Characterising the magnitude and breadth of T cell responses to SARS-CoV-2 vaccination

by

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This thesis is dedicated to my late father

Sollomon Morabane Masupye ∞

List of abbreviations

OAS	2'-5'-oligoadenylate synthetase
TMB	3,3′,5,5′-Tetramethylbenzidine
ADAM17	A disintegrin and metallopeptidase domain 17
ARDS	Acute respiratory distress syndrome
ACE-1	Angiotensin-converting enzyme
ADCC	Antibody-dependent T cell cytotoxicity
ADCP	Antibody-dependent T cell phagocytosis
CDC	Antibody-mediated complement-dependent cytotoxicity
BL	Baseline
BARC SA	Bio Analytical Research Corporation South Africa
BTI	Breakthrough infection
BFA	Brefeldin A
BALF	Bronchoalveolar lavage fluid
Tcm	Central memory T cell
COVID-19	Coronavirus disease 2019
DC	Dendritic cell
DNase	Deoxyribonuclease
DMSO	Dimethyl sulfoxide
dH_2O	Distilled water
dsRNA	Double-stranded RNA
Tem	Effector memory T cell
EUA	Emergency use authorisation
ER	Endoplasmic reticulum
ER	Endoplasmic reticulum
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
ERGIC	ER-Golgi intermediate compartment
FMO	Fluorescence minus one
W4	Four weeks
FP	Fusion peptide
HCW	Healthcare workers
HI-FCS	Heat-inactivated foetal calf serum

HR	Heptapeptide repeat sequence
HLA	Human leukocyte antigen
HREC	Human Research Ethics Committee
IRF3	IFN Regulatory Factor 3
ISG	IFN-stimulated genes
IFN	Interferon
IFN-γ	Interferon-gamma
IFNAR	Interferon-α/β receptor
IFN-γ	Interferon-γ
IL-1β	interleukin-1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IQR	Interquartile range
JAK-STAT	Janus kinase-signal transducer and activator of transcription
LN_2	Liquid nitrogen
M	Membrane
µg/mL.	Microgram per milliliter
µL	Microliter
MERS-CoV	Middle East respiratory syndrome coronavirus
mL	Milliliter
NK	Natural Killer
NIR	Near-infrared
-ss	Negative-sense single-stranded
2019-nCoV	New coronavirus 2019
NSP	Non-structural protein
NTD	N-terminal domain
NF-κB	Nuclear Factor-κB
N	Nucleocapsid
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PRR	pattern recognition receptor
Pen-Strep	Penicillin-Streptomycin
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate-buffered saline
PMT	Photomultiplier tube
pp	Polyproteins
+ss	Positive-sense single-stranded
PSO	Post-symptom
PKR	Protein Kinase R
ROS	Reactive oxygen species
RBM	Receptor binding motif
RBD	Receptor-binding domain
RO	Replication organelle
RIG-I	Retinoic Acid-Inducible Gene I
RNP	Ribonucleoprotein
RdRp	RNA-dependent RNA polymerase
RPMI	Roswell Park Memorial Institute
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
STAT	signal transducer and activator of transcription
SHERPA	Sisonke heterologous mRNA-1273 boost after priming with Ad26.COV2.S
S	Spike
H_2SO_4	Sulfuric acid
TCR	T cell receptor
Tfh	T-follicular helper
Th1	T-helper 1
Trm	Tissue-resident memory T cell
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serine protease 2
TNF-α	Tumor necrosis factor-alpha
TNF	Tumor necrosis factor
Ad26	Type 26 adenovirus
VOC	Variant of concern

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Abstract

Spike protein which serves as the immunogen in the current vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is targeted by the CD4 and CD8 T cells. While most studies frequently measure the magnitude of spike-specific CD4 and CD8 responses, only a few studies have characterised their breadth of targeting across the spike. This characterization is crucial for predicting cross-reactivity in T cell responses against emerging variants of concern (VOC) that may have mutations in the targeted regions.

This study aimed to examine the magnitude and breadth of SARS-CoV-2 spike-specific memory CD4 and CD8 T cells induced by a heterologous prime-boost vaccination strategy consisting of Ad26.COV2.S and mRNA-1273 vaccines.

Twenty healthcare workers (HCW) who participated in the SHERPA study where participants who had previously received either one (n=8) or two (n=12) doses of the Ad26.COV2.S vaccine received the mRNA-1273 vaccine booster at baseline (BL) were selected. The median time since last Ad26.COV2.S vaccine dose was 340 days [IQR: 335-355] and 187 days [IQR: 340-458] for the one and two prior dose groups, respectively. Ninety-five percent (19/20) of the participants had a history of SARS-CoV-2 infection. CD4 and CD8 T cell responses to the SARS-CoV-2 spike protein were characterized using intracellular cytokine staining interferon-γ (IFN-γ) or interleukin-2 (IL-2) and flow cytometry. The magnitude of T cell responses to full spike was examined, as well as crude T cell breadth, using 7 peptide pools spanning the entire length of the spike protein at BL and 4 weeks (W4) post-vaccination.

Boosting with mRNA-1273 resulted in a significant increase in the magnitude of spike-specific CD4 responses (median % memory CD4 T cells: 0.085 vs. 0.157; p=0.04) and CD8 responses (median % memory CD8 T cells: 0 vs. 0.015; p=0.025). A strong positive correlation was observed between the magnitude of the full spike and the combined seven pools for both CD4 ($p < 0.0001$; $r = 0.78$) and CD8 ($p < 0.0001$; $r = 0.74$) responses.

On average, participants had CD4 responses targeted against five pools (range 2-7) at BL. Following mRNA-1273 vaccination, the number of pools targeted slightly increased to a median of 5.5 (range 4-7). Only 8/20 (40%) participants had CD8 responses against the spike pools at BL (median: 0; range 0-6). At W4, the median number of targeted pools increased to one (range 0-6) among the participants. There was no statistical difference in CD4 or CD8 responses against the pools between those who had received one or two initial doses.

All seven spike pools were targeted by the CD4 and CD8 cells at both timepoints. The highest median magnitude of CD4 responses was detected for pool 2 (0.025%) which covers the N-terminal domain (131-315 aa), pool 5 (0.023%) which covers the fusion peptide domain (686-890 aa), pool 6 (0.019%) which includes the heptapeptide repeat sequence and pool 3 (0.015%) covers a portion of the receptor binding domain (305-515 aa). Since most CD8 responders targeted only a single pool, no pool dominated in terms of the median magnitude.

The data suggest that CD4 cells exhibit a broader breadth of responses compared to CD8 responses which were narrowly targeted against one region of the spike. This is consistent with what other published studies found. The broad targeting of the CD4 responses suggests that cross-reactivity against emerging VOCs may be better preserved than the CD8 responses, especially when mutations occur in the targeted epitopes. As new variants continue to emerge, it is important to introduce vaccines that induce broader CD8 responses to bolster cross-reactivity.

Chapter 1: Literature review

1.1 Introduction

1.1.1 SARS-CoV-2 pandemic

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a respiratory virus responsible for causing coronavirus disease 2019 (COVID-19; Zhou et al., 2020b). It was initially dubbed the new coronavirus 2019 (2019-nCoV) before being renamed due to its genetic resemblance to SARS-CoV-1 (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Liu et al., 2020a). The first cases of the virus were documented on 18 December 2019, after some patients presented with atypical pneumonia (Chen et al., 2020b; Zhou et al., 2020b). The virus was then identified throughout China within a month before spreading to other countries with more than a thousand cases confirmed each day (Chauhan, 2020; Jhu et al., 2020; Wu et al., 2020). The global impact of the virus led to its declaration as an international public health emergency on 30 January 2020 before being declared as a global pandemic a month later (World Health Organization, 2020a, 2020b).

Since then, more than 773 million infections and 6.9 million deaths have been officially confirmed (World Health Organisation Coronavirus (COVID-19) Dashboard, 2023), but there have been vastly more undiagnosed and unconfirmed infections. The latest estimates of total deaths and infections are 27 million and 14 billion, respectively, including reinfections (COVID-19 Data Explorer, 2023; IHME COVID-19 Projections 2023). To contextualise this, the scale of this respiratory viral outbreak has not been witnessed in over a century, since the H1N1 pandemic caused by the influenza virus. That pandemic infected over 500 million people and resulted in an estimated 50 million deaths (Johnson & Mueller, 2002).

1.1.2 SARS-CoV-2 transmission and disease

Despite extensive research, the origin of SARS-CoV-2 remains elusive, leading to various theories, with the most plausible being zoonotic spillover (Ellwanger & Chies, 2021; Tan et al., 2022). It is likely that the virus may have transferred from animals to humans, with horseshoe bats serving as the primary reservoir and an unidentified animal serving as the intermediate host (Alwine et al., 2023; Domingo, 2022). This is supported by SARS-CoV-2 genome being 96.2% and 93.3% homologous to

horseshoe bat species *Rhinolophus affinis* and *Rhinolophus malayanus*, respectively (Lam et al., 2020; Zhou et al., 2020b). Furthermore, bats infected with coronaviruses exhibit no histopathological or clinical symptoms (Xiao et al., 2020). Lastly, patients admitted to the hospital were linked to a downtown wet market in Wuhan, which sold seafood and live animals, including wildlife (Zhou et al., 2020b)

SARS-CoV-2 primarily spreads through contact with mucosal membranes in the eyes, mouth and nose (Emrani et al., 2021). Infectious viral particles are released through coughing, sneezing, talking, singing or breathing, and they can also be transmitted via contact with surfaces contaminated with the virus (Chu et al., 2020; Echternach et al., 2020; Klompas et al., 2020; Liu et al., 2021; Wang et al., 2020d). The viral genome has been detected in blood, urine and stool, though the extent to which these serve as additional transmission routes remains unclear (Meyerowitz et al., 2021; Wang et al., 2020c). Some studies have also reported the presence of the SARS-CoV-2 genome in newborns born to infected mothers and in the amniotic fluid, suggesting potential transplacental transmission (Alamar et al., 2020; Patanè et al., 2020; Vivanti et al., 2020).

After contracting SARS-CoV-2, individuals may start experiencing clinical symptoms within 2 to 14 days (Guan et al., 2020; Salzberger et al., 2020; Wang et al., 2020a). The manifestation of symptoms depends on several factors, including age and pre-existing medical conditions (Zsichla & Müller, 2023). People without comorbidities and those aged under 60 years typically remain asymptomatic or develop mild to moderate disease, characterised by symptoms such as loss of taste or smell, coughing, pharyngitis, and malaise, with some variation in symptoms depending on the viral variant (Alimohamadi et al., 2020; Chen et al., 2020a; Zhou et al., 2020a). In contrast, the elderly and individuals with pre-existing medical conditions, such as chronic pulmonary, kidney, and liver ailments, diabetes, and heart disease, are more prone to severe disease. Severe cases are marked by pneumonia, sometimes progressing to critical disease presenting as acute respiratory distress syndrome (ARDS) and septic shock (Fung & Babik, 2021; Liu et al., 2020b; Zeng et al., 2020a; Zhou et al., 2020a).

The highest viral load is typically observed seven days after infection and gradually decreases thereafter (Cevik et al., 2020; Kawasuji et al., 2020). People are generally infectious a week after symptom onset (Zhou et al., 2020b). However, transmission

can occur one or two days before the appearance of clinical manifestations, and asymptomatic individuals can also transmit the virus (Zhou et al., 2020b). In contrast, immunocompromised individuals and those with severe disease may remain infectious for an extended period due to impaired viral clearance and may also be a source for viral variants to emerge due to prolonged infection and viral evolution (Karim et al., 2021; Yousaf et al., 2021).

1.1.3 SARS-CoV-2 taxonomy

One of the earliest accounts of coronaviruses, which belong to the order *Nidovirales* in the *Coronaviridae* family, was in the late 1930s following their isolation from chicken embryos (Beaudette & Hudson, 1937). Since then, more than 100 different species of coronaviruses have been isolated from humans and animals (Zhou et al., 2021). They are divided into four genera: *Alphacoronaviruses* and *Betacoronaviruses*, which infect mammals, and *Deltacoronaviruses* and *Gammacoronaviruse*s, which infect birds. (Decaro & Lorusso, 2020; Zhou et al., 2021). Before SARS-CoV-2, only six other coronaviruses were known to circulate amongst humans, including the highly pathogenic SARS-CoV-1 and Middle East Respiratory Syndrome coronavirus (MERS-CoV) that were discovered in 2003 and 2010, respectively (Drosten et al., 2003; Zaki et al., 2012). SARS-CoV-1 is now extinct, having been effectively controlled and eradicated during the 2003 outbreak. In addition, there are four endemic coronaviruses HKU1, OC43, NL63, and 229E, which are typically associated with mild respiratory symptoms such as the common cold (Syed, 2020). Similar to other coronaviruses, SARS-CoV-2 is pleomorphic with petal-like spike protrusions on the viral surface (Li et al., 2016; Sternberg & Naujokat, 2020; Turoňová et al., 2020; Zhu et al., 2020). It has a positive-sense single-stranded (+ss) RNA genome of approximately 30,000 base pairs with a 5' cap and 3' polyadenylated tail (Al-Qaaneh et al., 2021; Kim et al., 2020; Lu et al., 2020; **Figure 1.1**). Over 70% of the genome codes for 16 non-structural proteins (NSP) across two overlapping open reading frames (ORFs) 1a and 1b and the remaining 30% encodes four structural and 11 accessory proteins (Hachim et al., 2020; Lu et al., 2020; Wall et al., 2020; Zhou et al., 2020b). NSP 1-16 proteins primarily facilitate viral replication while the accessory proteins (ORF3a, ORF3b, ORF3c, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c and ORF10) modulate evasion of the immune response (Yadav et al., 2021). The structural proteins are spike,

envelope, membrane and nucleocapsid, which play a crucial role in viral entry into and egress from host cells (Satarker & Nampoothiri 2020; Shang et al., 2020).

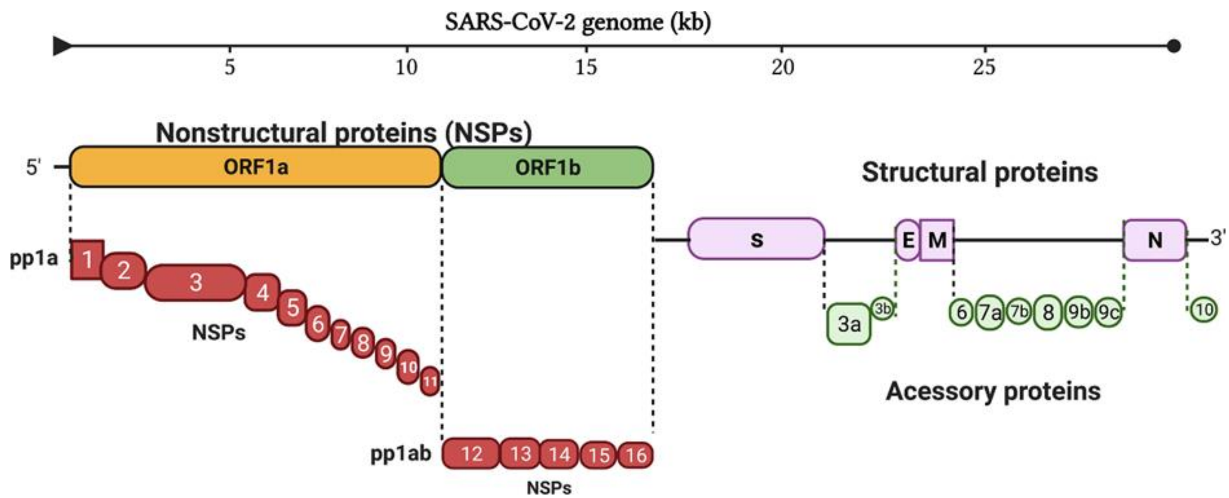


Figure 1.1: Illustration of SARS-CoV-2 genome organization. The genome encodes non-structural proteins (NSP), accessory proteins and four structural proteins, namely spike (S), nucleocapsid (N), envelope (E) and membrane (M). Adapted from Rashid et al., 2022.

