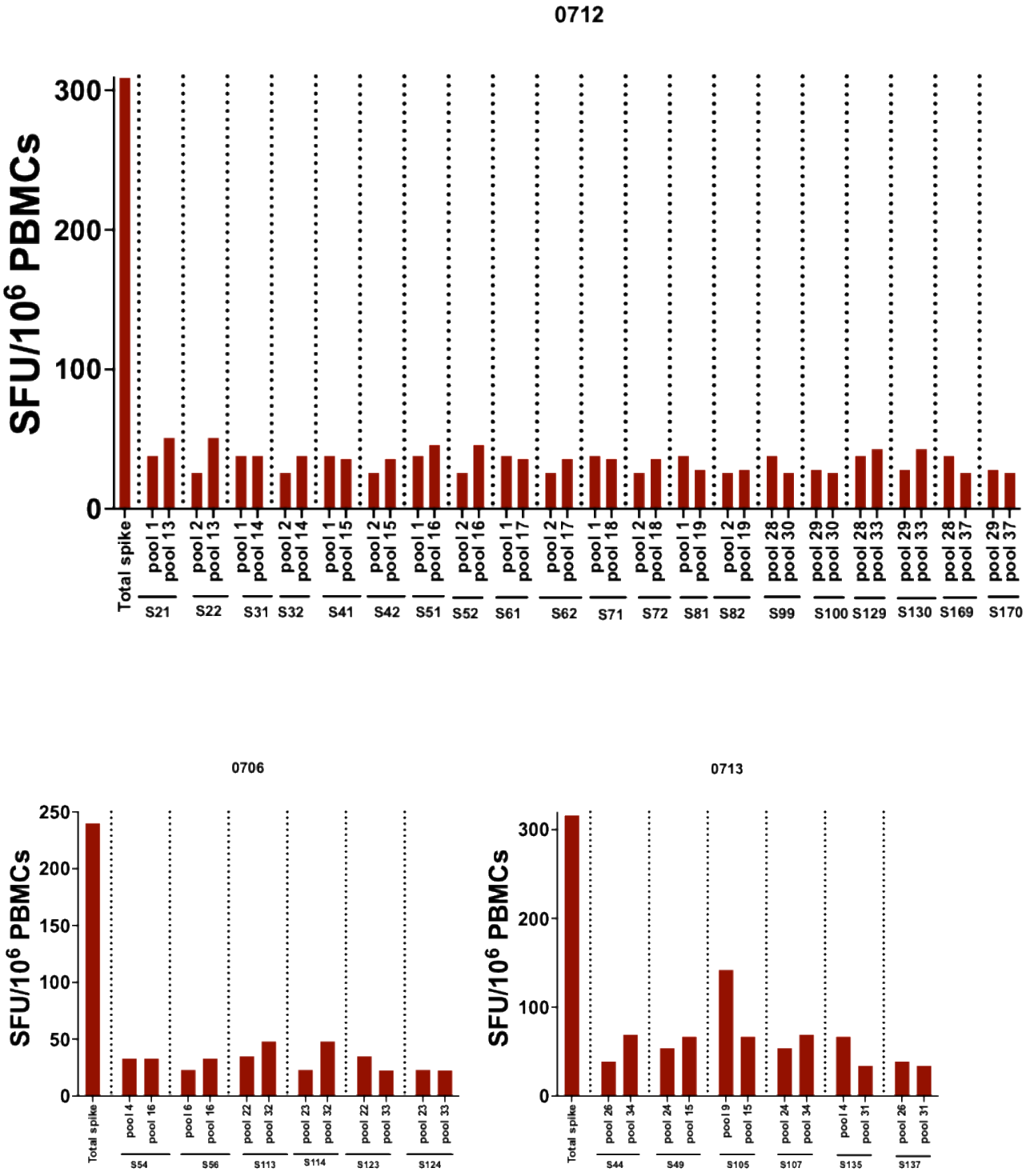


Figure 5.2.3. Breadth and percentage peptide responses to SARS-CoV-2 in vaccinated donors. (A) Overall summary of breadth of SARS-CoV-2 S1 and S2 responses in naïve (n=3, blue) and previously infected donors (n=14, red) after a third SARS-CoV-2 vaccine dose. Each column represents a donor, and each highlighted square represents a positive response as determined by the T cell IFN**γ** responses of the matrix (B) Among responses across donors, 65% of responses were in S1, 35% in S2, 29% in NTD, 22% in RBD and 14% in other S1 proteins. * NTD, RBD and other S1 proteins all lie within the S1 (C) Breakdown of T cell IFN**γ** responses per donor, showing the total spike (S1 + S2) responses and responses to their predicted peptide

pools. SARS-CoV-2 ancestral strain was used to assess T cell responses in all graphs. Data are presented as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

5.2.3 T cell responses maintained across ancestral and BA.2, BA.4 and BA.5 spike peptides

Following analysis of the T cell ELISpot responses by individual donors to the peptide matrix, responses were confirmed using a 14-day T cell line culture followed by ICS and flow cytometry. I identified 23 ancestral/wildtype peptides using the matrix approach for further exploration due to the presence of mutations in Omicron. T cell responses to these 23 ancestral/wildtype peptides, identified using the matrix, were maintained following expansion and stimulation with VOC peptides in ICS. Loss of cytokine secretion was seen in 18 antigenic targets, whilst an increase was seen in 13 using VOC in ICS (Figure 5.2.4). Interestingly, gain in function was only seen in those previously infected with Omicron. Despite these differences observed, none were statistically significant,. These results using a 14-day T cell culture followed by ICS further confirm the results in Chapter 4 using a T cell ELISpot assay that T cell responses are maintained in the presence of SARS-CoV-2 viral variants.

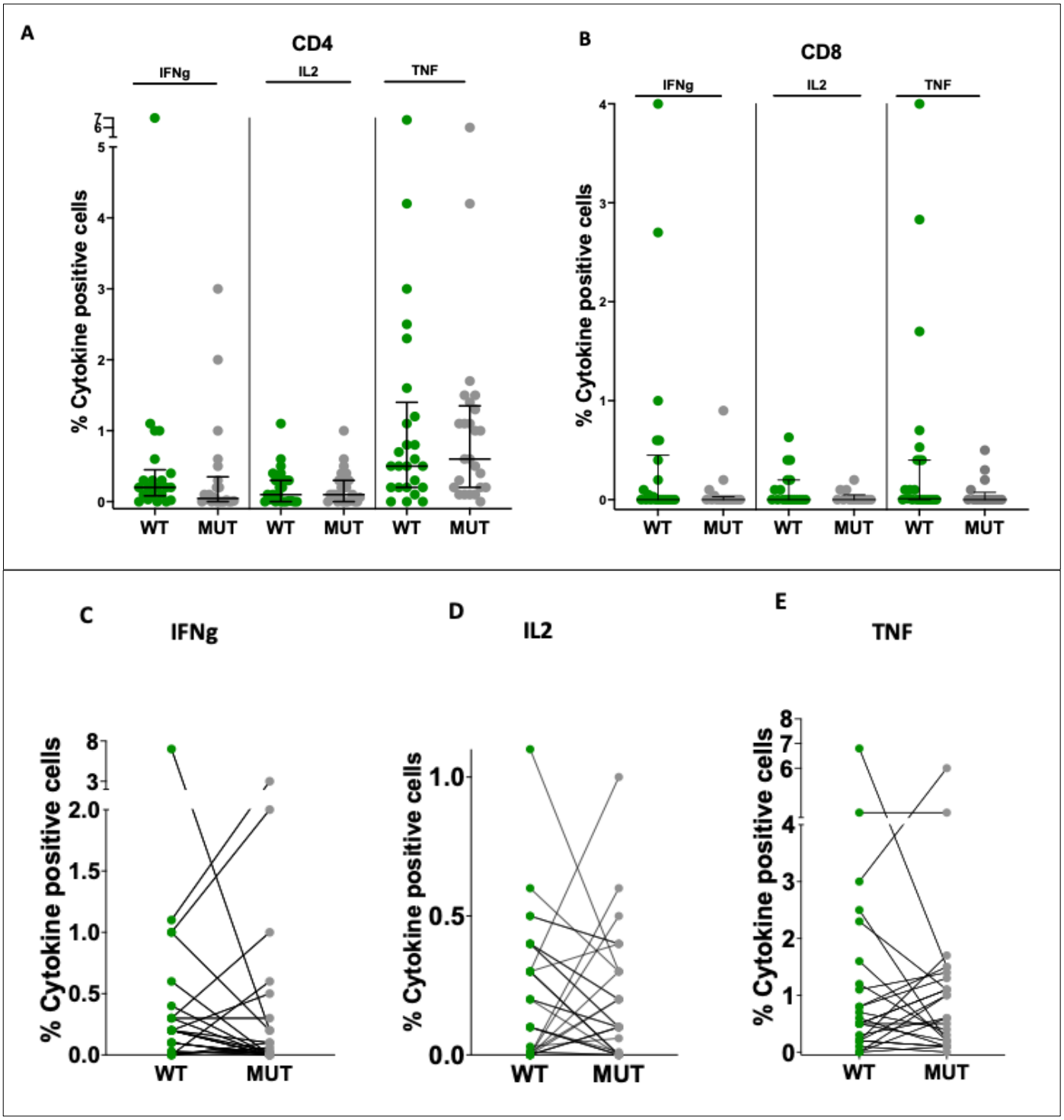


Figure 5.2.4. CD4 and CD8 T cell responses in SARS-CoV-2 ancestral and mutated peptides. SARS-COV-2 ICS responses after 14-day T cell culture. Comparison of (A) CD4 and (B) CD8 T cell responses to IFNg, IL2 and TNF. Green represents SARS-CoV-2 ancestral peptides and grey represent corresponding mutated peptides in BA.2, BA.4 and BA.5. n=17. Statistical significance assessed using Mann-Whitney test with only significant values shown. (C-E) Paired CD4 T cell responses among donors to IFNg, IL2 and TNF, n=17, statistical significance assessed using Wilcoxon test.

5.2.4 Comparison of Breadth of T cell responses to SARS-CoV-2 after bivalent vaccine

Another aim of this chapter was to compare the breadth of responses in donors after a third SARS-CoV-2 monovalent vaccine dose and after a fourth SARS-CoV-2 bivalent vaccine dose. The monovalent mRNA vaccine used until dose 3 (BNT162b2) consisted of the ancestral SARS-CoV-2 S protein, while the bivalent mRNA vaccine used for the fourth dose (Pfizer or Moderna) consisted of the ancestral SARS-CoV-2 S protein and the Omicron BA.1 S protein. To study the breadth across V3 and V4, I used PBMCs from 10 donors who had received a 3rd and 4th vaccine dose, running experiments concurrently. Figure 5.2.5 shows the summary of donors and the reported number of SARS-CoV-2 infections (by positive PCR and/or LFT).

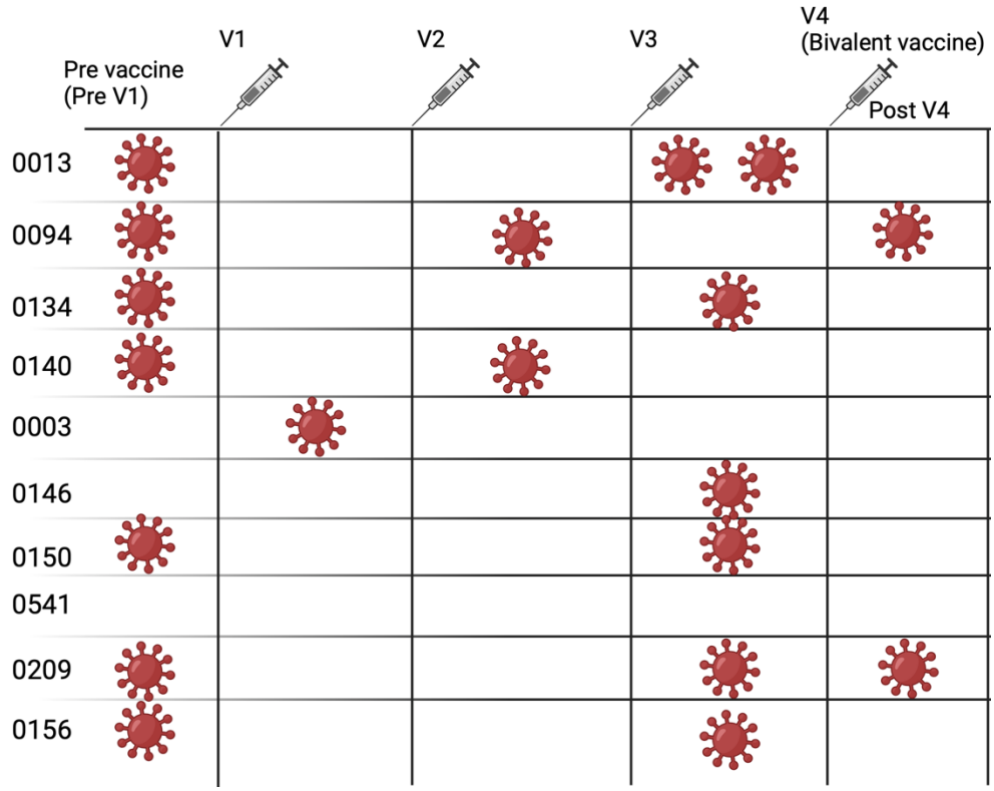


Figure 5.2.5. Number and timepoint of SARS-CoV-2 infections per donor. Number and timepoint of SARS-CoV-2 infections in 10 donors, as determined by positive PCR and / or LFT. Each virus symbol depicts a SARS-CoV-2 infection.

Prior to assessing the breadth of T cell responses to SARS-CoV-2 using the peptide matrix, SARS-CoV-2 total S responses to the ancestral strain were compared in all 10 donors using an IFNγ ELISpot assay. T cell responses were assessed using PBMCs collected 28 days after a third and fourth vaccine dose, V3+28 days and V4+28 days respectively. All donors produced

T cell responses to SARS-CoV-2 S protein, range 45 to 5108 SFUx10^6 PBMCs at V3+28 days and 44 to 1858 SFU x 10^6 PBMCs at the V4+28 days timepoint (Figure 5.2.6).

Interestingly, in 7 out of 10 donors, T cell response to S protein were higher at the V3+28 timepoint compared to the V4+28 timepoint, contradicting the evidence presented in previous chapters as well as several studies on T cells that suggest successive SARS-CoV-2 vaccinations generally increase the magnitude of T cell responses. This may just be a small numbers effect. However, this trend of higher responses after dose 3 than dose 4 was observed in donors who had experienced 2 to 3 SARS-CoV-2 infections prior to the fourth vaccine dose, and infection-induced responses could be waning regardless of the fourth vaccine boost. In contrast, for the 3 donors who had 0 to 1 prior SARS-CoV-2 infection, the magnitude of the T cell response was very low at V3+28 days, then higher at V4+28 days. These findings suggest that prior SARS-CoV-2 infections may influence the dynamics of T cell responses to subsequent vaccine doses. While successive vaccination is typically expected to boost T cell responses, pre-existing immunity from infection may result in a different immunological profile that warrants further investigation.

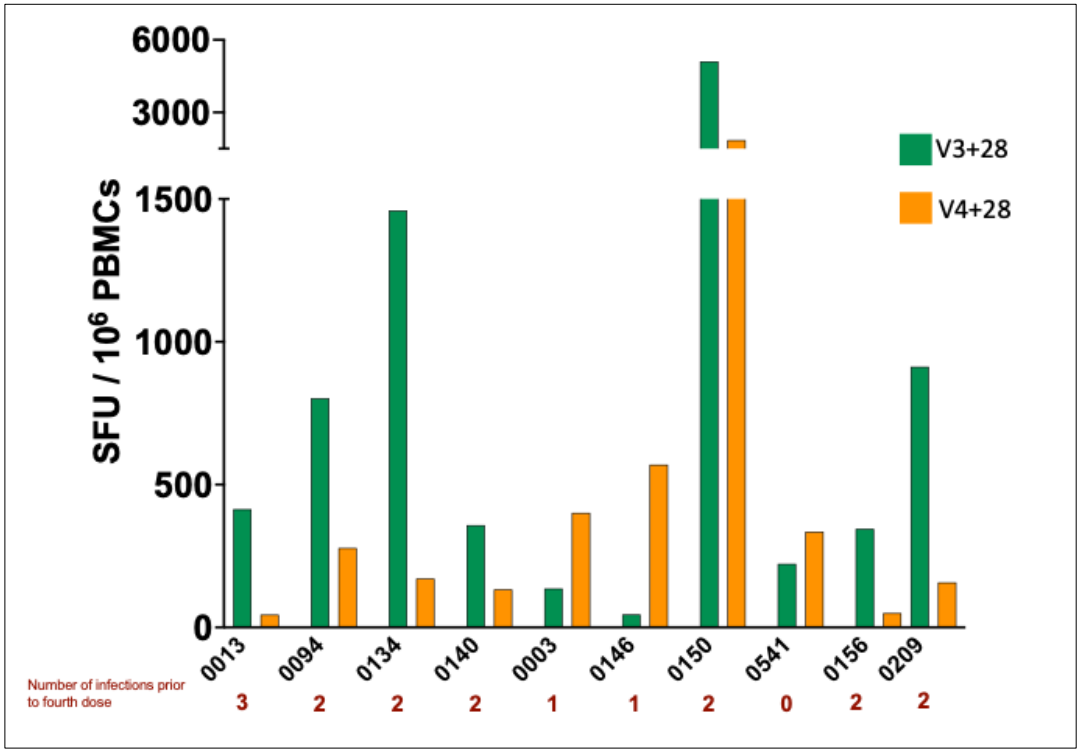
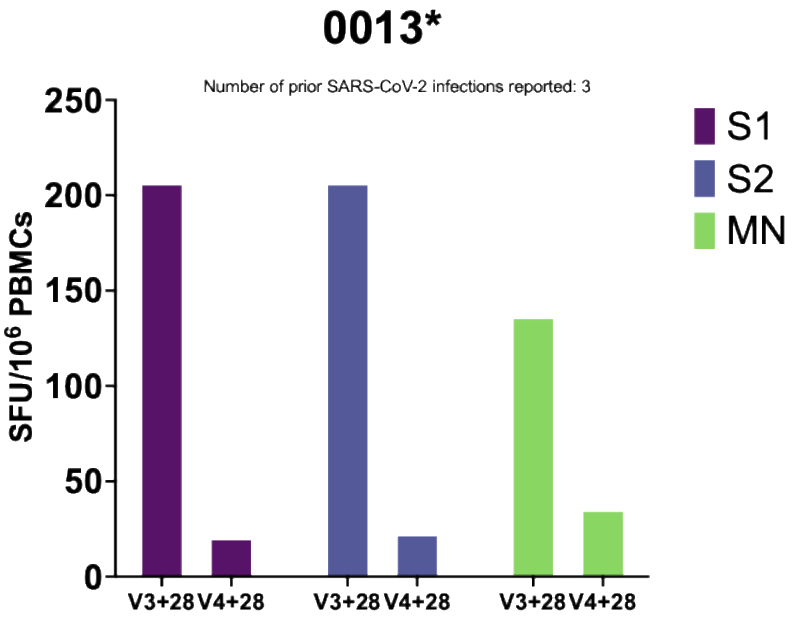
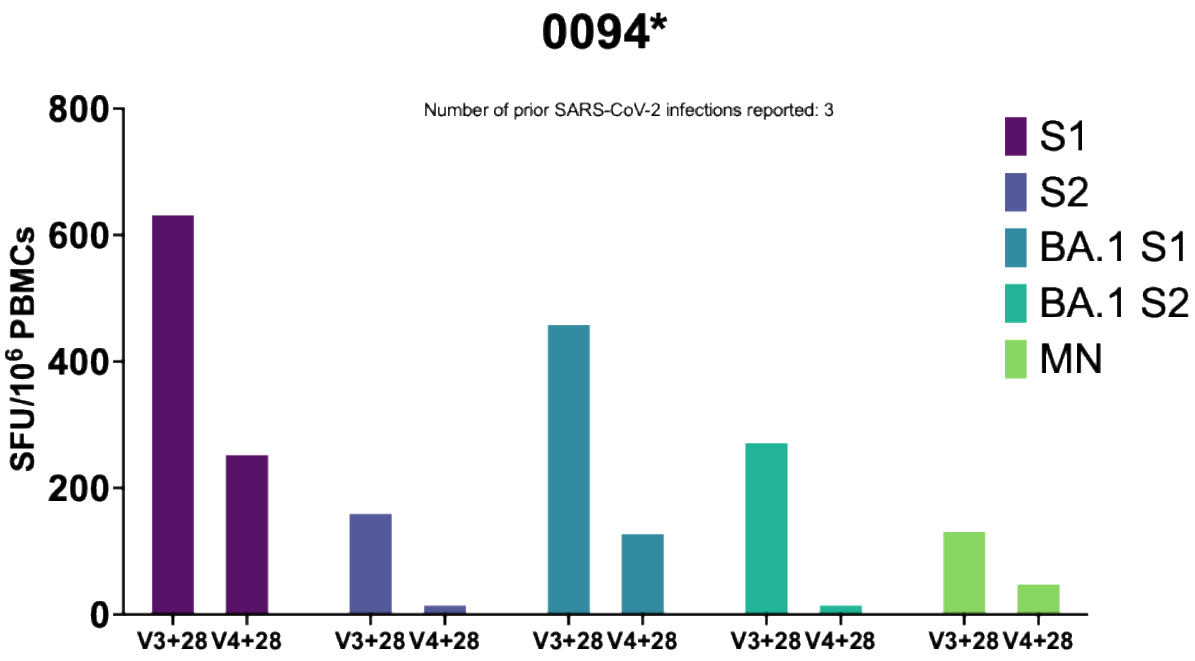


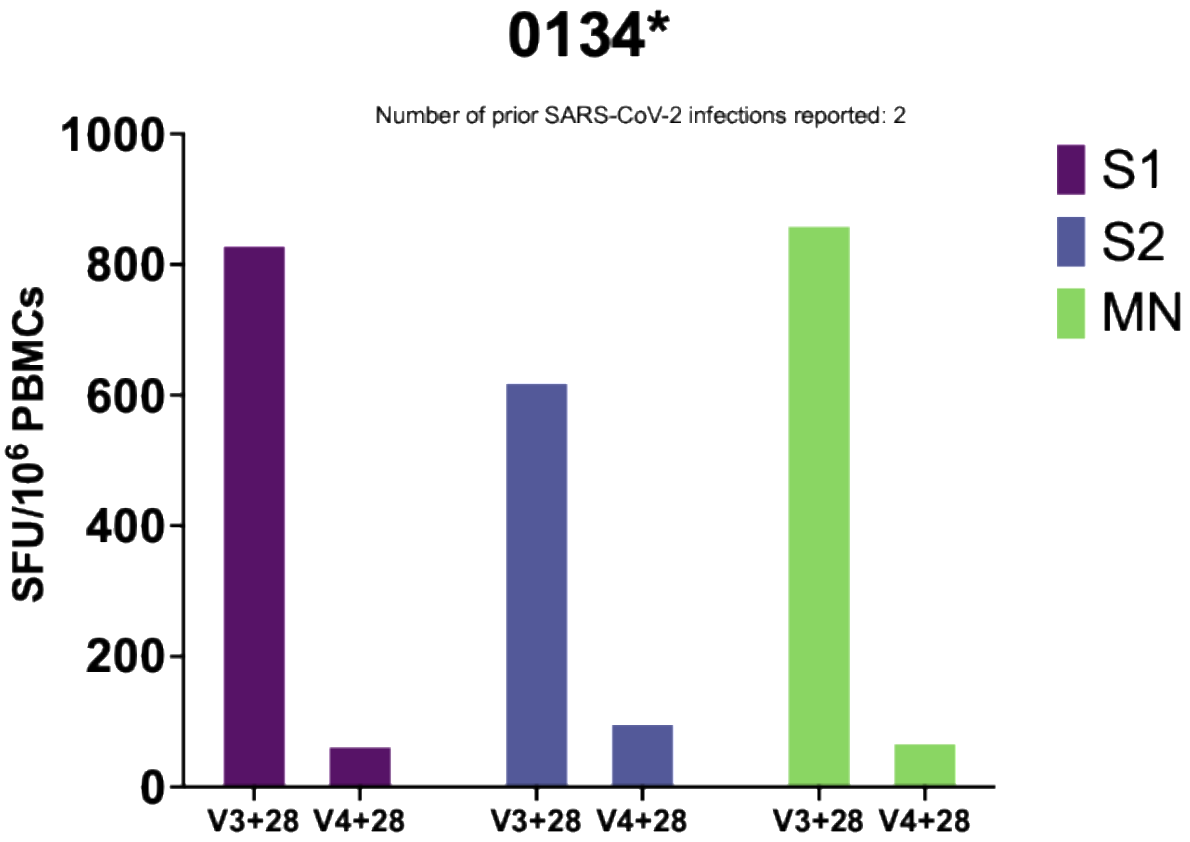
Figure 5.2.6. Magnitude of SARS-CoV-2 S responses to ancestral SARS-CoV-2 strain in donors after third and fourth COVID-19 vaccine. Total S protein SARS-CoV-2 responses measured using T cell IFN**γ** ELISpot in 10 donors at V3+28 (green) and V4+28 (orange) timepoints. Data are presented as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs). The number of infections prior to the fourth vaccine dose for each donor are shown in red.