1.1.4 SARS-CoV-2 life cycle

The main viral targets are cells that express angiotensin-converting enzyme 2 (ACE-2) receptors on their membrane surface (Samavati & Uhal, 2020). In humans, these receptors are predominantly expressed in tissues and cells such as cardiomyocytes, blood vessels, enterocytes, eyes, renal tubules, male reproductive cells and type II alveolar epithelial cells, which are the main viral targets (Hikmet et al., 2020, Zou et al., 2020). Viral entry is initiated by the SARS-CoV-2 spike protein, comprising S1 and S2 subunits (Ou et al., 2020; Walls et al., 2020; **Figure 1.2**). Before the virus can enter the cells, the two subunits need to be cleaved at the S1/S2 junction (Hoffmann et al., 2020a, 2020b; Lavie et al., 2022). The cleavage is performed by host proteases and occurs in two sequential steps. First, the S1/S2 junction is cleaved by

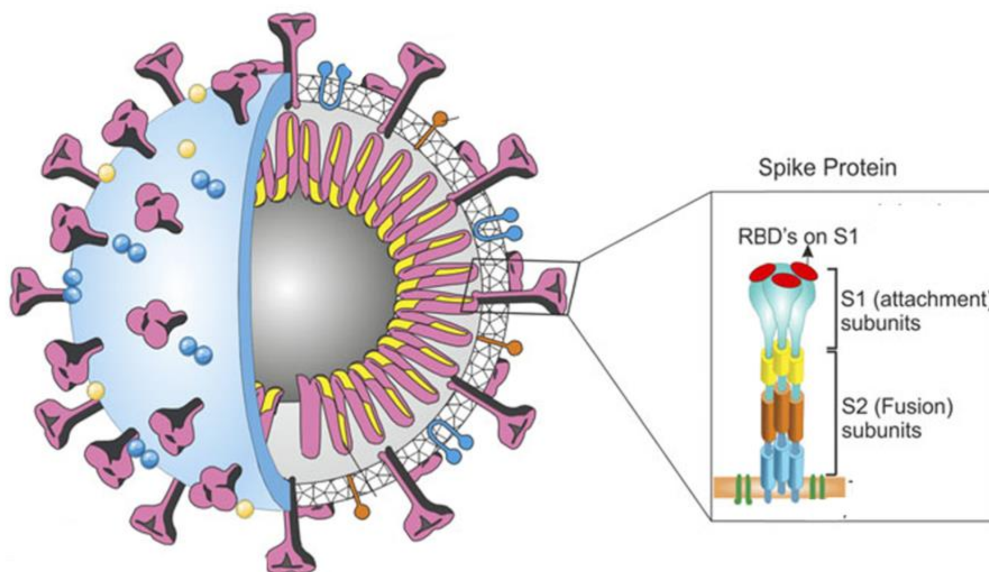


Figure 1.2: Schematic representation of the SARS-CoV-2 spike protein with the S1 and S2 subunits. Adapted from Rahbar-Saadat et al., 2021.

furin, which exposes the receptor-binding domain (RBD) found on the S1 subunit that is important for binding to the peptidase portion of the ACE-2 receptor (Hoffmann et al., 2020a; Millet & Whittaker, 2014).

The two subunits remain attached by non-covalent bonds before the second cleavage is carried out by host enzymes, a disintegrin and metallopeptidase domain 17 (ADAM17) and transmembrane protease serine protease 2 (TMPRSS2; Jackson et al., 2022; Samavati & Uhal, 2020). This cleavage enables a conformational change of the S2 subunit that results in the release of the fusion peptide and subsequent fusion with the host cell membrane (Belouzard et al., 2009; Walls et al., 2020). Alternatively, the virus may enter the host cell via an endocytic pathway. This pathway involves the activation of the S2 subunit by endosomal cathepsin proteases CatB and CatL in the absence of TMPRSS2 (Gomes et al., 2020; Padmanabhan et al., 2020; Zhao et al., 2021). This results in the fusion of the viral and endosomal membrane. The fusion of the viral and host membranes results in the release of the genome into the cell cytosol and subsequent viral replication.

To initiate replication, negative-sense single-stranded (-ss) RNA sub-genomic strands of the viral genome are synthesised. These strands serve as templates for producing positive-sense single-stranded (+ssRNA) genomes that are then translated into viral polyproteins (pp1a and pp1ab) in the host ribosomes (Kelly et al., 2020; Namy et al., 2006). Subsequently, viral NSP3 and NSP5 cleave these polyproteins to generate the 16 NSPs crucial for viral replication and protein synthesis (Gao et al., 2020; Peng et al., 2020a; Thoms et al., 2020).

Specifically, NSP3, NSP4 and NSP6 are responsible for forming a replication organelle (RO) that is comprised of interconnected membrane structures derived from the host cell endoplasmic reticulum (ER; Klatte et al., 2022; Namy et al., 2006; Ricciardi et., 2022). NSP13 is a helicase that unwinds the double RNA strand for replication and transcription (Jang et al., 2020). NSP7, NSP8, and NSP12 form the viral RNA-dependent RNA polymerase (RdRp) complex that is responsible for catalysing the production and proofreading of RNA transcripts (Imbert et al., 2008). NSP1 binds to the host ribosome to inhibit host mRNA production, promoting the translation of viral mRNA (Thoms et al., 2020). NSP10 complexes NSP14 and NSP16 to help induce mRNA 5' cap methylation and proofread mRNA transcripts to minimise the occurrence of mutations (Bouvet et al., 2014).

The synthesised viral proteins exit the replication site through the transmembrane before being assembled in the ER-Golgi intermediate compartment (ERGIC; Snijder et al., 2020). Membrane and envelope proteins promote the development of invaginations on the virion membrane for assembly and recruit the nucleocapsid protein to the viral assembly site (De Haan et al., 2000; Sarkar and Saha, 2020). Newly synthesised virions are then released from the host via exocytosis (Chen et al., 2021; Yang & Rao, 2021). Accessory protein ORF3a is also involved in viral replication and viral assembly and promotes the release of the virion (Chen et al., 2021; Zhang et al., 2022).

1.1.5 SARS-CoV-2 strategies for immune evasion

Interferon (IFN)-mediated innate immune responses serve as the first line of defence against most viral infections, including SARS-CoV-2. Notably, individuals with severe COVID-19 tend to exhibit lower levels of IFNs, while higher IFN levels are associated with better clinical outcomes, reduced viral loads, and quicker resolution (Arunachalam et al., 2020; Blanco-Melo et al., 2020; Hadjadj et al., 2020; Galani et al., 2021). The

initiation of IFN responses occurs following the detection of SARS-CoV-2-specific pathogen-associated molecular patterns (PAMPs). These PAMPs, such as double-stranded RNA (dsRNA) intermediates and +ssRNA, are detected by pattern recognition receptors (PRRs) within the host. Intracellular PRRs, such as Retinoic Acid-Inducible Gene I (RIG-I) and Melanoma Differentiation-Associated Protein 5 (MDA5), along with extracellular PRRs, including Toll-like receptors (TLRs) such as TLR3, TLR7/TLR8, and TLR9, have been implicated in detecting SARS-CoV-2 (Bortolotti et al., 2021; Costa et al., 2022; Thorne et al., 2021; Yang et al., 2021; Yin et al., 2021).

The recognition of PAMPs triggers the activation of IFN Regulatory Factor 3 (IRF3) and Nuclear Factor-κB (NF-κB) transcription factors (Diamond & Kanneganti, 2022; Onomoto et al., 2021; Rashid et al., 2022;). Subsequently, these transcription factors facilitate the production of IFN-I or -III, which then binds to the subunits (IFNAR 1 or 2) of IFN α and β receptors within the cells (Gonzales-van Horn et al., 2015; Panne et al., 2007; Rashid et al., 2022;). This binding initiates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, resulting in the phosphorylation of signal transducer and activator of transcription (STAT) 1 or 2 (Chen et al., 2021; Luo et al., 2021; Tolomeo et al., 2022). Phosphorylated STATs, in turn, activate IFN-stimulated genes (ISGs), which express various antiviral proteins, including Protein Kinase R (PKR), 2'-5'-oligoadenylate synthetase (OAS) and Mx proteins (Bignon et al., 2022; Greene & Zuniga, 2021; Li et al., 2021; Severa et al., 2021). Together, these proteins interfere with every step of viral replication, playing a crucial role in effectively containing infection.

Consequently, SARS-CoV-2 employs a range of strategies to avoid detection. For instance, during replication, the RO formed by NSP3, NSP4, and NSP6 creates a protected environment that prevents the detection of the viral intermediates (de Wilde et al., 2018; Klatte et al., 2022; Namy et al., 2006; Ricciardi et., 2022). NSP15 has an endonuclease activity and minimises the accumulation of dsRNA formed by the complementary RNA (Horrell et al., 2022; Pillon et al., 2021). The virus employs a dual strategy of capping and 2′-O-methylation of its RNA molecules, orchestrated by NSP13, NSP14, and NSP16 with NSP12 potentially serving as a co-factor (Bobrovs et al., 2021; Park et al., 2022; Wilamowski et al., 2021). These modifications mimic the features of host mRNA molecules, making the viral RNA less recognisable as foreign

by the immune system of the host. SARS-CoV-2 further utilises the N protein to form ribonucleoprotein (RNP) complexes that conceal the viral RNA and prevent its detection (Cubuk et al., 2021; Peng et al., 2020a; Wu et al., 2023; Zeng et al., 2020).

The virus has also evolved various mechanisms to interfere with IFN signalling at different stages. In particular, the membrane and nucleocapsid proteins inhibit the downstream activation of IRF3 by inhibiting RIG-1 and IFN-I transcription, potentially decreasing the phosphorylation of STAT1 (Zhao et al., 2021). NSP3, NSP6 and NSP12 along with accessory proteins such as ORF3a, ORF3b and ORF8 can also hinder the activity of IRF3 or its movement into the cell nucleus (Lei et al., 2020; Moustaqil et al., 2021). ORF3a, ORF6, ORF7a, ORF7b, NSP6 and NSP13 actively inhibit the phosphorylation of both STAT1 and STAT2 (Min et al., 2021; Miyamoto et al., 2022; Park et al., 2022; Redondo et al., 2021). They also directly impede the translocation of STAT proteins into the cell nucleus, effectively obstructing the expression of ISGs. NSP13 can prevent the binding of STAT1 with its receptor and subsequent phosphorylation (Feng et al., 2021; Guo et al., 2021).

Additionally, the accessory proteins of SARS-CoV-2 including ORF8 and ORF9c work together to delay viral clearance by preventing viral antigen presentation through MHC downregulation (Redondo et al., 2021; Zhang et al., 2021). This evasion strategy allows the virus to persist within the host, evading immune surveillance and prolonging infection duration. ORF9c is also responsible for downregulating the activation of the complement system, a key component of the innate immune response which aids in creating an environment conducive to viral survival and propagation within the host (Li et al., 2023b; Lu, 2020). ORF3a and ORF7 play a role in preventing apoptosis and autophagy, further enhancing the ability of the virus to evade host innate responses (Redondo et al., 2021). Although these studies have advanced our understanding of the virus's structure and function, and how its proteins contribute to viral replication and immune evasion, there are still controversies and gaps in the field. Research is ongoing.

1.1.6 Innate cellular responses to SARS-CoV-2 infection

In response to SARS-CoV-2 infection, various innate immune cells are recruited to the site of infection, playing crucial roles in protection and shaping the overall immune response. These cells include dendritic cells (DCs), macrophages, monocytes, natural killer (NK) cells, and neutrophils. DCs are pivotal in the immune response due to their ability to present viral antigens to T cells, thereby activating the adaptive immune system. They are also primary producers of type I IFNs cytokines, which are integral for antiviral responses (Tang et al., 2010). However, studies have shown that the presence of DCs is diminished in SARS-CoV-2-infected individuals compared to healthy controls and antigen presentation is impaired (Liao et al., 2020; Parackova et al., 2020; Saichi et al., 2021; Zhou et al., 2020c). This reduction can hinder the activation of adaptive immunity and contribute to a more severe disease progression.

Macrophages and monocytes help resolve infection and repair lung tissue by secreting both proinflammatory and anti-inflammatory factors (Brewer et al., 2022; Toor et al., 2020; Wang et al., 2020e). They produce elevated levels of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor (TNF), and interleukin-1 beta (IL-1β), as well as anti-inflammatory cytokines like IL-10 compared to normal controls (Konig et al., 2020; Toor et al., 2020; Zhang et al., 2021). However, their IFN production is limited in people with severe COVID-19 (Xu et al., 2020). The excessive release of proinflammatory cytokines, known as a cytokine storm, can lead to severe inflammation, tissue damage, and potentially fatal outcomes like ARDS (Konig et al., 2020).

NK cells play a crucial role in the innate immune response by directly eliminating virus-infected cells through the production of cytotoxic molecules such as granzyme B and perforin (Cunningham et al., 2020; Di Vito et al., 2022; Lee & Blish, 2023). They also release cytokines like IFN-γ and TNF, which enhance the antiviral response. During SARS-CoV-2 infection, increased levels of perforin and granzyme B in NK cells have been associated with markers of organ failure (Hammer et al., 2023). This suggests that while NK cells are essential for controlling the infection, their hyperactivation may contribute to disease severity and tissue damage.

Neutrophils also help resolve SARS-CoV-2 infection by promoting the death of infected cells through the production of reactive oxygen species (ROS) and the release of neutrophil extracellular traps (NETs). During infection, the upregulation of neutrophil

chemoattractants such as CXCL1, CXCL2, and CXCL8 enhances their recruitment to the infection site (Blanco-Melo et al., 2020; Xiong et al., 2020; Zhou et al., 2020d). However, dysregulated NETs formation has been implicated in driving inflammation and thrombosis (Ackermann et al., 2021; Li et al., 2023a; Zhu et al., 2022). This can result in organ damage and worsen disease outcomes. Additionally, dysregulated neutrophil activity can contribute to tissue damage and exacerbate the inflammatory responses (Ackermann et al., 2021; Li et al., 2023a; Zhu et al., 2022).

1.2 Adaptive immune responses to SARS-CoV-2 infection and vaccination

1.2.1 B cell and antibody responses post-infection

Upon detecting SARS-CoV-2, innate immune cells also swiftly initiate the activation of the adaptive immune response. The adaptive responses involve specialised subsets of immune cells, including B and T cells. B cells begin to generate both neutralising and non-neutralising antibodies targeting viral proteins, with detectable levels approximately one week after symptom onset (Ju et al., 2020; Lee & Oh, 2021; Qi et al., 2022; Wu et al., 2021; Yaugel-Novoa et al., 2022). Neutralising antibodies target the spike protein and inhibit viral infection by binding directly to the virus and preventing its interaction with the ACE-2 receptor (Gupta & Jaiswal, 2022). Such antibodies are associated with protection against infection (Khoury et al., 2021; Regev-Yochay et al., 2023). Conversely, non-neutralising antibodies mediate their protective effects by binding to infected cells, leading to a wide range of effector mechanisms such as antibody-dependent T cell cytotoxicity (ADCC), antibody-dependent T cell phagocytosis (ADCP) and antibody-mediated complement-dependent cytotoxicity (CDC) (Arvin et al., 2022; Heffron et al., 2021; Hong et al., 2022; Tso et al., 2021), likely contributing to protection from disease. Although antibody levels decline over time, memory B cells are maintained for a longer duration and can produce SARS-CoV-2 antibodies following reinfection (Abayasingam et al., 2021; Jeffery-Smith et al., 2022; Lumley et al., 2021; Ogega et al., 2021; Sokal et al., 2021).