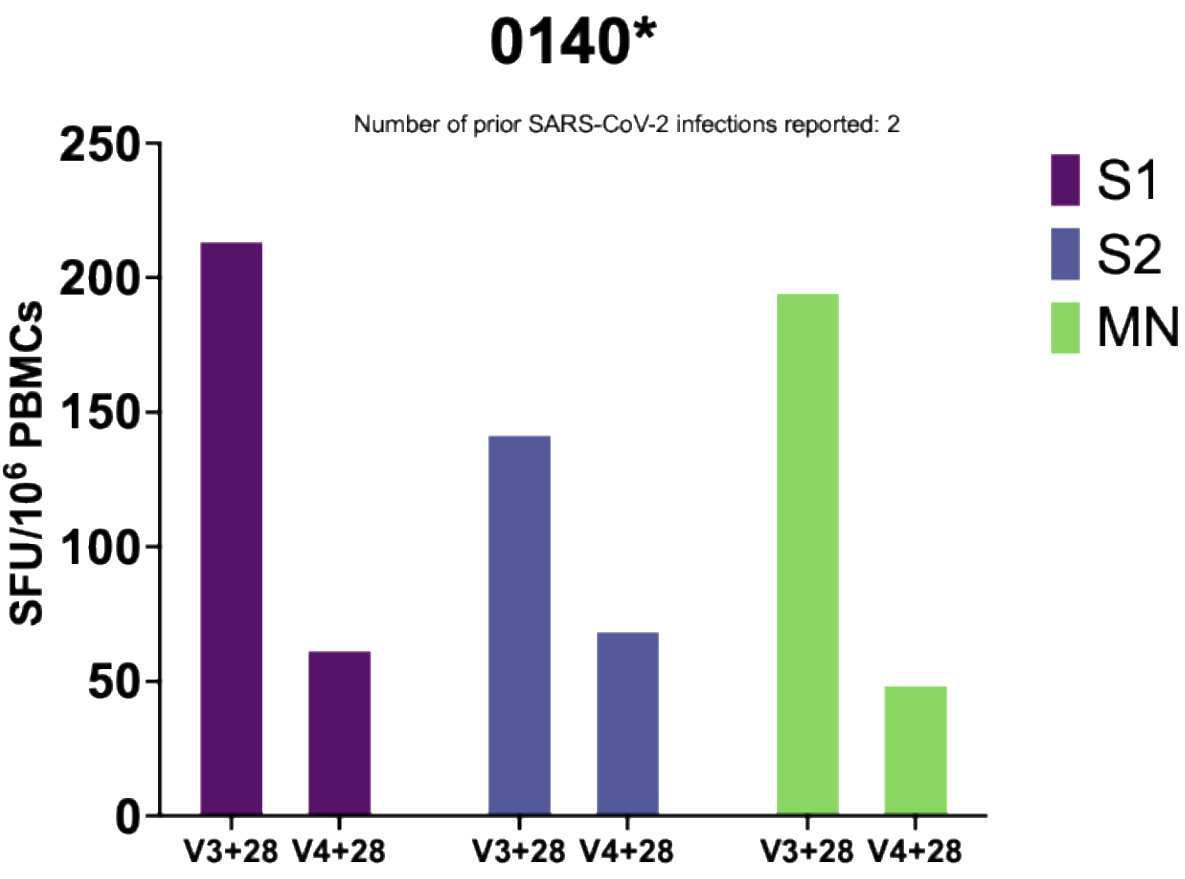
To further explore these findings, SARS-CoV-2-specific T cell responses to BA.1, BA.2, M, and N peptides were measured based on PBMC availability. Since the fourth bivalent vaccine dose included the BA.1 Spike backbone, I hypothesised that the observed decline in T cell responses following the fourth dose would not occur with variant-specific S peptides, as opposed to the ancestral strain. T cell responses to M and N peptides, which are robust markers of prior SARS-CoV-2 exposure [172], were included to trace infection histories among the donors.

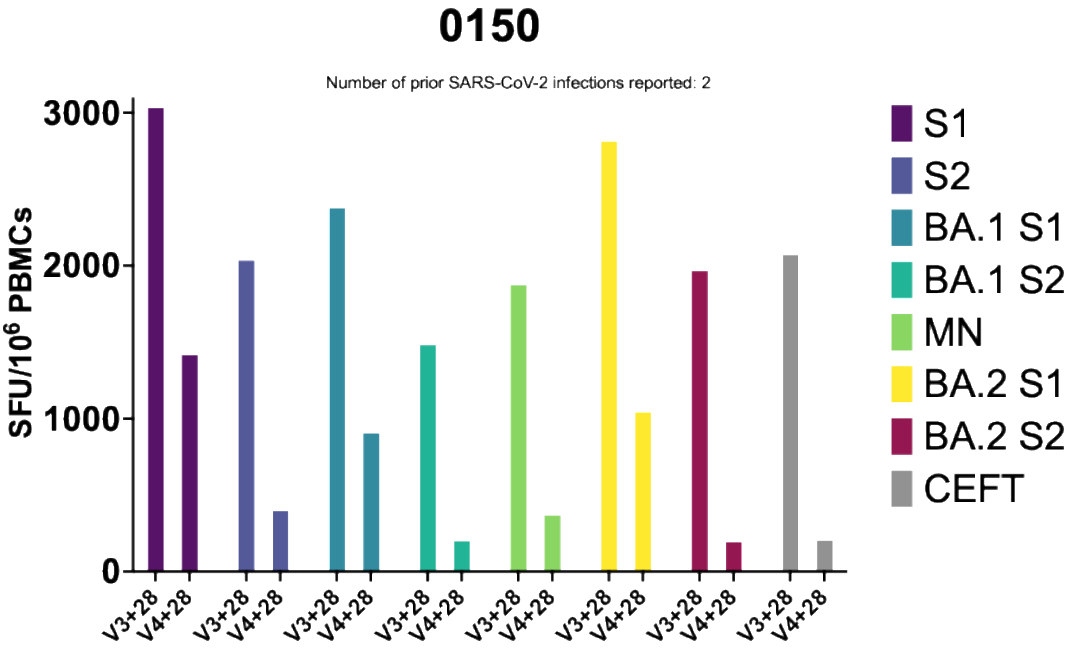
The results indicated that T cell response trends in donors against BA.1/BA.2 Spike peptides mirrored those observed with the ancestral strain (Figure 5.2.7). Specifically, donors with two or more prior SARS-CoV-2 infections showed a decrease in T cell responses after the fourth bivalent dose compared to the third dose. A possible explanation, supported by other studies [265,266] suggests that additional booster doses beyond the third may not significantly enhance T cell responses in individuals with prior SARS-CoV-2 infections.

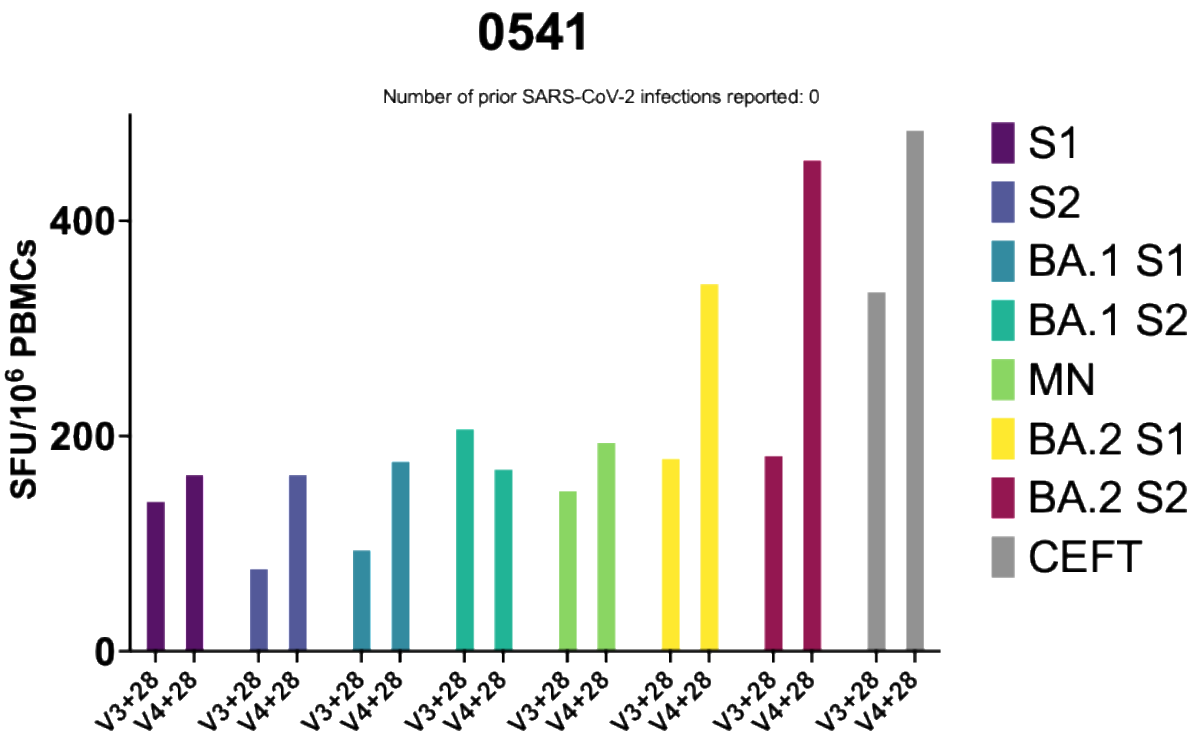


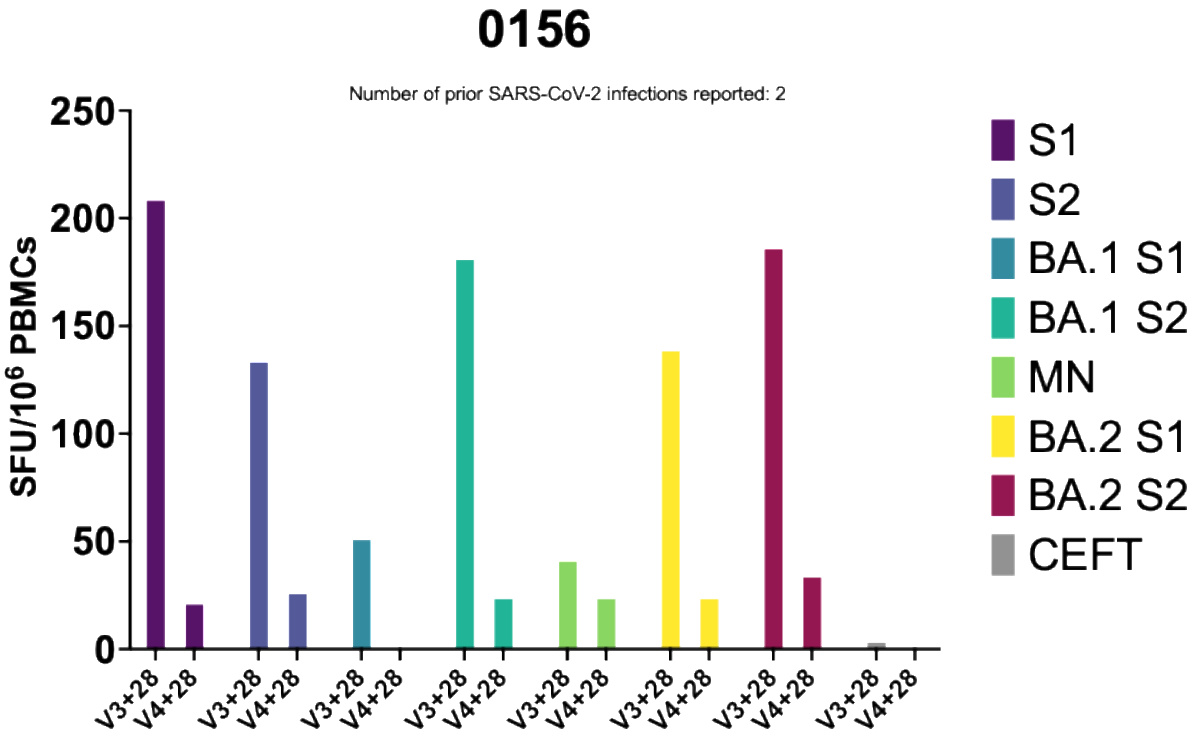

0094*
Number of prior SARS-CoV-2 infections reported: 3
SFU/10^6 PBMCs
800
600
400
200
0
V3+28 V4+28 V3+28 V4+28 V3+28 V4+28 V3+28 V4+28 V3+28 V4+28
S1
S2
BA.1 S1
BA.1 S2
MN


0134*
Number of prior SARS-CoV-2 infections reported: 2
SFU/10^6 PBMCs
1000
800
600
400
200
0
V3+28 V4+28 V3+28 V4+28 V3+28 V4+28
S1
S2
MN


0140*
Number of prior SARS-CoV-2 infections reported: 2
SFU/10^6 PBMCs
250
200
150
100
50
0
V3+28
V4+28
V3+28
V4+28
V3+28
V4+28
S1
S2
MN


0150
Number of prior SARS-CoV-2 infections reported: 2
SFU/10^6 PBMCs
3000
2000
1000
0
V3+28
V4+28
S1
S2
BA.1 S1
BA.1 S2
MN
BA.2 S1
BA.2 S2
CEFT


0541
Number of prior SARS-CoV-2 infections reported: 0
SFU/10^6 PBMCs
400
200
0
V3+28
V4+28
S1
S2
BA.1 S1
BA.1 S2
MN
BA.2 S1
BA.2 S2
CEFT


0156
Number of prior SARS-CoV-2 infections reported: 2
SFU/10^6 PBMCs
250
200
150
100
50
0
V3+28
V4+28
S1
S2
BA.1 S1
BA.1 S2
MN
BA.2 S1
BA.2 S2
CEFT

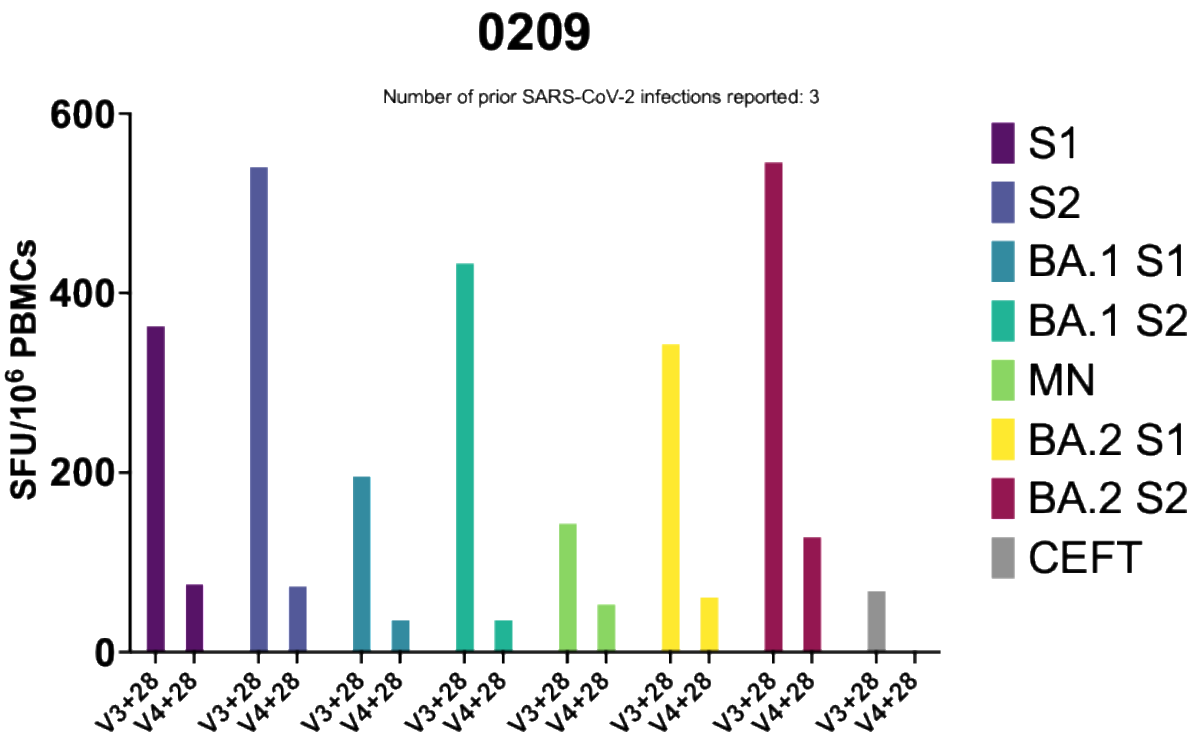
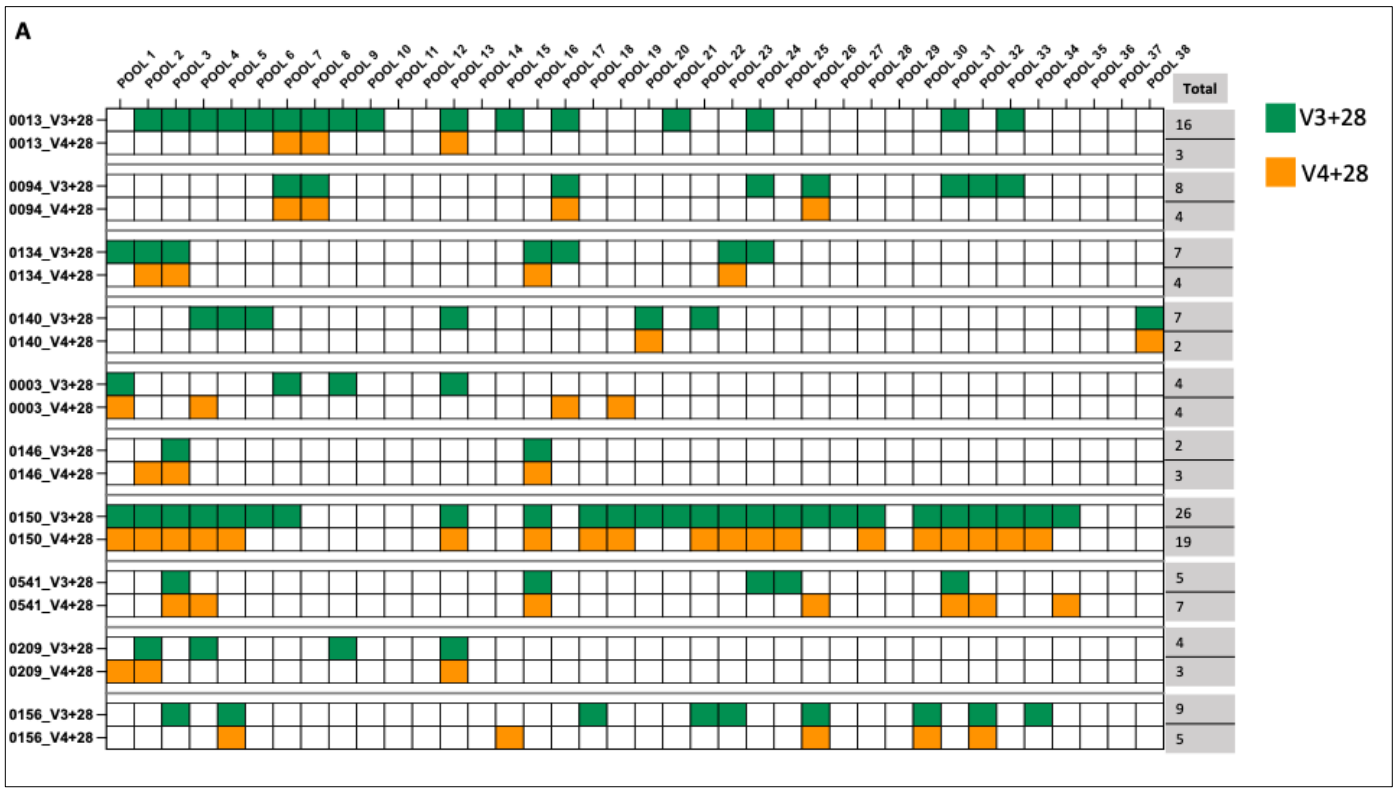


Figure 5.2.7. Magnitude of SARS-CoV-2 responses in donors after third and fourth COVID-19 vaccine dose. Responses are measured using IFN**γ** ELISpot assay in 8 donors to S1 (ancestral), S2 (ancestral), BA.1 S1, BA.1 S2, BA.2 S1, BA.2 S2, M, N and CEFT (Cytomegalovirus, Epstein-Barr Virus, Influenza Virus and Tetanus Toxoid peptide pool). Data are presented as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs). Number of prior SARS-CoV-2 infections are shown for each donor. *Due to limited PBMC numbers, all peptide conditions tested could not be measured.

Next, I used the peptide matrix to assess the breadth of responses to peptide pools spanning the SARS-CoV-2 S protein in all 10 donors at V3+28 days and V4+28 days. Responses to the peptide pools ranged from 2 to 26 pools, Figure 5.2.8 A. Assessing the breadth to the peptide pools, similar to the total S responses in Figure 5.2.6, there was a decline in breadth of responses in 7 out of 10 donors who had 2 to 3 SARS-CoV-2 infections prior to the fourth dose. However, there was no change or an increase in breadth in 3 out of 10 donors who had 0 to 1 infection prior to the fourth dose (donors 0003, 0146 and 0541). There were also new peptide pool responses at V4+28 days that were not present at V3+28 days in all 3 out of 10 donors who had 0 to 1 prior infection and 2 out 7 donors who had 2 to 3 prior infections. Fisher's Exact Test was conducted to determine if there was a statistically significant difference in the breadth of T cell responses after the 3rd and 4th vaccine doses. The test revealed no statistically

significant association between vaccine dose and response (P = 0.179). The observation of new peptide pool responses at V4+28 in donors with fewer prior infections (0 to 1) might suggest that these individuals were able to mount a broader response upon receiving the fourth dose, possibly due to the absence of pre-existing immune memory that may have focused the response in individuals with prior infections. In contrast, the fewer new peptide pool responses observed in donors with 2 to 3 prior infections suggest that their immune response may have been more focused on previously encountered epitopes, reducing the overall expansion of new responses.