1.2.2 T cell responses post-infection

T cell responses are readily detected following SARS-CoV-2 infection. SARS-CoV-2 specific CD4 T cells expand and differentiate into T helper 1 (Th1) and T follicular helper (Tfh) cells (Dan et al., 2021). Th1 cells produce antiviral cytokines such as interferon-gamma (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor-alpha (TNF-α) while Tfh cells support the maturation of B cells and antibody production (Balachandran et al., 2022; Jordan et al., 2021; Milne et al., 2021; Popescu et al., 2022). SARS-CoV-2-specific CD8 T cells are detectable a week post-symptom (PSO) and peak two weeks later (Bergamaschi et al., 2021). They are characterised by the production of effector molecules, such as perforin and granzyme, which induce apoptosis of infected cells, as well as antiviral cytokines such as IFN-γ, IL-2 and TNF-α (Mazzoni et al., 2020; Schulien et al., 2021). During infection, CD4 and CD8 T cells also exhibit a broad targeting of the SARS-CoV-2 proteome (Ferretti et al., 2020; Habel et al., 2020; Kared et al., 2020; Keller et al., 2020; Le Bert et al., 2020; Mateus et al., 2020; Nelde et al., 2020; Peng et al., 2020b; Tarke et al., 2020), with major viral proteins targeted being the spike, nucleocapsid and envelope. The magnitude of the T cell responses is based on the abundance of the proteins, with the highly expressed structural proteins inducing higher responses compared to the other proteins (Cohen et al., 2021; Grifoni et al., 2020; Sta et al., 2021). Notably, CD4 T cell responses are more dominant in peripheral blood mononuclear cells (PBMC) while CD8 T cells were found at higher frequencies in bronchoalveolar lavage fluid (BALF; Erdinc et al., 2021; Knudson et al., 2014; Kumar et al., 2022; Olea et al., 2022).

1.2.3 T cells protection in SARS-CoV-2

While the presence of antibodies is associated with protection, several studies have linked T cells to conferring protection against severe COVID-19. For instance, CD4 and CD8 T cells demonstrated the ability to protect against lung immunopathology in SARS-CoV-2 mouse models lacking neutralising antibodies (Fumagalli et al., 2024; Kingstad-Bakke et al., 2022; Pardieck, 2022). The depletion of CD8 T cells eliminated protection upon SARS-CoV-2 rechallenge in previously infected non-human primates (Liu et al., 2022; McMahan et al., 2021). Similarly, in humans, the early and coordinated activation of CD4 and CD8 T cells in infected people is associated with better clinical outcomes, while their absence has been reported in severe cases

(Bergamaschi et al., 2021; Braun et al., 2020; Gil-Etayo et al., 2020; Moderbacher et al., 2021; Notarbartolo et al., 2021; Roncati et al., 2020; Sekine et al., 2020). The presence of high antibody responses and low or absent T cell responses has been observed in patients with severe COVID-19 (Meng et al., 2020; Moderbacher et al., 2020; Tan et al., 2021; Wang et al., 2020b; Zhang et al., 2020a, 2020b). Supporting this, Meyts et al. (2021) reported that SARS-CoV-2-infected patients with X-linked agammaglobulinemia, characterised by low B cell levels, were able to clear the virus without medical interventions.

1.2.4 Memory T cell responses

SARS-CoV-2 infection also induces the development of memory T cells. These cells facilitate a robust response upon re-exposure to previously identified viral antigens, leading to accelerated disease resolution. Over 90% of individuals develop memory CD4 responses that are detectable up to a year post-infection (Cohen et al., 2021; Dan et al., 2021; Guo et al., 2022). The CD4 memory cells express both central memory (Tcm) and effector memory (Tem) phenotypes that primarily produce Th1-associated cytokines, including IFN-γ, IL-2, and TNF-α (Cohen et al., 2021; Dan et al., 2021; Law et al., 2022; Rodda et al., 2022). Notably, most asymptomatic individuals mount stronger T cell responses than symptomatic individuals (Le Bert et al., 2021; Samandari et al., 2021). Tissue-resident memory (Trm) CD4 T cells have also been isolated in tissues such as bone marrow, lung, lymph nodes and spleen (Poon et al., 2021). These cells are important as they provide rapid protection at the tissues including the site of infection.

In contrast, SARS-CoV-2-specific CD8 memory T cells are detectable in approximately 60% of individuals 12 months post-infection (Cohen et al., 2021; Dan et al., 2021; Guo et al., 2022). Memory CD8 cells mainly consist of Tem and Trm with fewer Tcm, and produce IFN-γ, TNF-α, IL-2 cytokines as well as granzymes (Cheon et al., 2021; Cohen et al., 2021; Poon et al., 2021; Zuo et al., 2021).

1.3 SARS-CoV-2 vaccines and vaccine responses

1.3.1 SARS-CoV-2 vaccines

The emergence of the pandemic was met with a swift response by the scientific community through the production of multiple vaccines using various platform technologies. These platforms include inactivated virus (Covaxin from Bharat Biotech, CoronaVac from Sinovac Biotech and BBIBP-CorV from Sinopharm), mRNA (BNT162b2 from Pfizer/BioNTech and mRNA-1273 from Moderna), protein subunit (Covovax from Novavax), and viral vectors (ChAdOx1-S from AstraZeneca, and Ad26.COV2.S from Johnson & Johnson/Janssen; Nagy & Alhatlani, 2021; Ndwandwe & Wiysonge, 2021). Most of the vaccines target the spike protein because it was shown to induce neutralising antibodies which were associated with protection from infection (Asamoah-Boaheng et al., 2023; Bergwerk et al., 2021; Dhama et al., 2020; Earle et al., 2021). The vaccines were either given emergency use authorisation (EUA) or approved during the pandemic in 2021 because they were at least 50% effective at protecting against severe COVID-19 (World Health Organization, 2021). Notably, vaccine distribution in the first year is estimated to have prevented 19.8 million COVID-19-related deaths (Watson et al., 2022).

To help combat the pandemic, over 28 million vaccine doses were administered in South Africa in 2021 (Cowling, 2023). While various vaccines were in use, this thesis will only focus on the Ad26.COV2.S (Johnson & Johnson/Janssen) vaccine which has been approved for use in South Africa, and the mRNA-1273 (Moderna/Spikevax) vaccine which was tested in clinical trials but was approved elsewhere (Bekker et al., 2022; Mathieu et al., 2023). The Ad26.COV2.S vaccine utilises a non-replicating type 26 adenovirus (Ad26) vector expressing the full-length spike protein of the SARS-CoV-2 Wuhan-Hu-1 strain (Bos et al., 2020). The spike features a S2 hinge loop with two successive proline substitution mutations (S2P) and a trimerisation domain conformation (Bos et al., 2020; Mercado et al., 2020). This conformation induces greater immune responses, and it is easier to construct (Hsieh et al., 2020; Mercado et al., 2020; Stephenson et al., 2021). Administered intramuscularly as a single-dose regimen, the vaccine demonstrated complete protection against infection in the lower respiratory tract of hamsters and rhesus macaques during preclinical trials (Mercado et al., 2020; Tostanoski et al., 2021). However, it provided only partial protection in the upper respiratory tract of rhesus macaques (Mercado et al., 2020). During phase 3

clinical trials, the vaccine exhibited an 85% effectiveness against severe to critical COVID-19 in adults (Sadoff et al., 2021b).

Unlike the Ad26.COV2.S vaccine, the mRNA-1273 uses a lipid nonparticipial to carry the viral spike mRNA, which is then translated by host cells (Baden et al., 2020). The spike protein is a prefusion stabilised S2P version of the Wuhan-Hu-1 strain. The mRNA-1273 is administered as a two-dose vaccine regimen (Jackson et al., 2020). The second dose is administered at least 28 days after the initial one. Similar to the Ad26.COV2.S, the mRNA-1273 vaccine conferred partial protection against SARS-CoV-2 in the upper and complete protection in the lower respiratory tract of rhesus macaques during the preclinical trials (Corbett et al., 2020). In clinical trials, the vaccine exhibited a 94% effectiveness against symptomatic infection and a 100% effectiveness against critical COVID-19 (Baden et al., 2021), before the emergence of variant viruses.

1.3.2 Neutralising responses post-vaccination

Several vaccines were given EUA based on their safety and ability to elicit robust neutralising and non-neutralising antibodies which were associated with protection against the original SARS-CoV-2 virus that emerged. This is also true for the Ad26.COV2.S and mRNA-1273 vaccines, as they both shown to induce high levels of neutralising and binding antibodies in animal models that were detectable 14 days post-vaccination (Bos et al., 2020; Laczkó et al., 2020; Mercado et al., 2020). In clinical trials, a single dose of Ad26.COV2.S vaccine or two doses of the mRNA-1273 vaccine elicited antibody responses that were detectable two weeks after vaccination without systemic adverse effects in adults (Sadoff et al., 2021a, 2022b; Widge et al., 2020). Notably, the Ad26.COV2.S vaccine induced lower antibody responses compared to mRNA-1273 vaccine, however the antibody responses were shown to be similar 6-8 months post-vaccination (Collier et al., 2021).

1.3.3 T cell responses post-vaccination

In addition to eliciting antibody responses, both the Ad26.COV2.S and mRNA-1273 vaccines have demonstrated the ability to stimulate spike-specific CD4 and CD8 T cells. These T cells exhibit polyfunctionality, producing antiviral cytokines such as IFN-

γ, IL-2, and/or TNF-α, similar to the response seen during infection (Liu et al., 2022; Stephenson et al., 2021). Detectable T cell responses emerge within two weeks post-vaccination, with CD4 cells generally outnumbering CD8 cells (Jackson et al., 2020; Sadoff et al., 2021a; Naranbhai et al., 2022).

Notably, mRNA-1273 induced higher CD4 responses compared to the Ad26.COV2.S vaccine (Hwang et al., 2022; Vespa et al., 2022; Zhang et al., 2022b). However, there are conflicting reports regarding the frequency of CD8 responses induced by the two vaccines, with some studies reporting similar responses and others suggesting higher CD8 responses following Ad26.COV2.S vaccination (Atmar et al., 2022; Collier et al., 2021; Tarke et al., 2022; Zhang et al., 2022b).

Both vaccines exhibited the capacity to generate memory CD4 cells, detectable for up to eight months in over 85% of recipients (Barouch et al., 2021; Goel et al., 2021; Zhang et al., 2022b). Additionally, memory CD8 cells remained detectable in at least 60% of individuals 6-8 months post-vaccination (Barouch et al., 2021; Goel et al., 2021; Zhang et al., 2022b).

1.3.4 Boosted and hybrid immunity

In response to declining vaccine efficacy over time, recommendations were proposed for administering booster vaccinations to individuals who had previously received one or two vaccine doses (Alter et al., 2021; Collie et al., 2022; Khoury et al., 2021; Sablerolles et al., 2022; Sadoff et al., 2022a; 2022b; Naranbhai et al., 2022b). The shortage of vaccines in certain countries and the limitation of giving multiple doses of viral vectors prompted the exploration of heterologous prime-boost strategies, involving the administration of different primary and booster vaccines (Sapkota et al., 2022). This approach has proven effective in inducing antibody or T cell responses at levels equal to or higher than those observed after infection or some homologous vaccine strategies (Atmar et al., 2022; Sapkota et al., 2022, Shaw et al., 2021).

SARS-CoV-2 immune responses can also be induced by a combination of infection and vaccination, known as hybrid immunity (Crotty, 2021). This type of immunity has become especially important in the context of COVID-19 because so many people have been exposed to SARS-CoV-2 proteins from infection and have also been vaccinated. Interestingly, individuals who were previously infected and then received

a vaccine often have more robust antibody, CD4 and CD8 T cell responses and potentially greater protection against severe COVID-19 and reinfection (Crotty, 2021; Gazit et al., 2022; Keeton et al., 2021; Mantus et al., 2022; Naranbhai et al., 2022a; Reynolds et al., 2021; Urbanowicz et al., 2022; Suryawanshi & Ott, 2022). In addition, individuals with hybrid immunity have a greater magnitude of memory T cell responses compared to those who were only vaccinated or infected (Collier et al., 2022; Lim et al., 2022; Mantus et al., 2022; Naranbhai et al., 2022; Vespa et al., 2022).

1.4 Adaptive immunity and variants

1.4.1 Antibody responses against variants

RNA viruses exhibit higher mutation rates compared to DNA viruses (Drake & Holland, 1999; Duffy, 2018; Smith et al., 2013). These mutations can arise randomly or under selective pressure from the host immune system as the virus spreads and replicates within a population. SARS-CoV-2 is no exception, as evidenced by the emergence of several variants dubbed variants of concern (VOC; Janik et al., 2021; Parums, 2021). The VOC are characterised by their high transmissibility, affinity for the ACE-2 and virulence compared to the wild-type strain. Since December 2020, five VOC have been identified and are named in order of appearance: Alpha (B.1.1.7 lineage; GR/501Y.V1), Beta (B.1.351 lineage; GH501Y.V20), Gamma (B.1.1.28 lineage; P1), Delta (B.1.617.2 lineage), and Omicron (B.1.1.529 lineage) variant (Choi & Smith, 2021; Jung et al., 2022; Winger & Caspari, 2021). Because each VOC is more transmissible than the previous one, they were able to rapidly supplant each other and circulate globally (Winger & Caspari, 2021; Jung et al., 2022).

The VOC have mutations across the genome and importantly in the spike protein, the main target of infection and vaccine-induced responses (Salehi-Vaziri et al., 2022; Liu et al., 2022; Taha et al., 2023). While the first four VOC had between nine to 12 amino acid mutations in spike, Omicron (BA.1, the first of the lineage to emerge) had 37 mutations, 15 of which were found in the RBD (Chakraborty et al., 2022; Kannan et al., 2021; Thakur & Ratho, 2022; **Figure 1.3**). This is important since the RBD is the primary target for neutralising antibodies (Gupta & Jaiswal, 2022; Piccoli et al., 2021). Consequently, these mutations have resulted in a dramatic reduction in neutralising

efficacy against the Omicron variant and subsequent Omicron lineages that now circulate, with decreases of over 45- and 85-fold observed in previously infected and vaccinated individuals, respectively, compared to the ancestral strain (Bekliz et al., 2022).

This decrease in neutralising ability against Omicron lineages is far greater than what was observed for earlier variants (Bekliz et al., 2022). The responses are also not durable, with many individuals having low or completely losing the ability to neutralise Omicron 6 to 12 months later (Garcia-Beltran et al., 2022; Planas et al., 2022; Zhang et al., 2022a). Additionally, this variant has shown the ability to evade neutralisation by all approved anti-SARS-CoV-2 monoclonal antibodies (Planas et al., 2022). This has resulted in re-infections and the attempt to prevent new infections with COVID-19 vaccines based on new Omicron lineages.

1.4.2 Cross-reactivity of the T cell responses to variants

Unlike antibodies, which are affected by mutations in VOC, T cells have been shown to maintain cross-reactivity against all VOC (Binayke et al., 2022; GeurtsvanKessel et al., 2022; Karsten et al., 2022; Keeton et al., 2021; Li et al., 2022; Mantus et al., 2022; Meyer et al., 2023; Riou et al.; 2022; Röltgen et al., 2022; Wratil et al., 2022). This may be due to broad T cell responses across the viral genome (Tarke et al., 2021, 2022). A larger breadth means that T cells that target more epitopes, which may reduce the ability to escape mutated variants. The breadth of T cells may be different between populations due to differences in HLA alleles, and more studies are needed in diverse populations.

The breadth of T cell responses towards the SARS-CoV-2 proteome spike protein following infection has been characterised in several studies (Ferretti et al., 2020; Gangaev et al., 2020; Grifoni et al., 2020; Habel et al., 2020; Kared et al., 2020; Keller et al., 2020; Lani et al., 2022; Le Bert et al., 2020; Mateus et al., 2020; Meyer et al., 2023 Nelde et al., 2020; Tarke et al., 2020). However, studies on the breadth of SARS-CoV-2 spike-specific T cell responses following vaccination are more limited (Karsten et al., 2022; Khoo et al., 2022; Lim et al., 2022; Sedegah et al., 2022). Moreover, it is still unclear whether repeated exposures to the spike protein through vaccination enhance the breadth of T cells. Documenting the breadth of responses against the SARS-CoV-2 is important for potentially predicting the impact of future variants on T cell responses, due to their importance in contributing to protection against severe

COVID-19 (Binayke et al., 2022; GeurtsvanKessel et al., 2022; Keeton et al., 2021; Li et al., 2022; Mantus et al., 2022; Meyer et al., 2023).

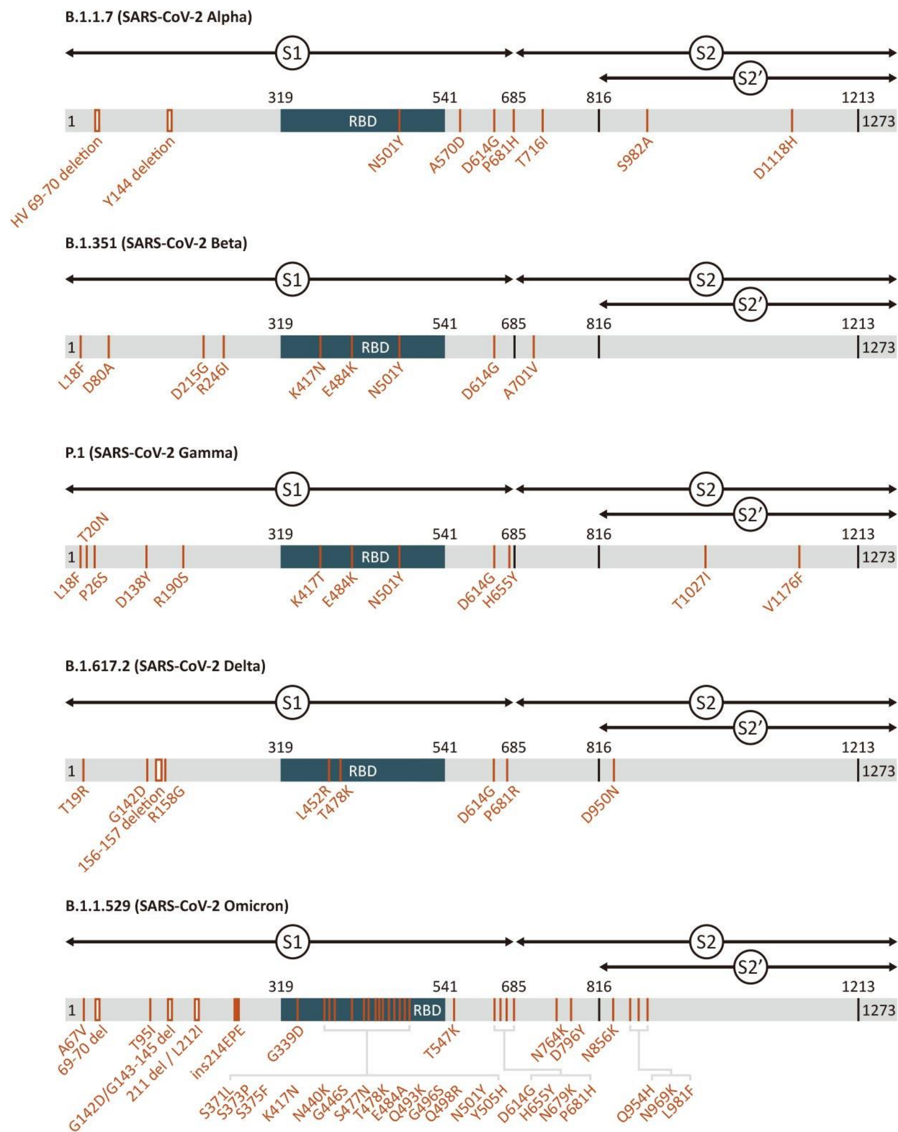


Figure 1.3: Illustration of SARS-CoV-2 VOC spike mutations in the Alpha, Beta, Gamma, Delta and Omicron (BA.1) variants. Adapted from Labclinics, 2024.

1.5 Research Aim:

This study aimed to examine the magnitude and breadth of SARS-CoV-2 spike-specific CD4 and CD8 T cell responses induced by a heterologous prime-boost vaccination strategy consisting of Ad26.COV2.S (Johnson & Johnson/Janssen) and mRNA-1273 (Moderna) vaccines in the context of hybrid immunity.

1.5.1 Objectives

1. To assess the magnitude of the SARS-CoV-2 spike-specific T cells in healthcare workers (HCW) after vaccination. This was performed by stimulating PBMC with a peptide pool covering the full-length viral spike protein and measuring T cell responses by flow cytometry.
2. To determine whether vaccination expands the breadth and specificity of the T cells across the spike by comparing the CD4 and CD8 T cell responses of the HCW before and after mRNA-1273 vaccination. This was performed by stimulating PBMC with seven peptide pools spanning the length of the spike protein and measuring T cell responses by flow cytometry.

1.5.2 Rationale

COVID-19 vaccines induce spike-specific CD4 and CD8 T cell responses. Multiple exposures to spike through infection, vaccination, or both can lead to a higher magnitude of T cell responses compared to either exposure alone. However, thus far, the breadth of CD4 and CD8 T cell responses following COVID-19 vaccination has not been thoroughly examined. We do not know whether two or more exposures to vaccines in individuals with a history of infection expands the breadth of T cell responses. Understanding which regions of the spike protein are targeted will provide information about the preservation of T cell responses for future variants. This is particularly important given that T cell responses likely confer protection against severe COVID-19 disease, despite the continued emergence of SARS-CoV-2 variants of concern.

Chapter 2: Materials and Methods

2.1 Study participants and ethical approval

The study participants were recruited from a cohort of 11,000 healthcare workers (HCW) from various clinical research sites across South Africa. These HCW were enrolled in the Sisonke heterologous mRNA-1273 boost after priming with Ad26.COV2.S (SHERPA) study, which was an open-label, phase 3 longitudinal study investigating the immunogenicity of the mRNA-1273 vaccine in participants who previously received the Ad26.COV2.S vaccine during the Sisonke study. The Sisonke study was a phase 3b clinical trial that evaluated the effectiveness and tolerability of one dose or two doses of Ad26.COV2.S (Johnson & Johnson/Janssen) COVID-19 vaccine among 50,000 South African HCW.
The Ad26.COV2.S vaccine utilises a non-replicating Ad26 vector expressing the full-length spike protein from the SARS-CoV-2 Wuhan-Hu-1 strain (Bos et al., 2020). Another COVID-19 vaccine, mRNA-1273 (Moderna/Spikevax), manufactured by Moderna, Inc., employs lipid nanoparticles to deliver spike mRNA from the same SARS-CoV-2 Wuhan-Hu-1 strain (Jackson et al., 2020).

Recruitment occurred through community engagement activities at various clinical research sites nationwide, national communication channels and the National Department of Health's electronic vaccination data system. During recruitment, a research nurse provided comprehensive information to the HCW about the study objectives, the procedures involved, and the potential risks and benefits. To be eligible for inclusion in the study, participants had to be at least 18 years old, and have previously received one or two doses of the Ad26.COV2.S vaccine. The second dose of the Ad26.COV2.S vaccine had to be administered at least three months before receiving the mRNA-1273 vaccine boost administered in the SHERPA study. The participants also had to express willingness to adhere to the study's procedures and vaccine schedule. The exclusion criteria for the study participants were participating in other COVID-19 intervention studies, receipt of other COVID-19 vaccines in addition

to the Ad26.COV2.S, unresolved acute infections, significant acute or chronic medical conditions, a prior history of severe adverse reactions to a vaccine, or a history of severe adverse reactions or allergic responses to any component of the mRNA-1273 vaccine. Additionally, individuals with a history of heparin-induced thrombocytopenia or thrombosis and thrombocytopenia syndrome were excluded. Before enrollment, all the participants provided written informed consent. This research project received ethical approval by the Human Research Ethics Committee (HREC) of the Faculty of Health Sciences at the University of Cape Town. It is a sub-study (HREC Ref no. 291/2020) under the parent protocol HREC Ref no. 548/2023.
The twenty participants included in this substudy were selected based on the availability of peripheral blood mononuclear cells (PBMC) in the sample repository.

2.2 Sample collection and processing

The blood samples were collected using heparin tubes (Lasec) and were processed by Bio Analytical Research Corporation South Africa (BARC SA) service laboratory. The processing occurred within four hours of collection and followed the Ficoll-Paque density gradient centrifugation method. Initially, the ficoll was allowed to reach room temperature, and then 15 mL was added to leucosep tubes (Lasec) with a separation disc. The ficoll was centrifuged at 1000x g for 1 minute. This centrifugation step was to enable the ficoll to move below the separation disc. Thereafter, 30 mL of blood was poured directly onto the separation disc before centrifuging for 15 minutes at 1000x g. This centrifugation step allowed for the division of blood contents into four layers based on their density with the plasma settling on top followed by PBMC (lymphocytes, monocytes and platelets), ficoll-layer and granulocytes and erythrocytes.

The plasma was aspirated and aliquoted into 2 mL screw cap tubes (Whitehead Scientific) and stored at a -80℃ freezer. PBMC were carefully collected and transferred into a 50 mL falcon tube (Whitehead Scientific) and washed with 45 mL of R1 media by centrifuging at 370x g for 10 minutes. Subsequently, cells were dislodged and resuspended in 5 mL of R1 media. A 10 µL aliquot of the cell suspension was mixed with 10 µL of trypan blue and the cells were counted using a TC20 automated cell counter. The wash step was repeated before the cells were then added into cryovials that were kept on ice and 1mL of freezing media which was kept on ice was

added. This media allows for a gradual decrease in the temperature of the cells to prevent their death. The cells were then transferred into CoolCell freezing containers (BioCision) and stored at a -80℃ freezer overnight before transferring into liquid nitrogen (LN_2; -192℃) until shipping. PBMC vials were shipped in dry ice and were stored in LN_2 upon reaching the lab until use.

2.3 Laboratory operations and reagents

All experiments were carried out in a Biosafety Class II cabinet, with appropriate personal protective equipment in use. The cabinets were thoroughly disinfected both before and after each use using 70% ethanol, and all biohazardous materials were disposed of in a designated waste bucket treated with Biocide. Details of the reagents, storage temperatures, manufacturers, and constituents (where applicable) used for PBMC stimulation and staining are summarised below in **Table 2.1.**

Table 2.1: Laboratory reagents and solutions used in this study

Reagent	Storage	Manufacturer
Roswell Park Memorial Institute (RPMI) 1640	4°C	Sigma
Deoxyribonuclease (DNase)	-20°C	Sigma
Dimethyl sulfoxide (DMSO)	4°C	Sigma
Penicillin-Streptomycin (Pen-Strep)	-20°C	Merck
Distilled water (dH_2O)	Room temperature	YMS
1x Phosphate-buffered saline (PBS)	4°C	Thermofisher
Heat-inactivated foetal calf serum (HI-FCS)	-20°C	Separations
Perm/Wash buffer (10x)	4°C	BD Biosciences
CellFIX (10x)	4°C	BD Biosciences
Cytofix/Cytoperm buffer	4°C	BD Biosciences
Brefeldin A (BFA)	-20°C	Sigma
Brilliant stain buffer	4°C	BD Biosciences
Ficoll	4°C	Amersham Biosciences
Trypan blue	Room temperature	Sigma
Sulfuric acid (H_2SO_4)	Room temperature	Thermofisher
Skimmed milk powder	Room temperature	Merck
Tween 20	4°C	Merck

3,3′,5,5′-Tetramethylbenzidine (TMB) substrate	4°C	Thermofisher
Blocking buffer	4°C	1x PBS supplemented with 5% skimmed milk powder and 0.05% Tween 20
Freezing media	4°C	10% DMSO in FCS
R1 Media	4°C	RPMI 1640 supplemented with 1% HI-FCS and 0.5% pen-strep
R10 Media	4°C	RPMI 1640 supplemented with 10% HI-FCS and 0.5% pen-strep
DNase I Solution	-20°C	1:10 dilution in R1 media
1x Perm/Wash	4°C	1:10 dilution in dH_2O
1x CellFIX	4°C	1:10 dilution in 1x PBS
FACS wash solution	4°C	2% FCS in 1x PBS

2.4 SARS-CoV-2 Peptides

2.4.1 Full spike peptide pool

This preparation comprises of 15-mer peptides with a 10-amino acid overlap, designed to span the full length of the SARS-CoV-2 spike protein from the Wuhan-Hu-1 strain including the D614G mutation. The stock was diluted with dH2O and 0.5% DMSO and was used at 1 ug/mL. All peptides were provided by our collaborator Prof. Alessandro Sette, La Jolla Institute for Immunology.

2.4.2 Seven spike peptide pools

The peptides were arranged into seven spike pools spanning the entire spike protein. They were designed based on previously identified functional regions within the S1 and S2 subunits of the spike protein (Huang et al., 2020; **Figure 2.1**).

The S1 subunit is divided into four pools:

- Pool 1, which contains N-terminal domain 1 (NTD1) (peptides 1-26; amino acids 1-140)
- Pool 2, which contains NTD2 (peptides 27-61; amino acids 131-315)
- Pool 3, comprising receptor binding domain 1 (RBD1), including the receptor binding motif (RBM) (peptides 62-101; amino acids 306-515)

- Pool 4, consisting of RBD2 or the C-terminal of S1 (peptides 102-137; amino acids 506-695)

The S2 subunit is divided into three pools:

- Pool 5, contains the fusion peptide (FP) (peptides 138-176; amino acids 686-890)
- Pool 6, containing a heptapeptide repeat sequence (HR) (peptides 881-1085; amino acids 177-215)
- Pool 7, which includes cytoplasmic and transmembrane domains (peptides 1076-1273; amino acids 216-253)

All peptide stocks were diluted with dH2O and 0.5% DMSO and were subsequently used at a final concentration of 1 µg/mL.