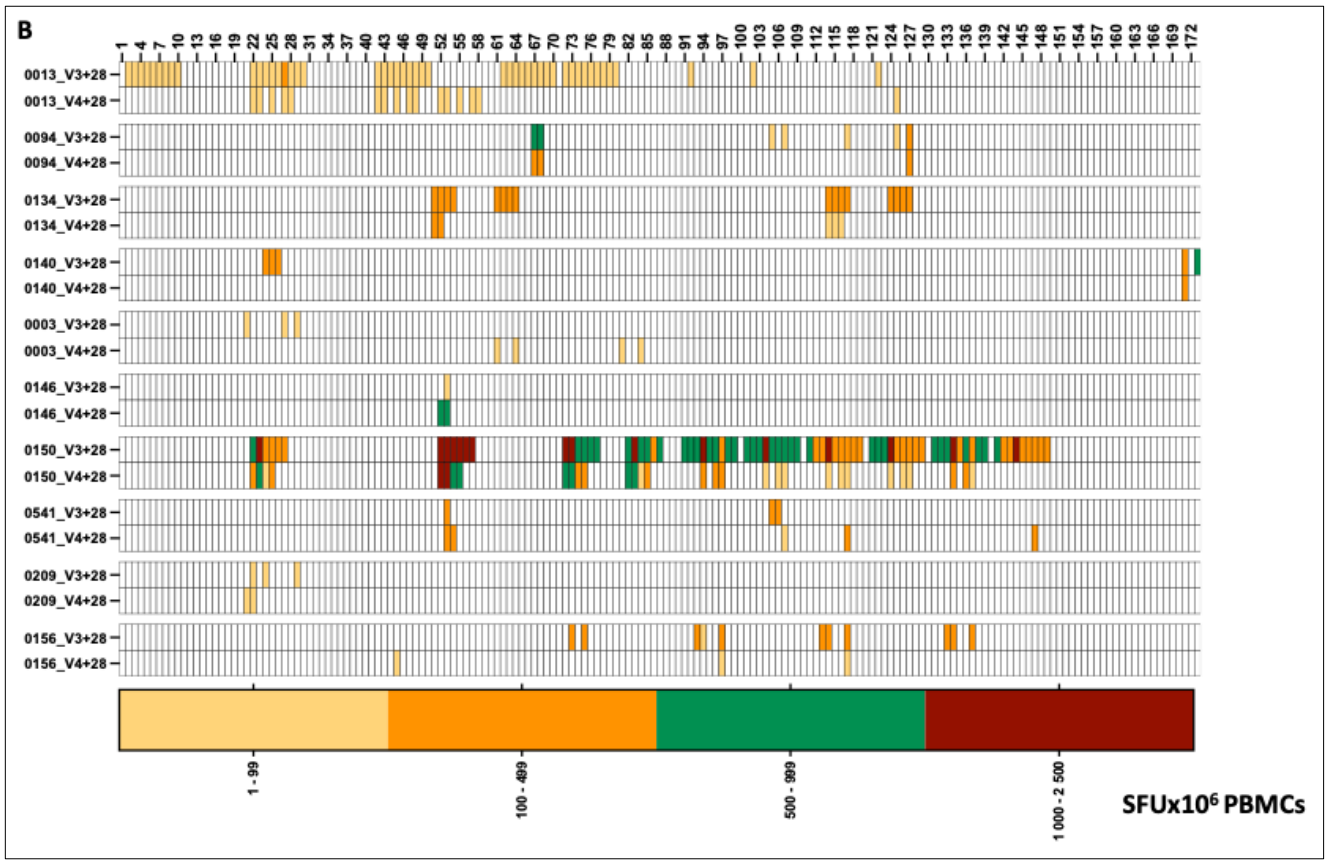


Figure 5.2.8. Magnitude of SARS-CoV-2 spike responses to ancestral strain donors after third and fourth doses of SARS-CoV-2 vaccine. Individual donors are represented by grouped rows, each depicting the V3+28 days and V4+28 days timepoints. (A) Heatmap showing number of peptide responses per donor measured using T cell ELISpot to 38 peptide pools 28 days after 3rd (green) and 4th (orange) doses of SARS-CoV-2 vaccines. The rightmost column shows the total number of peptide pools that elicited a response for each donor at each time point (V3+28 and V4+28). (B) Predicted individual peptide responses based on peptide matrix, showing the T cell magnitude. For visual clarity, not all 178 peptides are labelled. Data are presented as spot-forming units per million peripheral blood mononuclear cells (SFU/10^6 PBMCs).

The possible individual T cell peptides based on the peptide pool responses are highlighted in Figure 5.2.8 B and the respective magnitudes of the T cell responses from the peptide pools. These results also suggest a decline in magnitude of T cell responses at V4+28 compared to V3+28 which is more pronounced in individuals with more than 1 prior SARS-CoV-2 infection. Although the decline in breadth and magnitude seems counterintuitive compared to general vaccine boosting effects, repeated exposure (through infection or vaccines) may lead

to T cell exhaustion, where the immune system has already reached its peak response and does not further expand.

These results suggest that prior SARS-CoV-2 infections may have a dampening effect on both the breadth and magnitude of T cell responses following the fourth vaccine dose, particularly in donors with multiple previous infections. The appearance of new peptide pool responses at V4+28 in donors with 0-1 prior infections suggests that fewer prior exposures allow for a more diverse T cell response upon additional vaccination. These findings indicate that the immune system's capacity to expand its T cell repertoire may be influenced by prior immunity, potentially leading to a narrowed and focused response in individuals with more extensive SARS-CoV-2 exposure. This highlights the complexity of immune memory and its impact on subsequent vaccine-induced T cell responses, pointing to the need for further investigation into how infection history alters vaccine-induced immunity.

5.2.5 Assessment of T cell epitopes associated with a protective HLA allele

Finally, I explored the peptide targets identified and determined the HLA types of donors. As previously discussed, the HLA-DQB1*06 allele has been associated with asymptomatic as opposed to symptomatic infection [261]. Using the peptide matrix results, I observed that a notable difference exists in the immune response to the SARS-CoV-2 S protein between individuals with and without the HLA-DQB1*06 allele. Specifically, 6 out of 9 donors who were HLA-DQB1*06 positive showed a positive response to the SARS-CoV-2 Spike 125 peptide, while only 1 out of 13 non-HLA-DQB1*06 donors exhibited a similar response (P=0.007, Fisher’s exact), Table 5.1. Spike 125, a peptide located in the S2 region of the SARS-CoV-2 S protein, has the sequence TFGAGAALQIPFAMQMAY. This finding highlights a potential association between the HLA-DQB1*06 allele and an enhanced immune response to this peptide, suggesting that HLA-DQB1*06 may play a role in modulating immune recognition of SARS-CoV-2 epitopes within the S2 region.

Donor	HLA DQB1:06	S_125 response
0003	negative	negative
0013	positive	positive
0094	positive	positive
0134	negative	positive
0140	positive	negative
0146	negative	negative
0151	negative	negative
0604	negative	negative
0614	positive	positive
0699	negative	negative
0700	positive	positive
0702	positive	positive
0703	negative	negative
0704	positive	positive
0706	negative	negative
0707	negative	negative
0708	negative	negative
0709	negative	negative
0710	positive	negative
0711	negative	negative
0712	negative	negative
0713	positive	negative

Table 5.1. Table of the association between HLA-DQB1*06 status and matrix responses to SARS-CoV-2 Spike 125 peptide (S_125) across 22 donors. Each donor is categorized based on their HLA-DQB1*06 status (positive or negative) and their response to the S_125 peptide (positive or negative).

Secondly, using peptide prediction tools (NetMHCII and NetMHCIIpan), 16 potential strong-binding T cell epitopes for SARS-CoV-2 were identified specific to HLA-DQB1*06. The criteria for strong binding was set for peptides with an IC50 value of less than 50 nM. Among these 16 identified epitopes, one features a peptide binding core sequence, “GAGAALQIP,” which is present within our S125 peptide. This core sequence alignment suggests a possible cross-reactivity or similarity in immune recognition, underscoring the relevance of these epitopes in the context of SARS-CoV-2 immune response in HLA-DQB1*06 carriers.

peptide	core	Of	1-log50k(aff)	affinity(nM)	%Rank	Relia	Identity	Bind Level
TQQLIRAAEIRASANLAA	IRAAEIRAS	4	0.8208	7.0	0.04	0.74	Sequence	SB
QQLIRAAEIRASANLAAT	IRAAEIRAS	3	0.8197	7.0	0.04	0.69	Sequence	SB
QLIRAAEIRASANLAATK	IRAAEIRAS	2	0.8190	7.1	0.04	0.62	Sequence	SB
YVTQQLIRAAEIRASANL	IRAAEIRAS	6	0.8167	7.3	0.04	0.75	Sequence	SB
VTQQLIRAAEIRASANLA	IRAAEIRAS	5	0.8167	7.3	0.04	0.77	Sequence	SB
TYVTQQLIRAAEIRASAN	IRAAEIRAS	7	0.8165	7.3	0.04	0.77	Sequence	SB
QTYVTQQLIRAAEIRASA	IRAAEIRAS	8	0.8024	8.5	0.08	0.79	Sequence	SB
LIRAAEIRASANLAATKM	IRAAEIRAS	1	0.7956	9.1	0.12	0.53	Sequence	SB
IRAAEIRASANLAATKMS	IRASANLAA	5	0.7421	16.3	0.70	0.23	Sequence	SB
LQTYVTQQLIRAAEIRAS	IRAAEIRAS	9	0.7190	20.9	1.20	0.52	Sequence	SB
GDSSSGWTAGAAAYYVGY	TAGAAAYYV	7	0.7098	23.1	1.50	0.53	Sequence	SB
ITPGTNTSNQVAVLYQDV	NQVAVLYQD	8	0.7080	23.6	1.60	0.31	Sequence	SB
DSSSGWTAGAAAYYVGYL	TAGAAAYYV	6	0.7073	23.7	1.60	0.56	Sequence	SB
TPGTNTSNQVAVLYQDVN	NQVAVLYQD	7	0.7054	24.2	1.70	0.34	Sequence	SB
AEIRASANLAATKMSECV	LAATKMSEC	8	0.6989	26.0	1.90	0.26	Sequence	SB
SGWTFGAGAALQIPFAMQ	GAGAALQIP	5	0.6984	26.1	1.90	0.31	Sequence	SB
SSSGWTAGAAAYYVGYLQ	TAGAAAYYV	5	0.6971	26.5	2.00	0.60	Sequence	WB
EIRASANLAATKMSECVL	LAATKMSEC	7	0.6971	26.5	2.00	0.36	Sequence	WB
PGTNTSNQVAVLYQDVNC	NQVAVLYQD	6	0.6969	26.6	2.00	0.34	Sequence	WB
TSGWTFGAGAALQIPFAM	GAGAALQIP	6	0.6948	27.2	2.50	0.35	Sequence	WB
VITPGTNTSNQVAVLYQD	SNQVAVLYQ	8	0.6916	28.1	2.50	0.28	Sequence	WB
AAEIRASANLAATKMSEC	IRASANLAA	3	0.6914	28.2	2.50	0.28	Sequence	WB
GWTFGAGAALQIPFAMQM	GAGAALQIP	4	0.6912	28.3	2.50	0.30	Sequence	WB
PGDSSSGWTAGAAAYYVG	TAGAAAYYV	8	0.6903	28.5	2.50	0.51	Sequence	WB
RAAEIRASANLAATKMSE	IRASANLAA	4	0.6881	29.2	2.50	0.37	Sequence	WB
SSGWTAGAAAYYVGYLQP	TAGAAAYYV	4	0.6872	29.5	2.50	0.64	Sequence	WB
ITSGWTFGAGAALQIPFA	GAGAALQIP	7	0.6855	30.1	2.50	0.36	Sequence	WB
GTNTSNQVAVLYQDVNCT	NQVAVLYQD	5	0.6837	30.7	3.00	0.37	Sequence	WB
YYPDKVFRSSVLHSTQDL	FRSSVLHST	6	0.6824	31.1	3.00	0.47	Sequence	WB
WTFGAGAALQIPFAMQMA	GAGAALQIP	3	0.6816	31.3	3.00	0.32	Sequence	WB
YPDKVFRSSVLHSTQDLF	FRSSVLHST	5	0.6815	31.4	3.00	0.47	Sequence	WB
VYYPDKVFRSSVLHSTQD	FRSSVLHST	7	0.6812	31.5	3.00	0.47	Sequence	WB
IRASANLAATKMSECVLG	LAATKMSEC	6	0.6808	31.6	3.00	0.41	Sequence	WB

Figure 5.2.9. Figure showing the predicted binding affinities of SARS-CoV-2-derived peptides to HLA-DQB1*06, generated using NetMHCII tools. Peptides are ranked based on their binding strength, with an IC50 threshold of <50 nM indicating strong binders (SB) and >50 nM indicating weak binders (WB). Each peptide is shown with its core binding motif, affinity (in nM), and binding reliability score. The highlighted peptide "SGWFGAGAALQIPFAMQ" has a core sequence "GAGAALQIP," contained within our S125 peptide.

Next, I sought to confirm the findings of the peptide matrix and bioinformatic prediction using a 14-day T cell line culture of HLA DQB1*06 positive and negative donors stimulated with S125 followed by an ICS assay. I selected this assay to maximise sensitivity and to cross validate our findings with a different technique to ELISpot. Data from two HLA-DQB1*06 positive donors (0614 and 0700) are shown in Figures 5.2.8 A and B while data from a HLA-DQB1*06 negative donor (0712) is shown in 5.2.8 C. In the control condition, minimal cytokine expression was observed, indicating a low baseline activation. However, upon stimulation with the S125 peptide, T cells from the HLA-DQB1*06 positive donors exhibited marked increases in all cytokines

Comparatively, the HLA-DQB1*06 negative donor showed little to no increase in cytokine response to S125 compared to control, further suggesting that the immunogenicity of S125 may be specific to individuals with the HLA-DQB1*06 allele. These results corroborate our earlier predictions and indicate that the S125 peptide may serve as a potential SARS-CoV-2 epitope, particularly for HLA-DQB1*06 carriers, who might exhibit a stronger immune response to this epitope.

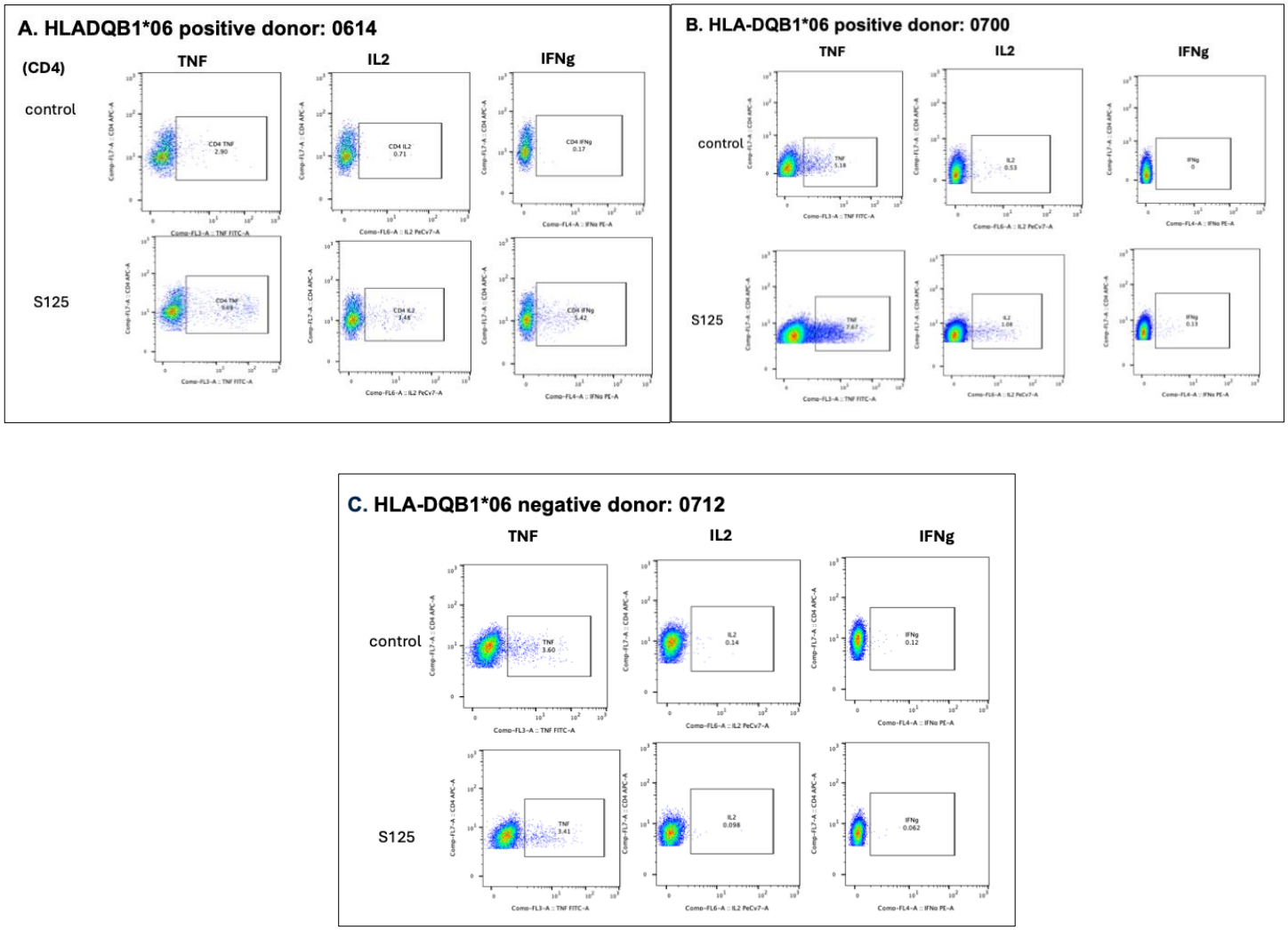


Figure 5.2.10. Intracellular cytokine responses following 14-day stimulation with S 125 peptide. ICS responses to TNF, IL2 and IFNγ in HLA-DQB1*06 positive donors, A and B and HLA-DQB1*06 negative donor, C. Top panels show the controls while bottom panel shows S125 responses.

These findings, though based on data from only three donors, highlight the potential specificity of the S125 peptide in eliciting a T cell response in HLA-DQB1*06 positive individuals, supporting its role as a candidate epitope for SARS-CoV-2. Results are shown in three donors only as other donors tested had high control backgrounds, one of the challenges of T cell line cultures. Despite the limited sample size, the observed increase in cytokine production in the two HLA-DQB1*06 positive donors, compared to the minimal response in the HLA-DQB1*06 negative donor, underscores the potential relevance of HLA allele-specific immune recognition in SARS-CoV-2 infection. This differential response suggests that certain HLA alleles may contribute to stronger or more targeted immune responses. While preliminary, these results offer valuable insights into HLA-dependent immunogenicity in SARS-CoV-2, pointing to the need for larger studies and potentially informing the development of personalized immunotherapies or vaccines that account for individual HLA profiles.

Finally, I decided to confirm these findings using tetramers specific for HLA-DQB1*06. However, I encountered challenges in sourcing these reagents, as it was initially difficult to find a company willing to produce tetramers for HLA-DQB1*06. The tetramers eventually arrived five months after the order was placed, but they did not bind to any targets tested, even after conducting multiple titration experiments (Figure 5.2.11). The company has since agreed to remake the tetramers using a different fluorochrome at no additional cost, and future work will involve testing these newly synthesized reagents.

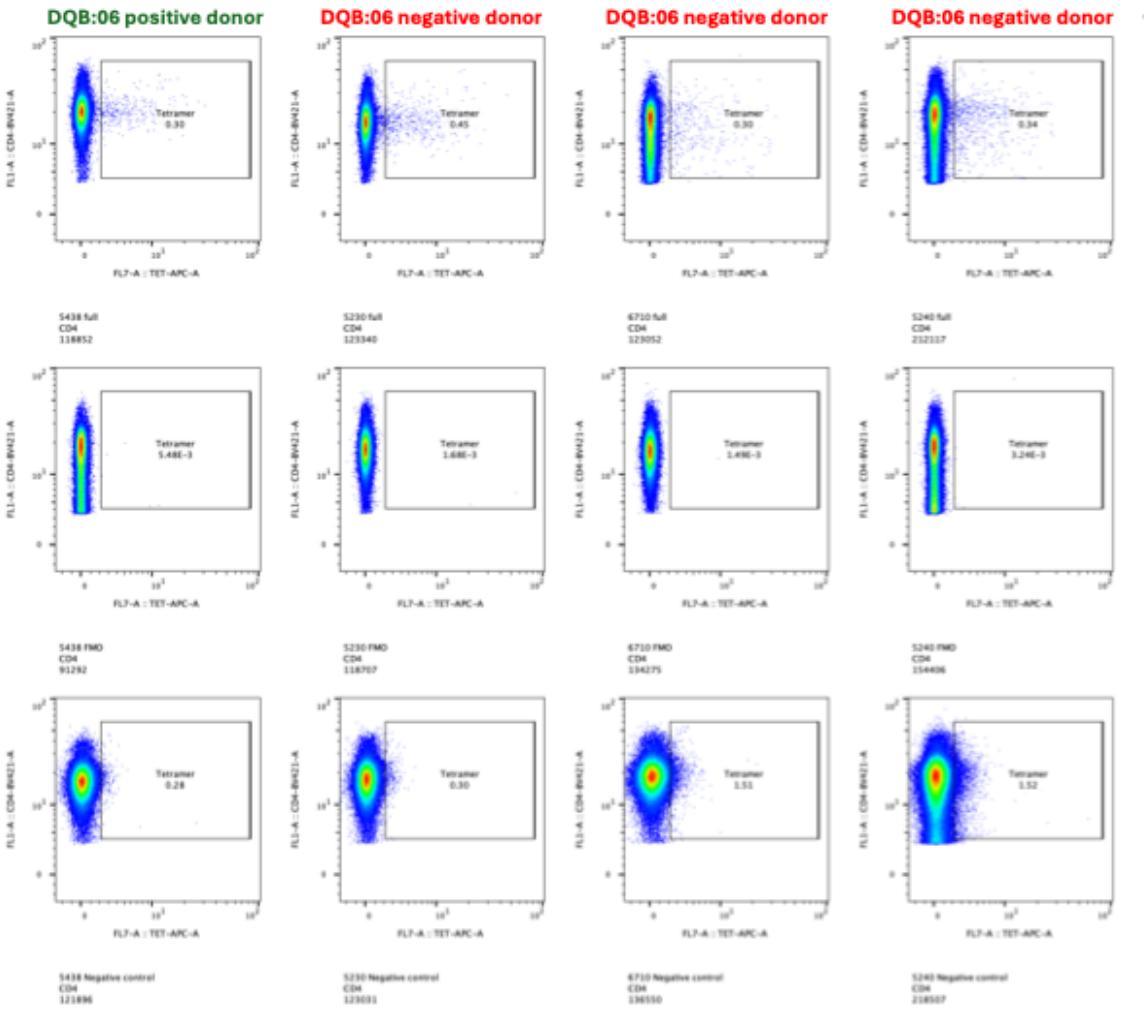


Figure 5.2.11. Tetramer assay in four donors using HLA-DQB1*06 S 125 tetramer. Tetramer responses to S 125 tetramer in one HLA-DQB1*06 positive and three HLA-DQB1*06 negative donors. Each column represents a donor. Top row represents the S125 tetramer, middle row represents the fluorescence minus one (FMO) control and bottom row represents a negative control (a class II unrelated peptide on the same fluorochrome as the tetramer).

5.3 Discussion

In this chapter, I explored the breadth of SARS-CoV-2-specific T cell responses in vaccinated individuals, comparing those with and without prior infections. My findings provide novel insights into the role of pre-existing immunity in shaping T cell responses following successive SARS-CoV-2 vaccine doses. These results reveal important implications for understanding how prior infections and booster doses influence the breadth, magnitude, and functional quality of T cell responses, particularly in the context of emerging variants.

One of the key findings from this study is that SARS-CoV-2-specific T cells exhibit a broad range of epitope recognition in vaccinated individuals. I observed robust T cell responses across all donors, particularly in regions within the NTD and RBD of the SARS-CoV-2 S protein. This breadth of response highlights the capability of T cells to target multiple regions of the SARS-CoV-2 proteome, even in the face of viral mutations, underscoring their importance in long-term immunity. The observation that a substantial proportion of these T cell epitopes remain conserved in Omicron subvariants (BA.2, BA.4, BA.5) further emphasizes the potential of T cells to provide cross-variant protection, even as the virus accumulates mutations that may evade antibody responses.

T cell responses remained broadly intact across ancestral and variant peptides tested, consistent with previous reports that indicate immune evasion mechanisms are more pronounced in antibody responses. The persistence of T cell responses to conserved regions within the S protein highlights the critical role of T cells in offering protection where antibodies might fall short. An unexpected finding from our study was the decline in both the breadth and magnitude of T cell responses following the fourth vaccine dose compared to the third. This contrasts the expectation that successive vaccinations would enhance T cell responses. Notably, this decrease was more pronounced in donors with a history of multiple SARS-CoV-2 infections, suggesting that pre-existing immunity may influence the dynamics of vaccine-induced T cell responses. However, these findings should be interpreted with caution due to the relatively low number of donors included in the study, which limits the generalizability of the results. Additionally, the interplay between waning immunity from prior infections and the boosting effect of vaccines adds further complexity, underscoring the need for future studies with larger cohorts to confirm these observations. It is possible that repeated SARS-CoV-2 exposures may influence the T cell responses observed with additional booster doses [267,268]. This is a critical point for future vaccine strategies, as it suggests that individuals with prior infections

may have a more focused, and potentially narrower, T cell repertoire following additional vaccine doses.

In contrast, donors with fewer prior infections (0–1) exhibited an increase in breadth and even generated new peptide-specific responses after the fourth dose. This finding suggests that the absence of extensive pre-existing immunity may allow for a more diverse and flexible T cell response upon subsequent vaccination. These data highlight the importance of considering individual infection histories when assessing vaccine efficacy and the potential need for tailored booster strategies. The decline in the breadth and magnitude of SARS-CoV-2 T cell responses after a fourth bivalent vaccine dose, compared to a third monovalent dose can also be explained by several additional factors. One of the central concepts that emerges from this study is immunological imprinting, also known as "original antigenic sin." This concept describes how the immune system's first exposure to a pathogen or vaccine can influence its response to future encounters [269]. A study by Reynolds et al. demonstrated that repeated antigen exposures with homologous and heterologous spike proteins influence the breadth and durability of immune responses to SARS-CoV-2 variants [270]. They observed that while initial exposures boosted immunity, subsequent exposures to variant antigens sometimes resulted in a plateau or decline in neutralization and T cell responses, highlighting the complexities of immune responses after multiple exposures.