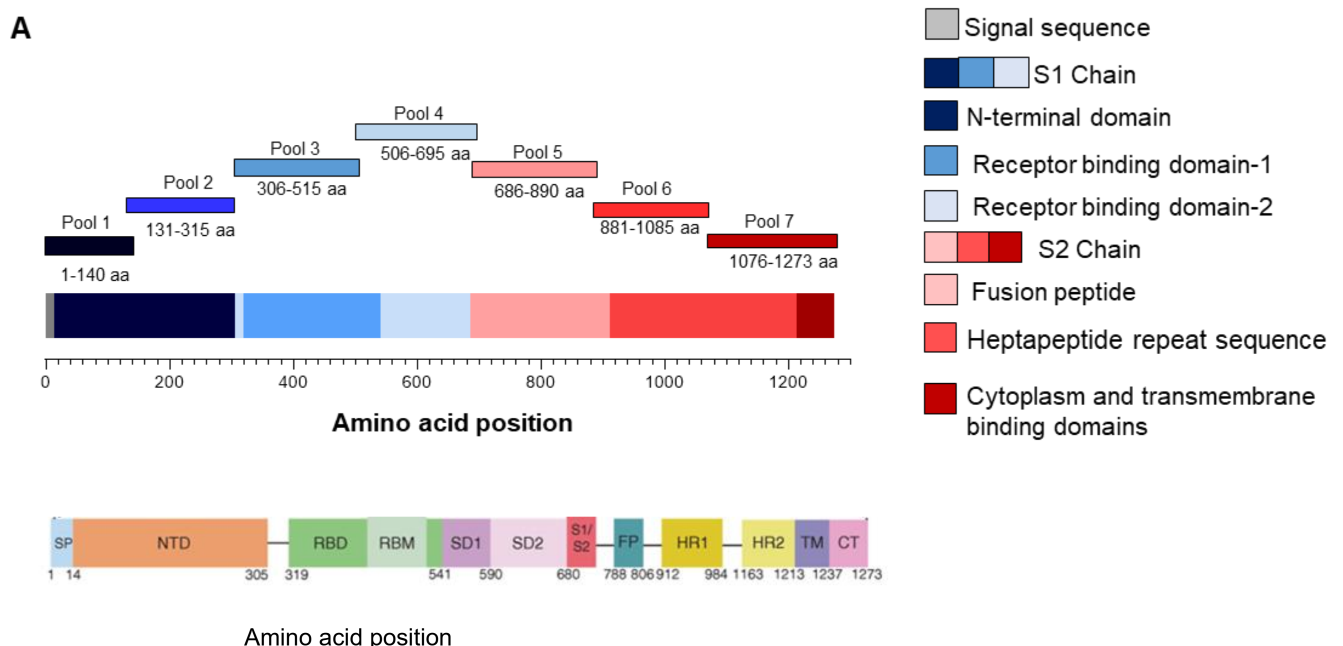


Figure 2.1: SARS-CoV-2 spike protein. (A) Schematic representation of the location of the seven Spike-specific peptide pools containing 15-mer overlapping peptides spanning the entire spike protein. Pools 1-4 represent the S1 region and pools 5-7 represent the S2 region of the spike (B) Functional domains of the spike protein. Adapted from Rajpal et al., (2022).

2.5 PBMC thawing

Cryopreserved peripheral blood mononuclear cells (PBMC) vials were retrieved from the LN2 and kept on dry ice before they were thawed in a 37°C waterbath. The freezing media contains DMSO, which prevents the formation of ice crystals during freezing process, however this can also be harmful to cells at temperatures above 5°C during thawing, therefore the sample was not completely thawed to maintain cell viability. The cells were then poured into 50 mL falcon tubes and 10 mL of pre-warmed R1 media was added dropwise before topping up to a final volume of 50 mL. The cells were then centrifuged at 300x g for 10 minutes at room temperature, and the R1 media was poured out, being careful not to disturb the cell pellet. Subsequently, the cells were resuspended in 500 μL of DNase solution and incubated for 3 minutes. The purpose of using DNase is to prevent cell aggregation caused by DNA released from dead cells. Cells were then washed with 50 mL of R1 at 300x g for 10 minutes, and the supernatant was decanted. The cells were resuspended in 5 mL of R10 media and a 10 uL cell suspension was taken for counting before resting the incubator for 4 hours at 37°C and 5% CO_2.

2.6 Cell stimulation

After resting, PBMC were centrifuged at 300g (Eppendorf centrifuge 5804) for 10 minutes and resuspended in R10 media. They were then dispensed into a 96-well U-bottom plate, with an approximate cell count of 2 million cells per well, in a final volume of 100 μL per well. PBMC were stimulated with a 100 μL stimulation mix, which was prepared using one of the seven spike sub-pools or the complete spike peptide pool (both at 1 μg/mL), along with CD28 and CD49d co-stimulatory antibodies (1 μg/mL each; BD Biosciences), and BFA (10 μg/mL; Sigma-Aldrich), all in R10 media. The anti-CD28 and CD49d antibodies provide co-stimulatory signals to the T cells for cytokine production while BFA prevents cytokine trafficking, enabling intracellular detection (Freer & Rindi, 2013; Nunès et al., 1993; Sciaky et al., 1997). PBMC incubated with brefeldin A and co-stimulatory antibodies alone served as a negative control. PBMC were then incubated overnight for 16 hours at 37°C and in 5% CO2.

2.7 Flow cytometry

Flow cytometry is an immunological technique that uses physical and chemical characteristics of fluorescently labelled single cells in a fluid suspension to identify the phenotype and function of immune cells. In this study, flow cytometric analysis was performed using a Fortessa instrument (BD Biosciences) equipped with blue, red, green-yellow, and violet lasers (**Figure 2.2).** During this process, lasers excite the fluorochromes within and on the outside of the labelled cells as they pass through the sheath fluid in single-file. This excitation results in the emission of light at different wavelengths, which is subsequently detected by photomultiplier tubes (PMTs) and photodiode light detectors after reflection by dichroic mirrors. The captured light signals are then converted into electronic and digital signals.

2.7.1 Monoclonal antibodies

The surface and intracellular antibodies utilised in the study are listed in **Table 2.2**. All the antibodies used in the study were previously titrated to establish the optimal staining titre for detecting each marker. This determination was based on staining index, saturation, and signal-to-noise ratio. Additionally, fluorescence minus one (FMO) controls were used to guide the gating strategy and assess the extent of fluorochrome spillover, consisting of samples stained with all antibodies except the one of interest.

2.7.2 Cell staining and acquisition

After overnight incubation, cells were washed for 10 minutes at 900x g with 1x PBS before staining with 50 µL of amine-reactive near-infrared (NIR) fixable dye (1/10000 dilution) in 1x PBS, Molecular Probes) for 20 minutes in the dark. The NIR fluorescent dye works by binding to the amines on proteins, which are more abundant within dead cells. This allowed for the exclusion of dead cells, which can bind non-specifically to antibodies, leading to the detection of false positive responses.

The cells were then washed twice with FACS wash to a final volume of 200 µL at 2100 rpm and stained with surface antibodies: CD14 APC-Cy7, CD19 APC-Cy7, CD4 BV786, CD8 FITC, CD45RA BV570 and CD27 PECy5. This was done in a final volume of 50 µL in brilliant stain buffer. Staining was carried out for 30 minutes in the dark at room temperature. Next, the cells were washed twice with 200 µL FACS wash at 900x g before being permeabilised for 15 minutes with 100 µL of Cytofix/Cytoperm buffer.

This contains saponin, a chemical that effectively forms pores in the cell membrane, facilitating access of the fluorescently labelled antibodies to the intracellular targets. To maintain membrane permeability, the cells were washed twice with 200 µL Perm/Wash buffer, which also contains saponin, at 900x g before staining intracellularly with antibodies anti-CD3-BV650, IFN-γ BV711, TNF-α PECy7, and IL-2

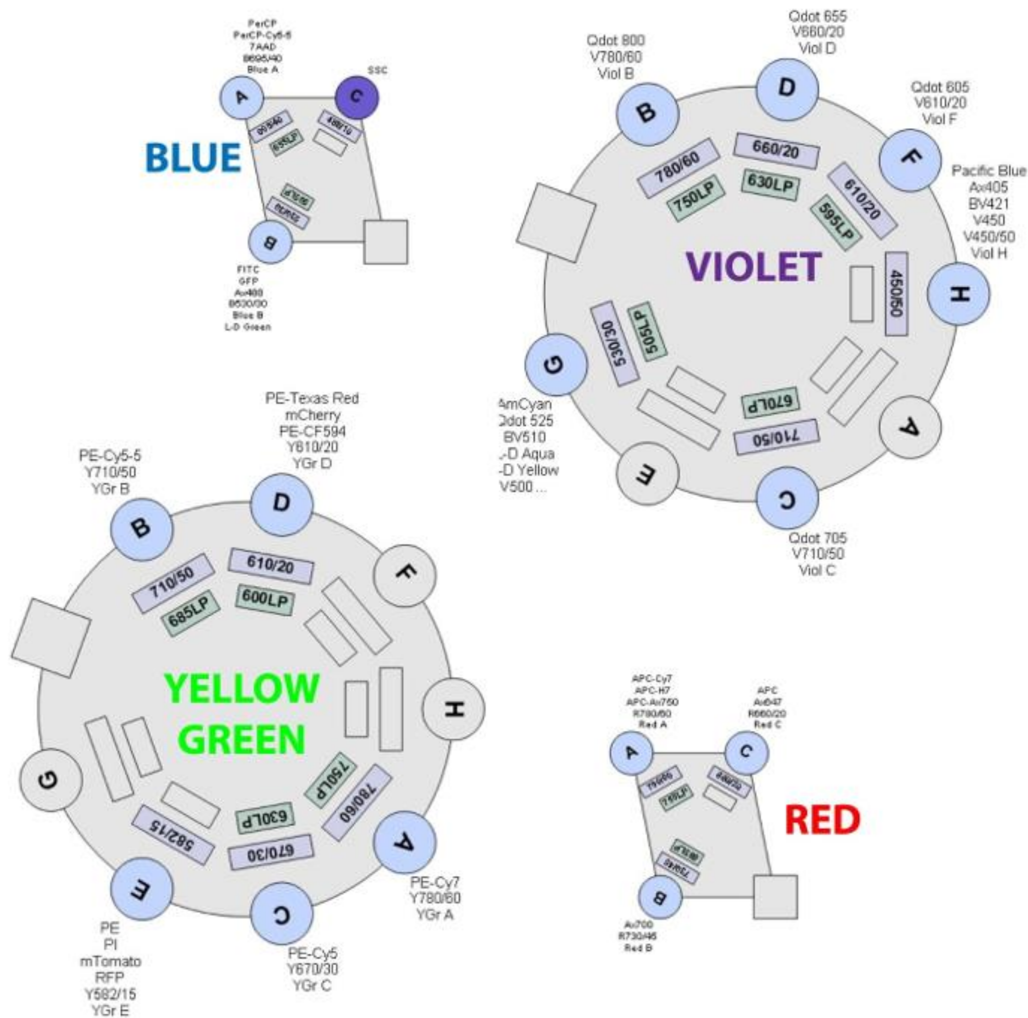


Figure 2.2: Illustration of the BD Fortessa filter configuration utilised in the study. The arrangement of the blue, violet, red and yellow-green lasers with two octagons and two trigons.

PE-CF594. This was done in a final volume of 50 µL in Perm/Wash and incubated for 30 minutes. After the incubation, the cells were washed twice with 200 µL Perm/Wash buffer at 900x g and fixed in 150 µL of 1x CellFIX. The cells were then transferred into cluster tubes (Corning) and stored at 4°C wrapped in foil until acquisition, which was performed on the same day.

Table 2.2: Antibodies used for flow cytometry

Marker	Fluoro-chrome	Clone	Volume (µl) per 50 µl stain	Function	Staining	Manufac-turer	Lot no.
CD14	APC-Cy7	HCD14	1	Exclusion (dump)	Surface	Biolegend	B363389
CD19	APC-Cy7	HIB19	1	Exclusion (dump)	Surface	Biolegend	B361547
CD3	BV650	OKT3	0.75	Defines lineage	Intra-cellular	Biolegend	B366735
CD4	BV786	OKT4	0.5	Defines lineage	Surface	Biolegend	2150833
CD8	FITC	RPA-T8	0.5	Defines lineage	Surface	Biolegend	B336469
CD45RA	BV570	HI100	0.5	Memory Marker	Surface	Biolegend	B348404
CD27	PECy5	1A4CD27	1	Memory Marker	Surface	Beckman Coulter	7621069
IFN-γ	BV711	4S.B3	1.2	Cytokine response	Intra-cellular	Biolegend	B352527
IL-2	PE-CF594	MQ117-H12	0.5	Cytokine response	Intra-cellular	Biolegend	B351592

2.7.3 Compensation

To address spectral overlap, which occurs when emitted light from fluorochromes spills over into adjacent fluorescent channels, potentially leading to false positives or false negatives, a corrective measure was implemented. This involved using individually stained compensation beads (BD Biosciences) specific to each antibody-conjugate used for cell staining in every flow cytometry run. The compensation beads were prepared by adding 1 µL of each antibody to separate tubes containing 80 µL of FACS wash and 20 µL of anti-mouse Ig-κ or anti-rat Ig-κ beads. The tubes were then

centrifuged for 3 minutes at 600x g before vortexing and incubating for 10 minutes in the dark at room temperature. Afterwards, the beads were washed with 2 mL of FACS wash and centrifuged for 8 minutes at 600x g. The supernatant was discarded, and the beads were resuspended in 200 µL of 1x CellFIX, wrapped in tin foil, and stored at 4°C until acquisition on the same day. Finally, the cells and compensation beads were acquired using a BD LSR-II flow cytometer, and the data were analysed using FlowJo version 10 (BD Biosciences).

2.8 Enzyme-linked immunosorbent assay (ELISA)

The cohort's seroprevalence was previously assessed through ELISAs, with data provided by collaborator Prof Penny Moore from the National Institute of Communicable Diseases in Johannesburg, South Africa. These assays are used to detect the presence of antigens or antibodies by using enzyme-conjugated antibodies. In this case, they were used to measure the presence of IgG antibody responses against the SARS-CoV-2 nucleocapsid proteins which are indicative of an infection. The process involved coating 96-well plates (Thermofisher) with nucleocapsid protein (final concentration: 2 µg/mL), followed by overnight incubation at 4 °C (BioTech Africa). After five washes with an automated washer, non-specific binding sites were blocked for an hour using a blocking buffer. Serum samples, diluted to 1:100 in blocking buffer, were then added and incubated for two hours at room temperature. After five washes, plates were incubated with 100 uL of enzyme-conjugated secondary antibody (diluted 1:3000 in blocking buffer) for an hour. Subsequently, plates were washed five times before the addition of TMB chromogenic substrate. The reaction was halted with 1 M H_2SO_4, and the absorbance at 450 nm was measured using a plate reader. Controls included 1A6 and palivizumab monoclonal antibodies.

2.9 Analysis

A common gating strategy was consistently applied to all samples for the identification of cytokine-producing T cells (**Figure 2.3**). First, time gates were applied to monitor and exclude any shifts in fluorescence. Following this, gating procedures were implemented to isolate singlets (doublet exclusion) and lymphocytes based on forward and side scatter parameters. Subsequently, live CD3+ T cells were gated to ensure the exclusion of B cells, dead cells, and monocytes stained with the APC-Cy7 dye. CD4+ and CD8+ markers were then employed to define their respective T cell

populations. CD4+ and CD8+ T cells were further gated based on their memory phenotype, with all naïve (CD27+CD45RA+) T cells being excluded. This allowed for the identification of IL-2 and/or IFN-γ cytokine-producing cells within these populations. The cutoff for cytokine production was set at ≥5 events above the background (unstimulated samples) and ≥1.5x the cytokine responses in the background.