Our data indicate that individuals with prior SARS-CoV-2 infections may be "imprinted" with immune responses that were shaped by their initial viral exposure, which could explain why their T cell responses following a fourth vaccine dose were narrower and less expansive than in individuals with fewer prior infections. These results align with the idea that pre-existing immune memory may limit the immune system's ability to adapt to new exposures, resulting in a more focused response on epitopes encountered during previous infections [271,272]. With repeated exposures, memory cells may have limited expansion capacity with successive exposures, leading to a plateau in response [273]. Thus, the T cell responses may plateau, where each subsequent vaccine dose does not generate a significantly broader or stronger response. After three doses of a monovalent vaccine, the immune system has likely already mounted a robust response to the ancestral spike protein. When a bivalent booster is given, instead of expanding the T cell repertoire to the Omicron variant, the T cell responses may be biased towards the ancestral strain, resulting in less breadth. The bivalent vaccine presents two S proteins (ancestral and Omicron). It’s possible that the immune system may focus more heavily on one set of epitopes, such as those in the ancestral strain, leading to epitope dominance, where

only a few dominant epitopes are recognised, and the overall T cell repertoire does not expand significantly to cover new variant epitopes [225,274], reducing the breadth of the T cell response.

Overall, my results highlight the breadth of T cell responses, also providing additional evidence that T cell responses are maintained against VOCs. It will be interesting to further explore the implications of this and how this may contribute to long-term protection. While vaccination consistently generates broad and durable T cell responses, the role of prior infection in shaping these responses is complex and requires further investigation. These findings underscore the need for exploration of tailored booster strategies that take into account prior SARS-CoV-2 exposures. In conclusion, this study provides valuable insights into how SARS-CoV-2 vaccines, particularly after multiple doses, modulate T cell responses. The observation that prior infections may dampen the expected boosting effect of subsequent vaccine doses is of particular interest and suggests that future vaccination strategies may need to account for individuals' infection histories. Further research is needed to clarify the long-term implications of these findings, especially as the global effort to control COVID-19 continues through both natural and vaccine-induced immunity.

This chapter also explored T cell epitopes that may be associated with a previously identified protective allele in SARS-CoV-2, HLADQB1*06. I used a 14-day T cell line culture and ICS to validate findings from the peptide matrix. A key limitation of my T cell line culture assay is the potential for high background responses, which can obscure specific reactivity to the target peptide. In my study, while I initially tested 10 additional donors, high background responses limited our analysis to just three individuals (two HLA-DQB1*06 positive and one HLA-DQB106 negative). This limitation in sensitivity and specificity of the T cell line culture prompted me to consider using HLA-peptide tetramers, which allow for more precise detection of antigen-specific T cells by binding directly to T cell receptors. However, I encountered a significant challenge with tetramers, specifically in the synthesis of an HLA class II peptide tetramer compatible with our HLA-DQB1*06 allele. This process faced unexpected delays, taking approximately five months, ultimately preventing us from completing additional experiments. The synthesis challenges highlight an obstacle in applying tetramer technology to HLA class II peptides, which can impact the feasibility of timely and effective experimentation. Overall, these findings, along with evidence from B cell studies and epidemiological data, underscore the potential importance of HLA-DQB1*06 in SARS-CoV-

2 immunity. While my study faced challenges in T cell line culture sensitivity and tetramer synthesis, which limited the scope of our experiments, the observed responses in HLA-DQB1*06 positive individuals suggest that further research in this area is warranted. Future studies with larger sample sizes and optimized detection methods, such as refined tetramer technology or alternative assays, could provide more definitive insights into the role of HLA-DQB1*06 and other protective alleles in immunity against SARS-CoV-2. Despite the limitations, these preliminary findings contribute to a growing body of evidence supporting the relevance of specific HLA alleles in immune responses, potentially guiding the development of targeted immunotherapies and informing vaccine design tailored to individual HLA profiles.

Chapter 6: General discussion

The COVID-19 pandemic has underscored the complex dynamics of immune responses to emerging viral pathogens, particularly SARS-CoV-2. This thesis explored the adaptive immune response, with a specific focus on T cell responses elicited by both infection and vaccination. Through successive studies, I examined the factors influencing the breadth, durability, and effectiveness of T cell responses against SARS-CoV-2 and its variants, addressing key questions about immune memory, booster efficacy, and variant-specific reactivity. The findings of this thesis contribute to a deeper understanding of T cell immunity and offer insights for optimizing vaccination strategies to ensure long-term protection in diverse populations.

One of the primary insights from this work is the role of dosing intervals in shaping T cell responses. The study on varied dosing intervals indicated that extended intervals between vaccine doses enriched for more polyfunctional T cell responses, particularly in infection-naïve individuals, as seen with increased CD4+ IL-2 T cells. However, the magnitude of T cell responses was lower with extended intervals compared to a short dosing interval, and by six months after the second dose, differences in T cell magnitude or functionality were no longer detectable. While the extended interval was associated with better B cell responses, there was no sustained advantage for T cell immunity in the long term either in T cell response magnitude or polyfunctionality. This finding suggests that while adjusting the timing of vaccine doses can enhance specific aspects of immune responses, it may not necessarily result in sustained long-term advantages for T cell immunity. For previously infected individuals, however, the impact of dosing intervals was less pronounced, likely due to the presence of pre-existing immunity. These results indicate that hybrid immunity provides a baseline level of T cell response, which may make dosing schedules less critical in individuals with prior exposure to the virus. Optimizing dose intervals could be a key consideration for countries designing booster campaigns to balance immune enhancement with logistical feasibility.

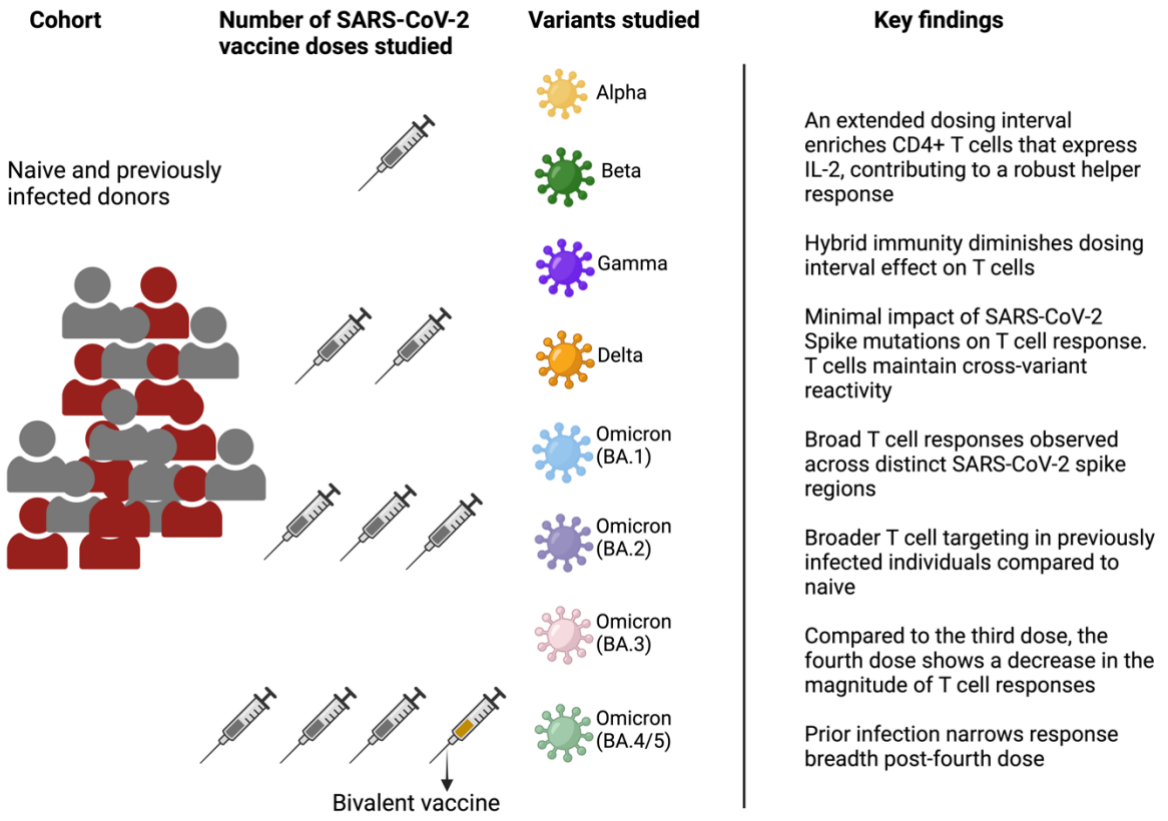


Figure 6.1. Summary of thesis findings. Naïve and previously infected donors were studied across various SARS-CoV-2 variants and vaccine doses, including the bivalent SARS-CoV-2 vaccine. Key findings highlight the impact of dosing intervals, hybrid immunity, variants, and the influence of prior infection and booster doses on T cell response magnitude and breadth.

The exploration of T cell responses to different SARS-CoV-2 variants further highlighted the adaptability of T cell immunity. While neutralizing antibody responses were significantly affected by mutations in the viral spike protein, T cells demonstrated a broader capacity for cross-variant recognition. The T cell responses observed across Alpha, Beta, Gamma, Delta, and Omicron variants underscore the importance of cellular immunity in providing stable protection, even as the virus evolves. These findings support the view that T cell-mediated immunity is an essential component of effective vaccine design, offering a secondary line of defence in cases where antibody-mediated protection may be compromised by viral mutation. In real-world settings, this broad T cell response may explain the continued protection against severe disease, even as antibody titres wane and variants with immune escape mutations - leading to reduced neutralising antibody function - emerge.

In examining the effects of booster doses, my work revealed a nuanced picture of T cell response dynamics. While the third dose of vaccine generally enhanced T cell responses, the

fourth bivalent dose had a more variable effect, with responses declining after a fourth dose, particularly in individuals with multiple prior SARS-CoV-2 infections. Although the sample size studied was relatively small, these findings nevertheless challenge the widely held assumption that additional booster doses consistently enhance the magnitude of T cell responses. This observation is important for informing future booster strategies, indicating that additional doses may provide diminishing returns, particularly in individuals with robust immune memory from prior infections or vaccinations. These insights imply that a more targeted booster strategy might be beneficial, focusing on populations with lower baseline immunity or assessing the effectiveness of additional doses in eliciting robust responses, even with updated vaccines targeting emerging variants.

Similarly, the finding that prior infection history impacts T cell breadth and magnitude is also important. The findings showed that donors with multiple prior infections showed a narrower T cell response after the fourth dose, while those with fewer exposures showed increased breadth after the fourth dose. These insights underscore the importance of considering individual immune histories when designing booster policies, as repeated exposures may have unique effects on immune flexibility and durability. Such findings could influence real-world public health decisions, guiding differentiated vaccination strategies based on prior infection status to optimize immune responses across the population.

While our results offer valuable insights, it is important to consider some limitations. Most of our studies were conducted in a healthcare worker cohort, a group with higher potential exposure to SARS-CoV-2 than the general population and potentially unique immune profiles due to occupational risks. In addition, there is the “healthy worker effect” where employed people cannot fully represent the immune health of the background population. Although our findings in this cohort have been replicated across several populations, caution should be exercised in generalizing the results to other groups, especially those with different demographic or health characteristics. For example, older adults or immunocompromised individuals may exhibit distinct immune responses that require tailored vaccination strategies. Further research involving diverse populations would enhance our understanding of how these findings translate across various demographic and clinical contexts.

Another limitation is that our studies primarily focused on the spike protein as the main antigenic target, given its central role in current SARS-CoV-2 vaccines. While T cell responses

to non-spike proteins were briefly assessed, a more comprehensive analysis of T cell reactivity to the entire SARS-CoV-2 proteome would provide deeper insights, particularly for designing vaccines that generate broader immune responses. Ongoing efforts are underway to develop vaccines encoding RTC T cell antigens using mRNA and ChAd platforms, as well as broader work on pan-coronavirus vaccines, which aim to achieve more comprehensive and durable immunity. As variants with significant spike mutations emerge, conserved non-spike targets could become increasingly relevant, and it is important that future studies consider evaluating the immune response to these conserved regions.

Looking forward, there are several avenues for further investigation. First, further validating the identified S125 peptide, which exhibited promising T cell reactivity, using a tetramer assay would confirm its potential as a robust target for T cell responses. Tetramer assays allow for precise measurement of T cell responses to specific epitopes, providing a valuable tool for understanding the role of this peptide in immunity.