A median of 482,000 CD3+ events (interquartile range (IQR) 380,534-572,500) were acquired, in addition to a median of 304212 (IQR 219,765-378,776) and 144,485 (IQR 101,385-184,056) CD4 and CD8 events, respectively, for each sample. Statistical analyses were performed using Prism version 9.5.2 (GraphPad Software Inc). All comparisons were made using non-parametric tests, namely one-way ANOVA and Dunn's multiple comparison tests for multiple comparisons, Mann-Whitney U-test for unmatched samples, Wilcoxon signed-rank test for paired samples, and Spearman's rank correlation for correlations. A p-value of > 0.05 was considered statistically significant.

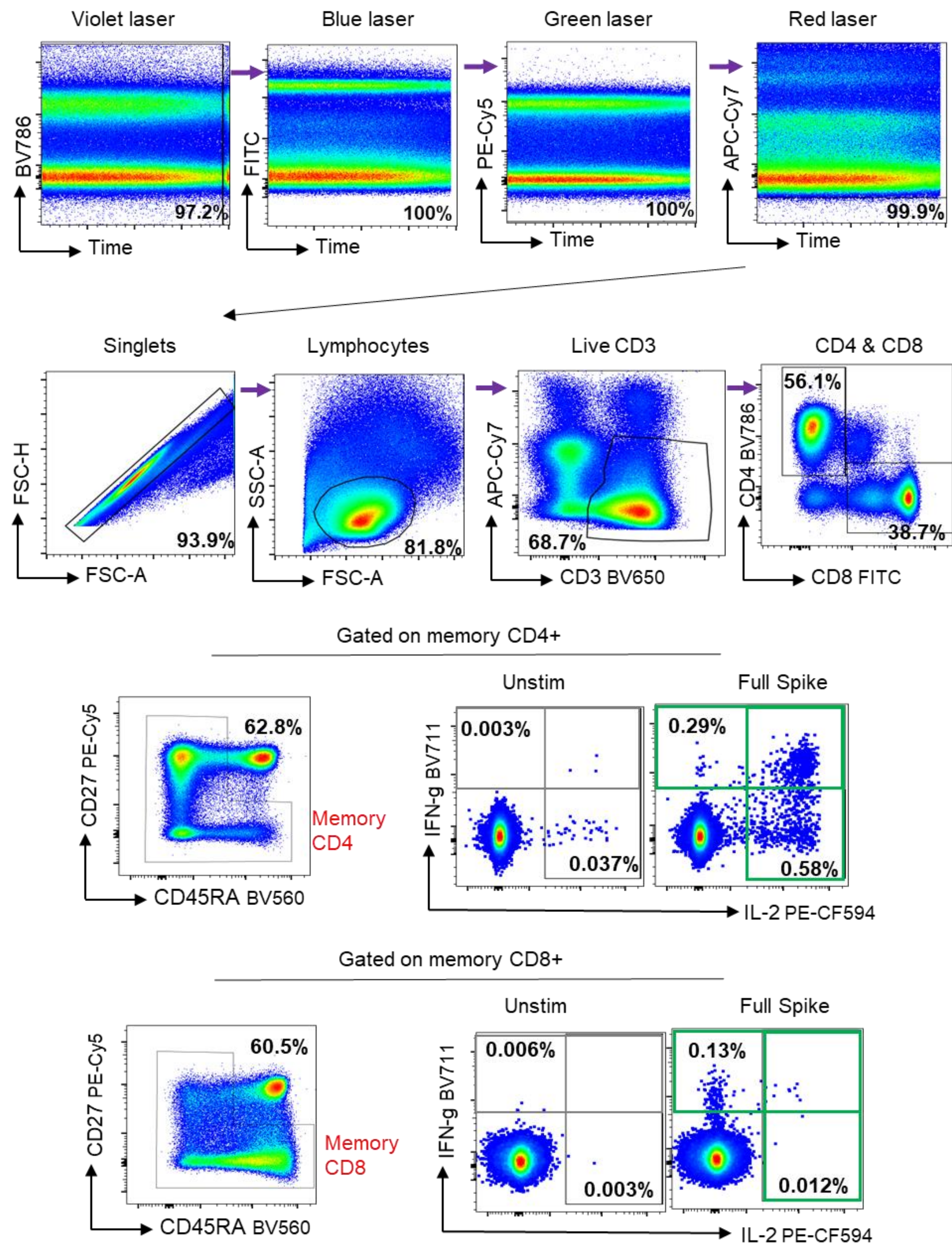


Figure 2.3: Gating strategy used for flow cytometry data analysis. Time gates were used to detect and remove potential shifts in the fluorescence. Singlet cells and lymphocytes were selected for using FSC-H vs. FSC-A and SSC-A vs. FSC-A plots, respectively. Live CD4 and CD8 T cells were isolated after by gating for cells expressing CD3+ marker. Naïve cells expressing CD27+CD45RA+ were excluded to leave out memory CD4 and CD8 cells. The cells were then gated for IFN-γ and IL-2 cytokines.

Chapter 3: Results

3.1 Study cohort

To assess the impact of a heterologous vaccine prime-boost strategy on SARS-CoV-2 cellular immunity, T cell responses were studied in a group of 20 healthcare workers (HCWs) from the Phase 3 SHERPA trial, who had previously been vaccinated with the Ad26.COV2.S vaccine in the Sisonke Phase 3b clinical trial in South Africa (**Figure 3.1**). Of the selected participants, n=8 had received a single prior dose of the Ad26.COV2.S vaccine and n=12 had received two prior doses of the Ad26.COV2.S vaccine, before being boosted with mRNA-1273 vaccine (**Table 3.1**). Ninety percent (18/20) of the participants were female. Both groups showed similar age ranges, with a median age of 40 years (interquartile range, IQR: 34-46 years). The median interval between the first Ad26.COV2.S dose and the administration of the mRNA-1273 vaccine was similar between the two sets of participants, at approximately a year or 345 days (IQR: 335-411) days for those who received a second Ad26.COV2.S vaccine dose, mRNA-1273 occurred a median of 187 days (IQR: 172-202) later. In each group, three participants were living with HIV-1 (PWH). Viral load data were available for only two participants from the two-dose Ad26.COV2.S group, and both of them had a viral load below detectable levels.

To determine how many participants were seropositive for SARS-CoV-2, indicating prior viral infection, plasma IgG specific for the SARS-CoV-2 nucleocapsid protein was measured using an I 3ndirect ELISA assay at baseline (BL) and four weeks (W4) post-vaccination with mRNA-1273 vaccine. These data were provided by our collaborator Prof Penny Moore, National Institute of Communicable Diseases, Johannesburg, South Africa. The majority of participants had a prior SARS-CoV-2 infection; 90% (18/20) were seropositive at both BL and W4 post mRNA-1273 vaccination, and one individual who had previously tested positive for SARS-CoV-2 by PCR a year prior did not exhibit an IgG response. There was a significant decrease in the nucleocapsid IgG response from BL to W4 (median OD490: 2.267 vs. 1.896; $p = 0.0215$). There was one participant who had a 3.2-fold increase in nucleocapsid IgG during the two timepoints, which indicates a probable breakthrough infection (BTI). It is important to note that the occurrence of a BTI between BL and W4 cannot be ruled out in the rest of the participants, as PCR testing was not performed routinely during this period and an

increase in nucleocapsid IgG is not always detectable despite infection (Riou et al., 2023). One participant required hospitalization for COVID-19 prior to the SHERPA study, while the rest were either asymptomatic or reported only mild COVID-19 symptoms.

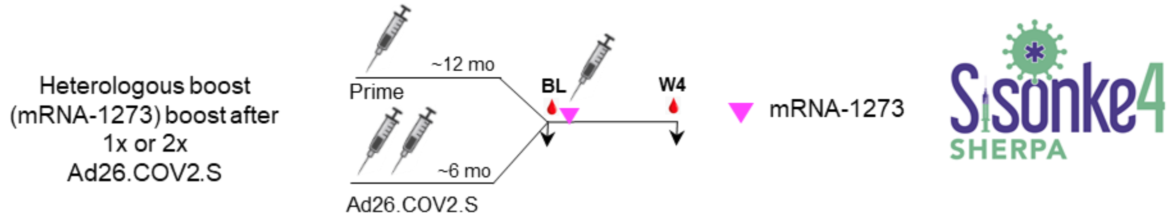
Table 3.1: Clinical characteristics of participants

	mRNA-1273 vaccination		
	All vaccinees	Ad26.COV2.S (1 dose prior)	Ad26.COV2.S (2 doses prior)
N	20	8	12
Gender (% female)	90% (18)	88% (7)	92% (11)
Age, years (median, IQR)	40 [34-46]	37 [28-55]	42 [36-45]
Time since prior vax (days)			
1x Ad26.COV2.S	345 [335-411]	340 [335-355]	355 [340-458]
2x Ad26.COV2.S	N/A	N/A	187 [172-202]
HIV+ (%) [a]	30% (n=6)	38% (n=3)	25% (n=3)
Prior SARS-CoV-2 infection[b]	95% (n=19)	87.5% (n=7)	100% (n=12)

[a]Viral loads were available for only two participants and were below the detectable limit.
[b]As measured at study entry by nucleocapsid ELISA or a prior PCR-confirmed infection

3.2 SARS-CoV-2 spike-specific CD4 and CD8 T cell responses at BL and W4 postvaccination.

Using intracellular cytokine staining and flow cytometry, SARS-CoV-2-specific T cell responses were quantified at baseline (BL) and 4 weeks (W4) after COVID-19 mRNA booster vaccination (**Figure 3.2**). The proportion of responders and the magnitude of spike responses were measured. **Figure 3.2A** illustrates representative flow cytometry plots depicting IFN-γ and IL-2 cytokine responses from CD4 and CD8 T cells to the full spike in three participants (PID#11, 15 and 20). All three participants had detectable



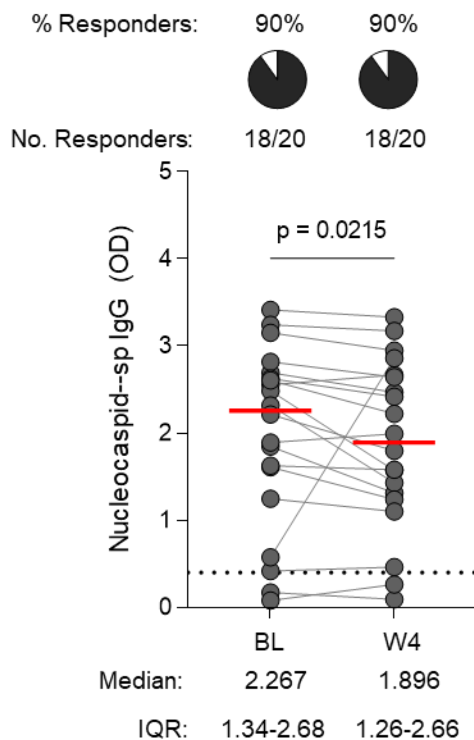


Figure 3.1: Study design and SARS-CoV-2-specific nucleocapsid antibody responses. (A) Twenty healthcare workers (HCWs) received the mRNA-1273 vaccine at baseline (BL) after having previously received one dose (n=8) or two doses (n=12) of the Ad26.COV2.S vaccine. The last Ad26.COV2.S vaccine doses were administered a median of 12 and 6 months prior for the one and two dose groups, respectively. Blood samples were collected at BL and W4 (four weeks) post-vaccination. (B) The frequency of SARS-CoV-2 nucleocapsid (N) IgG (OD490nm) was measured by ELISA at BL and W4. The proportion of seropositive participants is indicated on top of the graph. Horizontal bars indicate the median of responders. Median and interquartile ranges (IQR) of the antibody responses are indicated at the bottom of the graph. The dotted line indicates the cut-off for positivity which was at 0.4.

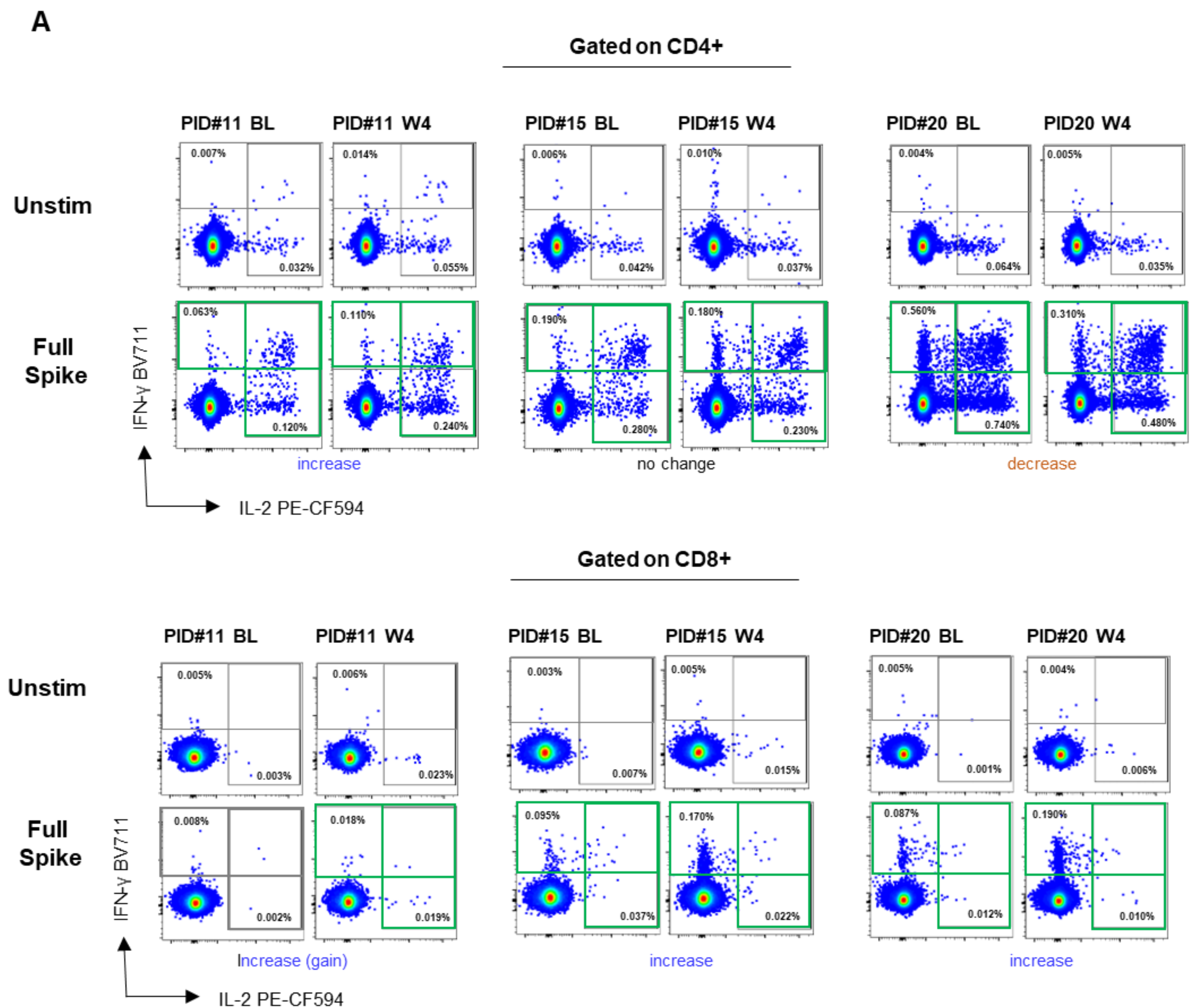
CD4 responses at BL and at W4 following mRNA-1273 vaccination. Participants varied in their response to the booster vaccination at W4. For example, PID#11 showed an

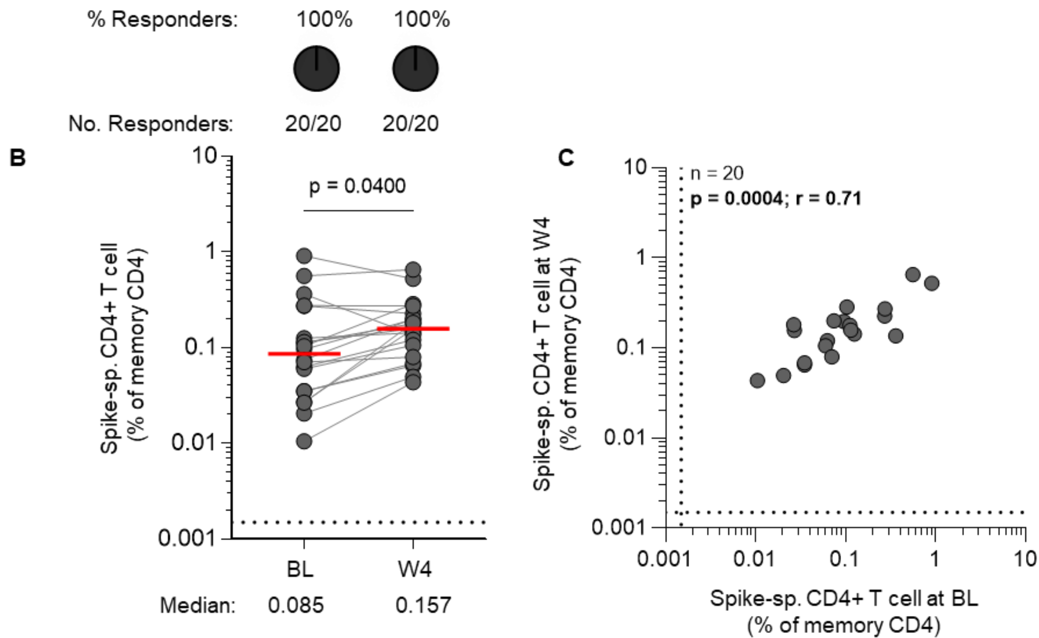
increased spike response, PID#15 experienced no change and PID#20 exhibited a lower CD4 response. For CD8 T cells, of the three participants, PID#11 and PID#20 had existing CD8 spike responses at BL, but all participants had higher (boosted) CD8 responses at W4 after mRNA-1273 vaccination.

Overall, all participants (20/20; 100%) exhibited CD4 responses to the full spike peptide pool at both time points assessed (**Figure 3.2B**). The magnitude of CD4 responses increased significantly from BL to W4, with a median spike-specific CD4 T cell response increasing from 0.085% to 0.157% (p= 0.04). Further analysis demonstrated a positive correlation between the magnitude of the BL and W4 responses (p= 0.0004; r= 0.71; **Figure 3.2C**). Unlike the abundant CD4 T cell responses, fewer participants had CD8 responses to spike, especially at BL (**Figure 3.2D**). Nevertheless, the proportion of participants with detectable spike-specific CD8 responses increased from 55% (11/20) to 80% (16/20) at W4 (**Figure 3.2D**). Seven participants gained a CD8 response, while two lost their CD8 responses between the two timepoints. There was a significant increase in the magnitude of responses from BL to W4 (median: 0% vs. 0.015%; p= 0.025). As for CD4 responses, a positive correlation was observed between the BL and W4 CD8 responses (p< 0.008, r= 0.57; **Figure 3.2E**), although this was weaker than the CD4 response owing to the new responses emerging after booster vaccination. Altogether, these data demonstrate the heterogeneity among participants in how they respond to vaccination.

Next, the magnitudes of CD4 and CD8 responses between the two timepoints were compared (**Figure 3.3**). CD4 responses were significantly higher than CD8 responses at BL (median: 0.085% vs. 0%; p< 0.0001; **Figure 3.3A**). This was the same at W4, where CD4 responses remained significantly higher than CD8 responses (median: 0.157% vs. 0.015%; p< 0.0001; **Figure 3.3B**), even though there was a greater number of CD8 responders, with most CD4 responses being approximately 10-fold greater in magnitude than CD8 responses. Despite this, positive correlations were observed between CD4 and CD8 responses at both BL (p= 0.0009; r= 0.57; **Figure 3.3C**) and W4 (p= 0.003; r= 0.63; **Figure 3.3D**). Of note, the 6 PLWH in the cohort displayed a similar T cell responses when compared to the HIV-negative people.

To determine whether the number of prior vaccinations influenced spike T cell response magnitudes, the number of responders and the frequency of spike-specific T cells following one or two doses of Ad26.COV2.S vaccine were compared (**Figure 3.4**). As described, all participants in both the one-dose group (8/8; 100%) and the two-dose group (12/12; 100%) exhibited CD4 responses at BL. Participants who had previously received two Ad26.COV2.S vaccines had a similar CD4 responses at BL as those who received a single dose (median: 0.104% and 0.048%; **Figure 3.4A**). This was also true after administering the mRNA-1273 vaccine. The single-dose group exhibited a 3-fold increase in CD4 response compared to BL (median: 0.144%), while the two-dose group showed a 1.5-fold increase (median: 0.157%).





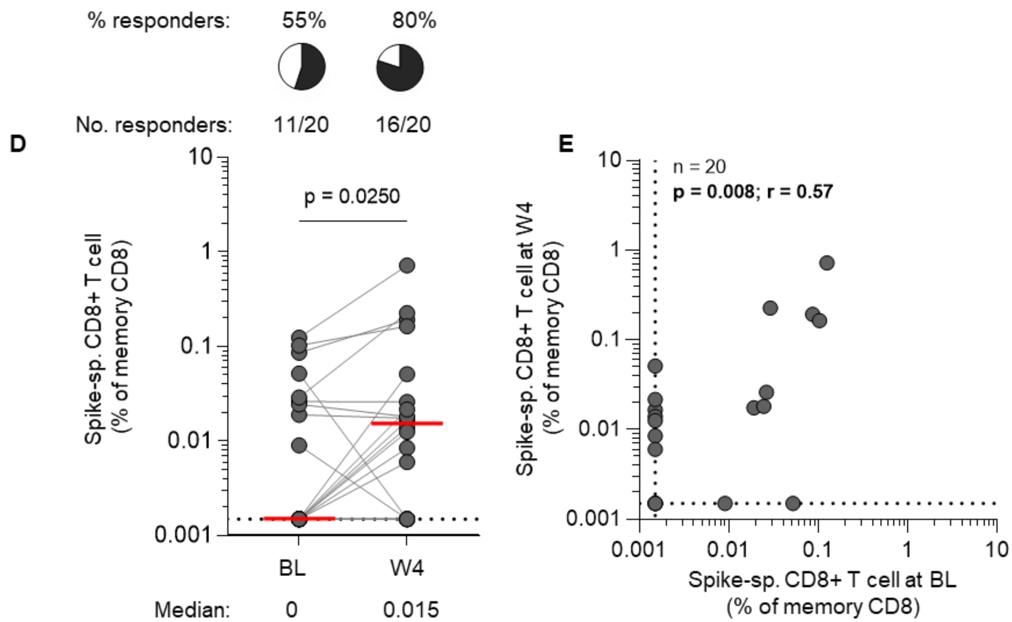


Figure 3.2: SARS-CoV-2-specific spike T cell responses. (A) Representative flow plots showing CD4 and CD8 cytokine (IL-2 and IFN-γ) responses following stimulation with the SARS-CoV-2 full spike peptide pool in three participants at the BL and W4 timepoints. (B and D) Frequency of spike-specific CD4 (B) and CD8 T cells (D) in paired samples (n=20). Proportion and number of responders are indicated on top of the graphs. Medians are indicated at the bottom of the graphs. Statistical comparisons were performed using the Wilcoxon matched-pairs signed rank test. (C and E)

Correlation between the frequency of SARS-CoV-2-specific CD4 (C) and CD8 T cells (E) at BL and W4. Analysis were performed using a two-tailed non-parametric Spearman rank test. p values <0.05 were considered statistically significant.

Regarding CD8 responses, the one-dose group had fewer BL CD8 responders than the two-dose group (38% vs. 50%; **Figure 3.4B**). The median magnitude of responses between those who received one dose and two doses was 0% and 0.01%, which was not significantly different. At W4, CD8 responders increased to 67% (8/12) for the one-dose group and 75% (6/8) for the two-dose group. Furthermore, there was an increase in the magnitude of CD8 responses in the one-dose group, reaching levels similar to those observed in the two-dose group (median: 0.013 and 0.017%).

Overall, these data suggest few differences in the response to booster vaccination between recipients of one or two prior doses of Ad26.COV2.S vaccine, in the context of hybrid immunity.

3.3 Validation of seven pool approach

An important analysis to perform was to ascertain whether T cell responses to the seven smaller spike peptide pools were representative of the entire spike peptide pool, to evaluate whether the experimental approach was valid. The cumulative responses from the seven pools were compared with those elicited by the full spike pool at both the BL timepoint and W4 after vaccination. **Figure 3.5A** shows representative flow cytometry plots of IFN-γ and IL-2 cytokine responses from CD4 and CD8 cells to the full spike and the seven spike pools in two selected participants (PID#15 and PID#19) at BL and after boosting. The administration of mRNA-1273 vaccine resulted in different T cell responses across the seven spike pools at both timepoints. Initially, PID#15 exhibited CD4 responses towards pool 1 to pool 6. At W4, the number of targeted pools decreased to five, with a loss of detectable responses against pools 1 and 4 and a newly detectable response to pool 7. There were no changes in the responses for pools 2 and 3, while responses against pools 5 and 6 increased. Notably, the majority of the CD4 response at BL and W4 was directed at pool 2.

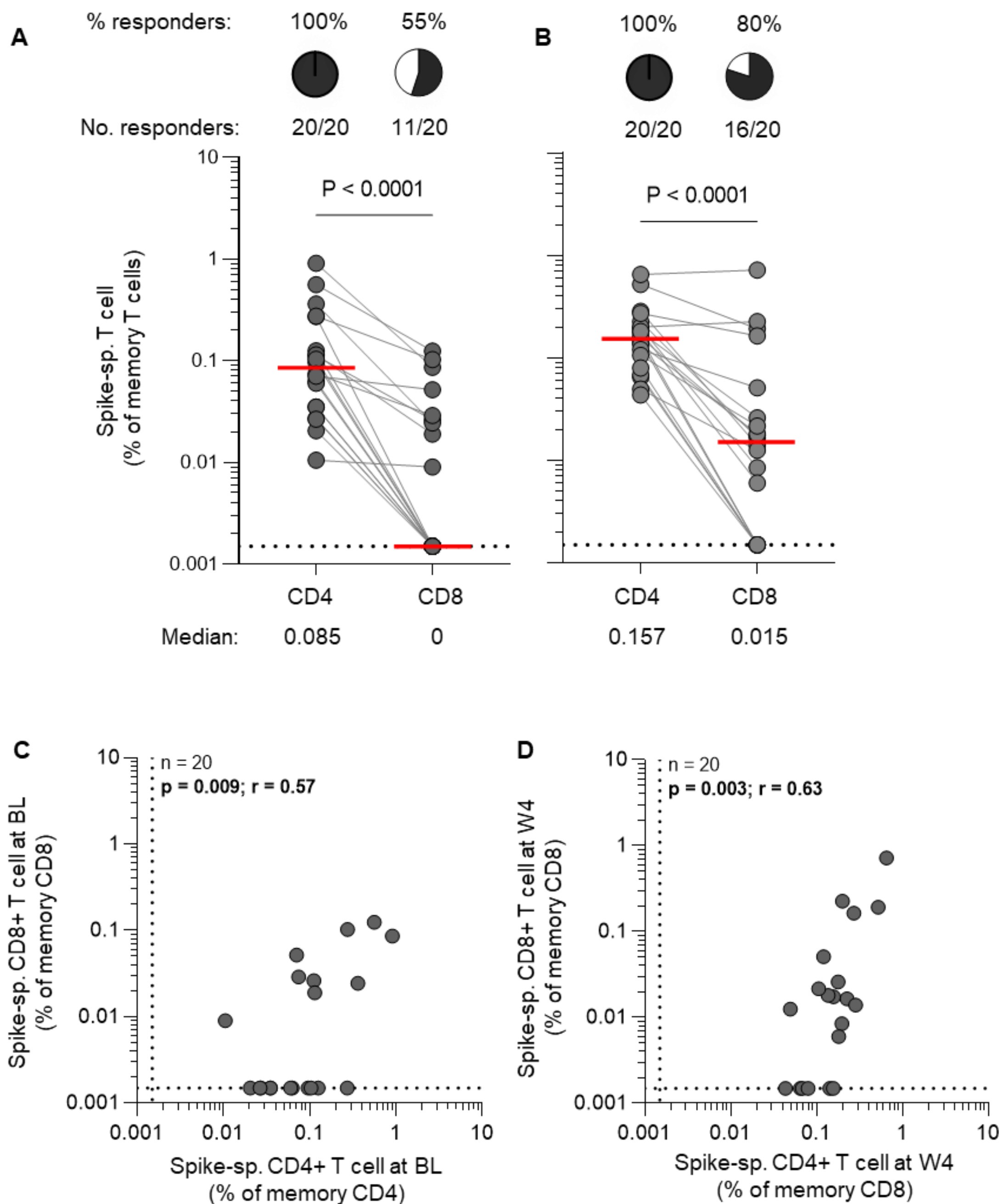


Figure 3.3: Comparison of SARS-CoV-2 spike-specific CD4 and CD8 T cell responses. (A) The magnitude of SARS-CoV-2 spike CD4 and CD8 T cell responses at BL. (B) The magnitude of SARS-CoV-2 spike CD4 and CD8 T cell responses at W4. (C) Correlation between the magnitude of SARS-CoV-2-specific CD4 and CD8 and T cells at BL. (D) Correlation between the magnitude of SARS-CoV-2-specific CD4 and CD8 and T cells at W4. Statistical comparisons for (A) and (B) were performed using the Wilcoxon test, and analysis for (C) and (D) were performed using a two-tailed non-parametric Spearman rank test. p values <0.05 were considered statistically significant.

As an example of CD8 full spike and seven pool responses, PID#19 is shown (**Figure 3.5A** bottom panel). Initially, participant PID#19 had CD8 responses against full spike and pool 1 only. This decrease involved the loss of responses in Pool 3 and an expansion of responses towards Pool 7. At W4 after mRNA boosting, responses against full spike and pool 1 increased, and de novo responses were detected to pools 2, 3, 4 5 and 7. Interestingly, most CD8 responses were directed towards pool 1 before and after vaccination, with the remainder of the responses at a low magnitude, apart from pool 7 with a higher magnitude than others.