Additionally, future work could focus on longitudinal studies tracking T cell responses over extended periods to determine the long-term sustainability of immune protection following successive vaccine doses, particularly within the context of updated vaccine formulations. Such studies would clarify whether the observed plateau or decline in T cell responses after multiple doses is a transient effect or represents a stable immune memory profile. Understanding the duration and stability of these responses will be critical for shaping booster recommendations and ensuring ongoing protection against SARS-CoV-2.

Moreover, exploring the interplay between T cell responses and other immune components, such as B cells and innate immunity, could provide a more holistic view of immune protection. While T cells play a critical role in limiting disease severity, their interactions with neutralizing antibodies and innate immune cells are essential for comprehensive immunity. Future research should aim to elucidate these interactions, particularly in the context of immune escape variants and waning antibody responses. Such insights would be valuable for developing next-generation vaccines that can engage multiple arms of the immune system.

Finally, advancements in tools such as single-cell sequencing and multi-omics approaches will enable deeper analyses of the immune response to SARS-CoV-2. These tools could help identify new targets and biomarkers for vaccine efficacy, facilitating the development of more

personalized immunization strategies. By understanding individual variations in immune responses, we can move toward a precision-medicine approach for those most in need due to immune suppression or advanced age, optimizing vaccine schedules and formulations based on both personal and population-level data. Moreover, these efforts provide a robust framework for addressing other infectious diseases with variant escape, ensuring preparedness for future pandemics.

Overall, this thesis highlights several key aspects of T cell immunity that are critical for shaping SARS-CoV-2 vaccination strategies. The findings on dosing intervals, booster responses, and variant cross-reactivity provide a framework for optimizing vaccination schedules to enhance both the durability and breadth of immune protection. Although the acute phase of the COVID-19 pandemic has passed, these insights remain valuable for guiding public health strategies aimed at achieving comprehensive and long-lasting immunity. Future research should continue to investigate the mechanisms underlying T cell memory and the factors influencing immune adaptation to successive exposures. By leveraging these insights, we can advance towards a more resilient immunization strategy that remains effective against both current and future SARS-CoV-2 variants, ultimately contributing to global pandemic preparedness and resilience.

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Annexe

SARS-CoV-2 Peptides

ID	Length	Sequence
S_1	18	MFVFLVLLPLVSSQCVNL
S_2	16	PLVSSQCVNLTTRTQL
S_3	18	CVNLTTRTQLPPAYTNSF
S_4	16	QLPPAYTNSFTRGVYY
S_5	16	TNSFTRGVYYPDKVFR
S_6	18	GVYYPDKVFRSSVLHSTQ
S_7	17	FRSSVLHSTQDLFLPFF
S_8	16	STQDLFLPFFSNVTWF
S_9	15	LPFFSNVTWFHAIHV
S_10	18	NVTWFHAIHVSGTNGTKR
S_11	18	HVSGTNGTKRFDNPVLPF
S_12	16	KRFDNPVLPFNDGVYF
S_13	18	VLPFNDGVYFASTEKSNI
S_14	16	YFASTEKSNIIRGWIF
S_15	17	KSNIIRGWIFGTTLDSK
S_16	17	WIFGTTLDSKTQSLLIV
S_17	18	DSKTQSLLIVNNATNVVI
S_18	17	IVNNATNVVIKVCEFQF
S_19	18	VVIKVCEFQFCNDPFLGV
S_20	17	QFCNDPFLGVYYHKNNK
S_21	18	LGVYYHKNNKSWMESEFR
S_22	18	NKSWMESEFRVYSSANNC
S_23	15	FRVYSSANNCTFEYV
S_24	18	SANNCTFEYVSQPFLMDL
S_25	18	YVSQPFLMDLEGKQGNFK
S_26	18	DLEGKQGNFKNLREFVFK
S_27	18	FKNLREFVFKNIDGYFKI
S_28	17	FKNIDGYFKIYSKHTPI
S_29	16	FKIYSKHTPINLVRDL
S_30	17	HTPINLVRDLPQGFSAL
S_31	18	RDLPQGFSALEPLVDLPI
S_32	17	ALEPLVDLPIGINITRF
S_33	18	LPIGINITRFQTLLALHR
S_34	18	RFQTLLALHRSYLTPGDS
S_35	18	HRSYLTPGDSSSGWTAGA
S_36	18	DSSSGWTAGAAAYYVGYL
S_37	18	GAAAYYVGYLQPRTFLLK
S_38	17	YLQPRTFLLKYNENGTI
S_39	18	LLKYNENGTITDAVDCAL

S_40	17	TITDAVDCALDPLSETK
S_41	18	CALDPLSETKCTLKSFTV
S_42	15	TKCTLKSFTVEKGIY
S_43	17	KSFTVEKGIYQTSNFRV
S_44	18	GIYQTSNFRVQPTESIVR
S_45	17	RVQPTESIVRFPNITNL
S_46	17	IVRFPNITNLCPFGEVF
S_47	18	TNLCPFGEVFNATRFASV
S_48	18	VFNATRFASVYAWNRKRI
S_49	17	SVYAWNRKRISNCVADY
S_50	18	KRISNCVADYSVLYNSAS
S_51	17	DYSVLYNSASFSTFKCY
S_52	17	SASFSTFKCYGVSPTKL
S_53	18	KCYGVSPTKLNDLCFTNV
S_54	18	KLNDLCFTNVYADSFVIR
S_55	17	NVYADSFVIRGDEVRQI
S_56	18	VIRGDEVRQIAPGQTGKI
S_57	17	QIAPGQTGKIADYNYKL
S_58	18	GKIADYNYKLPDDFTGCV
S_59	18	KLPDDFTGCVIAWNSNNL
S_60	18	CVIAWNSNNLDSKVGGNY
S_61	18	NLDSKVGGNYNYLYRLFR
S_62	17	NYNYLYRLFRKSNLKPF
S_63	18	LFRKSNLKPFERDISTEI
S_64	18	PFERDISTEIYQAGSTPC
S_65	16	EIYQAGSTPCNGVEGF
S_66	16	STPCNGVEGFNCYFPL
S_67	15	VEGFNCYFPLQSYGF
S_68	18	CYFPLQSYGFQPTNGVGY
S_69	18	GFQPTNGVGYQPYRVVVL
S_70	15	GYQPYRVVVLSFELL
S_71	16	RVVVLSFELLHAPATV
S_72	15	FELLHAPATVCGPKK
S_73	18	APATVCGPKKSTNLVKNK
S_74	16	KKSTNLVKNKCVNFNF
S_75	18	VKNKCVNFNFNGLTGTGV
S_76	18	NFNGLTGTGVLTESNKKF
S_77	18	GVLTESNKKFLPFQQFGR
S_78	18	KFLPFQQFGRDIADTTDA
S_79	17	GRDIADTTDAVRDPQTL
S_80	15	TDAVRDPQTLEILDI
S_81	18	DPQTLEILDITPCSFGGV
S_82	18	DITPCSFGGVSVITPGTN
S_83	18	GVSVITPGTNTSNQVAVL
S_84	18	TNTSNQVAVLYQDVNCTE

S_85	15	VLYQDVNCTEVPVAI
S_86	15	VNCTEVPVAIHADQL
S_87	17	VPVAIHADQLTPTWRVY
S_88	17	DQLTPTWRVYSTGSNVF
S_89	18	RVYSTGSNVFQTRAGCLI
S_90	15	VFQTRAGCLIGAEHV
S_91	18	AGCLIGAEHVNNSYECDI
S_92	16	HVNNSYECDIPIGAGI
S_93	18	ECDIPIGAGICASYQTQT
S_94	17	GICASYQTQTNSPRRAR
S_95	18	TQTNSPRRARSVASQSII
S_96	16	ARSVASQSIIAYTMSL
S_97	18	QSIIAYTMSLGAENSVAY
S_98	17	SLGAENSVAYSNNSIAI
S_99	18	VAYSNNSIAIPTNFTISV
S_100	17	AIPTNFTISVTTEILPV
S_101	17	ISVTTEILPVSMTKTSV
S_102	16	LPVSMTKTSVDCTMYI
S_103	18	KTSVDCTMYICGDSTECS
S_104	16	YICGDSTECSNLLLQY
S_105	17	TECSNLLLQYGSFCTQL
S_106	17	LQYGSFCTQLNRALTGI
S_107	16	TQLNRALTGIAVEQDK
S_108	16	LTGIAVEQDKNTQEVF
S_109	18	EQDKNTQEVFAQVKQIYK
S_110	17	VFAQVKQIYKTPPIKDF
S_111	18	IYKTPPIKDFGGFNFSQI
S_112	16	DFGGFNFSQILPDPSK
S_113	17	FSQILPDPSKPSKRSFI
S_114	18	PSKPSKRSFIEDLLFNKV
S_115	18	FIEDLLFNKVTLADAGFI
S_116	17	KVTLADAGFIKQYGDCL
S_117	18	GFIKQYGDCLGDIAARDL
S_118	16	CLGDIAARDLICAQKF
S_119	16	ARDLICAQKFNGLTVL
S_120	18	AQKFNGLTVLPPLLTDEM
S_121	18	VLPPLLTDEMIAQYTSAL
S_122	15	EMIAQYTSALLAGTI
S_123	16	YTSALLAGTITSGWTF
S_124	18	AGTITSGWTFGAGAALQI
S_125	18	TFGAGAALQIPFAMQMAY
S_126	17	QIPFAMQMAYRFNGIGV
S_127	16	MAYRFNGIGVTQNVLY
S_128	16	GIGVTQNVLYENQKLI
S_129	18	NVLYENQKLIANQFNSAI

S_130	17	LIANQFNSAIGKIQDSL
S_131	17	SAIGKIQDSLSSTASAL
S_132	17	DSLSSTASALGKLQDVV
S_133	17	SALGKLQDVVNQNAQAL
S_134	17	DVVNQNAQALNTLVKQL
S_135	17	QALNTLVKQLSSNFGAI
S_136	18	KQLSSNFGAISSVLNDIL
S_137	16	AISSVLNDILSRLDKV
S_138	18	NDILSRLDKVEAEVQIDR
S_139	16	KVEAEVQIDRLITGRL
S_140	17	QIDRLITGRLQSLQTYV
S_141	16	GRLQSLQTYVTQQLIR
S_142	15	QTYVTQQLIRAAEIR
S_143	15	QQLIRAAEIRASANL
S_144	15	AAEIRASANLAATKM
S_145	15	ASANLAATKMSECVL
S_146	18	AATKMSECVLGQSKRVDF
S_147	18	VLGQSKRVDFCGKGYHLM
S_148	18	DFCGKGYHLMSFPQSAPH
S_149	17	LMSFPQSAPHGVVFLHV
S_150	18	APHGVVFLHVTYVPAQEK
S_151	18	HVTYVPAQEKNFTTAPAI
S_152	18	EKNFTTAPAICHDGKAHF
S_153	17	AICHDGKAHFPREGVFV
S_154	18	AHFPREGVFVSNGTHWFV
S_155	16	FVSNGTHWFVTQRNFY
S_156	15	HWFVTQRNFYEPQII
S_157	17	QRNFYEPQIITTDNTFV
S_158	18	QIITTDNTFVSGNCDVVI
S_159	18	FVSGNCDVVIGIVNNTVY
S_160	17	VIGIVNNTVYDPLQPEL
S_161	17	TVYDPLQPELDSFKEEL
S_162	15	PELDSFKEELDKYFK
S_163	17	FKEELDKYFKNHTSPDV
S_164	18	YFKNHTSPDVDLGDISGI
S_165	17	DVDLGDISGINASVVNI
S_166	17	SGINASVVNIQKEIDRL
S_167	17	VNIQKEIDRLNEVAKNL
S_168	17	DRLNEVAKNLNESLIDL
S_169	16	KNLNESLIDLQELGKY
S_170	18	LIDLQELGKYEQYIKWPW
S_171	17	KYEQYIKWPWYIWLGFI
S_172	18	WPWYIWLGFIAGLIAIVM
S_173	18	FIAGLIAIVMVTIMLCCM
S_174	18	VMVTIMLCCMTSCCSCLK

S_175	18	CMTSCCSCLKGCCSCGSC
S_176	18	LKGCCSCGSCCKFDEDDS
S_177	18	SCCKFDEDDSEPVLKGVK
S_178	18	FDEDDSEPVLKGVKLHYT