All participants had detectable CD4 responses against the full spike pool and at least one of the seven peptide pools (**Figure 3.5B**). Although CD4 responses against the full spike pool and the sum of the individual peptide pool responses were quite similar in magnitude (median % memory CD4 T cells: 0.118 vs. 0.09), overall the sum of the smaller pools was significantly lower than full spike (p= 0.0002**; Figure 3.5B**). A minority (5/40; 12.5%) of the summed-up seven spike pools was higher in magnitude than the full spike peptide pool response. Importantly, even though the summed CD4 response to the seven pools was lower in magnitude than the full spike pool response, the two showed a strong positive correlation (p< 0.0001; r= 0.78; **Figure 3.5C**).

For CD8 responses, 23/40 (58%) and 21/40 (53%) participants had detectable CD8 responses against the full spike and at least one of the seven pools, respectively (**Figure 3.5D**). The overall CD8 responses against the full spike pool and the combined seven pools were similar (median % of memory: 0.011 vs. 0.008). However, 8/40 (20%) of participants had discordant CD8 responses to the full spike pools or spike pools, in that either participants exclusively targeted the full spike pool and not any of the pools (5/40), or responses were detectable to at least one pool but not to full spike. The latter group's responses were limited to at most two peptide pools. Despite these instances of discordance, a positive correlation was still observed between the full and summed spike responses (p< 0.0001; r= 0.74; **Figure 3.5D**).

In summary, CD4 and CD8 responses to the seven peptide pools generally reflected the full spike pool, indicating that the experimental approach was sound.

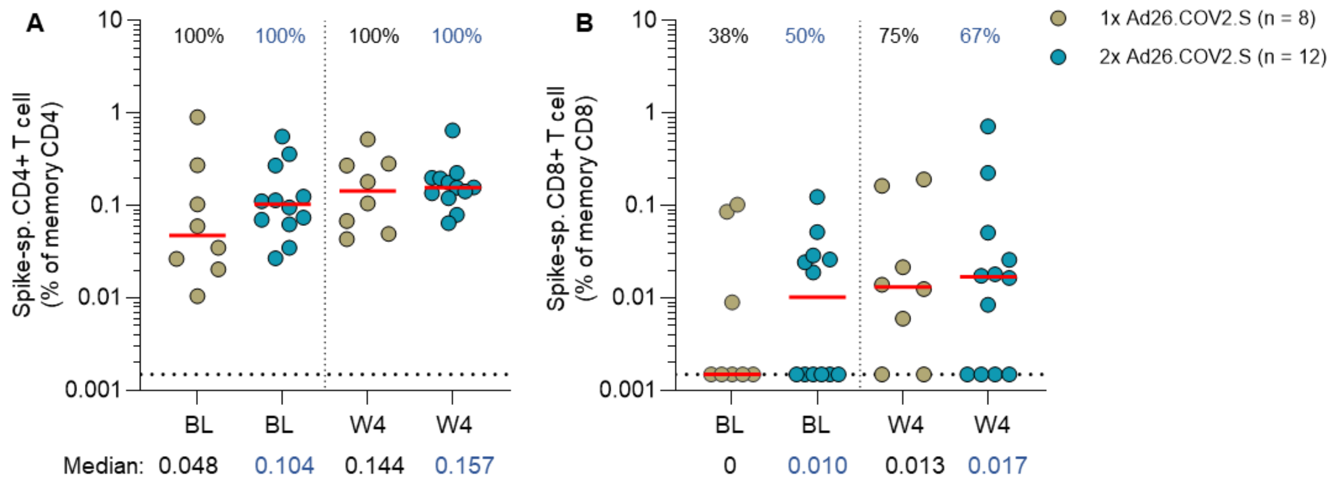


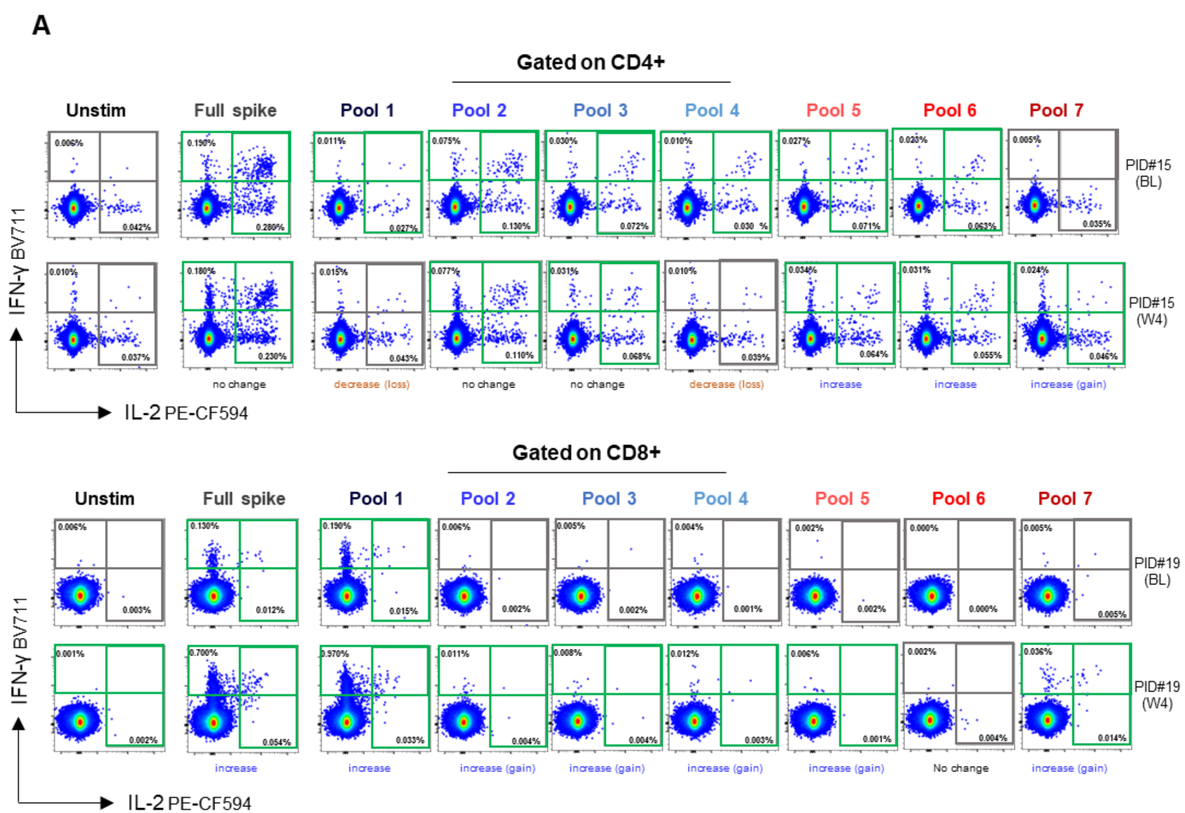
Figure 3.4: Frequency of SARS-CoV-2 spike T cell responses following one or two Ad26.COV2.S vaccine doses. (A and B) Comparison of the frequency of SARS-CoV-2 spike CD4 (A) and CD8 T cell (B) responses at BL and W4 following one (n=8; beige) or two (blue; n=12) Ad26.COV2.S vaccine doses. The proportion of responders is indicated at top of each graph. Horizontal lines indicate median values of responders. Medians are indicated at the bottom of the graphs. No statistically significant differences were observed after using the Kruskal-Wallis test with Dunn's multiple comparison test.

3.4 Breadth and specificity of the response to spike

To gain a comprehensive understanding of the breadth of CD4 and CD8 responses, the number of spike pools targeted at BL and W4 was investigated (**Figure 3.6**). Initially, 16/20 participants (80%) exhibited CD4 responses mounted against four to seven spike pools (**Figure 3.6A**). The remaining 4/20 (20%) participants had responses to two or three spike pools. By W4, all participants had multi-specific CD4 responses targeting at least four of the seven spike pools. In contrast,12/20 (60%) participants had no CD8 responses at BL, and 5/20 (25%) had responses to one or two pools. Following mRNA-1273 booster vaccination, almost half of the participants (9/20; 45%) targeted between one to three pools, and 7/20 (35%) had no detectable CD8 responses since they might have been low frequency below the limit of detection. Interestingly, only three (15%) and four (20%) participants had multi-specific CD8 responses targeting between four to six pools at BL and W4, respectively.

Next, I compared more quantitatively whether there was a difference in the number of CD4 and CD8 responses to the pools (**Figure 3.6B**). The participants exhibited CD4 responses targeted towards a median of five pools (range 2-7). At W4, the number of pools targeted modestly increased to 5.5 (range 4-7). On the other hand, only 8/20 (40%) of the participants had CD8 against the pools at BL (median 0; range 0-6). By W4, most participants (13/20; 65%) had CD8 responses against at least one pool (range 0-6). No significant differences were also observed between the number of targeted pools in the one and two-dose groups (**Supplementary Fig S3.1**).

In summary, most participants mounted broad CD4 responses and more narrowly-directed CD8 responses against spike which did not significantly differ following the mRNA-1273 vaccine boost.



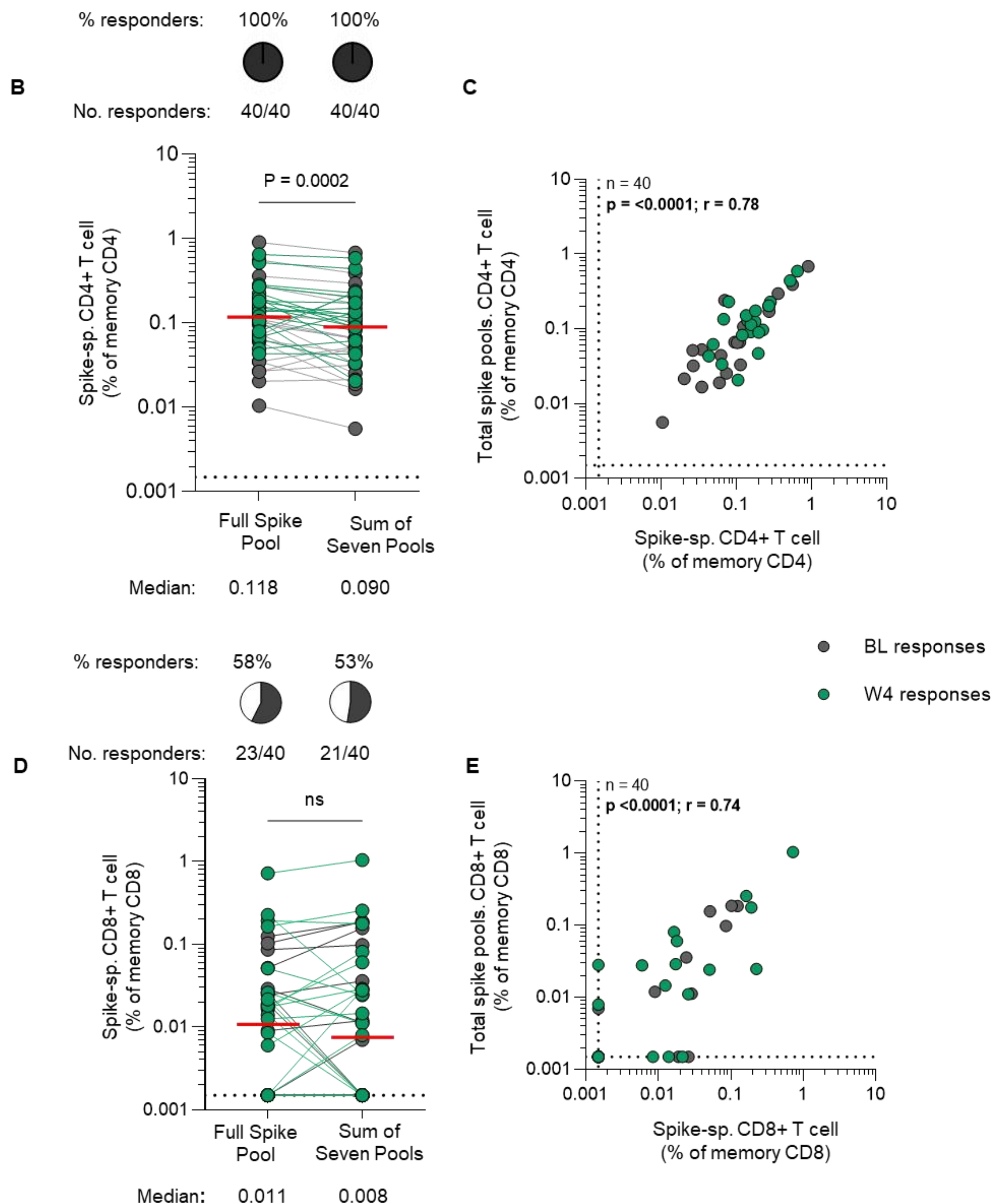


Figure 3.5: T cell responses to the full spike and summed seven peptide pools. (A) Examples of flow plots showing CD4 and CD8 cytokine (IL-2 and IFN-γ) responses following stimulation with the SARS-CoV-2 full spike and seven spike pools in two participants at the BL and W4 timepoints. (B and D) The magnitude of SARS-CoV-2 spike CD4 (B) and CD8 (D) T cell responses to the full spike and cumulative magnitude of the seven peptide pools in paired samples (n=40). Proportion and number of responders are indicated on top of the graphs. Medians are indicated at the bottom of the graphs. Statistical comparisons were performed using the Wilcoxon matched-pairs signed rank test. (C and E) Correlation between the magnitude of SARS-CoV-2-specific CD4 (C) and CD8 T cells (E) to the full spike and summed seven peptide

pools. Statistical analyses were performed using a two-tailed non-parametric Spearman rank test. P values <0.05 were considered statistically significant.

The seven peptide pools were designed to cover distinct regions of spike which differ in their sequence variability (Huang et al., 2020). To understand the specificity of T cell targeting in spike, **Figure 3.7A** provides a schematic representation of these pools, with pools 1 to 4 constituting the spike S1 subunit, and pools 5 to 7 constituting the spike S2 subunit. The S1 subunit comprises NTD1 (peptides 1-26, amino acids 1-140) in pool 1, NTD2 (peptides 27-61, amino acids 131-315) in pool 2, RBD1 (peptides 62-101, amino acids 306-515) in pool 3, and RBD2 (peptides 102-137, amino acids 506-695) in pool 4. The S2 subunit is represented by pool 5 containing FP (peptides 138-176, amino acids 686-890), pool 6 includes HR (peptides 881-1085, amino acids 177-215), and pool 7 contains CT (peptides 1076-1273, amino acids 216-253).

All functional domains of the spike protein were targeted by CD4 responses at both timepoints (**Figure 3.7B**). The majority of the participants (18/20; 90%) had CD4 responses targeted against the FP functional domain in S2, followed by NTD1, NTD2, RBD1 (all S1) and HR (S2), each recognized by between 15 (75%) to 16 (80%) participants. Post-vaccination, there was an increase in participants targeting some spike domains, with NTD2 (S1) and FP (S2) being frequently targeted (both 19/20; 95%), followed by HR (S2) region (18/20; 90%). Notably, RBD2 and CT were the least targeted domains at both timepoints, although they were still targeted by 13/20 (65%) participants.

While the number of CD8 responders for each pool was at least three times lower than that of CD4 responders, CD8 responses were also directed across the entire spike (**Figure 3.7C**). NTD1, RBD2, and FP emerged as the most frequently targeted domains, each by 5/20 (25%) participants. RBD2 had the second-highest number of responders, observed in 4/20 participants (20%). In contrast, NTD1, HR, and CT were the least targeted, with only 2/20 (10%) participants mounting CD8 responses to each. The specificity of CD8 responders increased at W4, with NTD1, NTD2, and RBD1

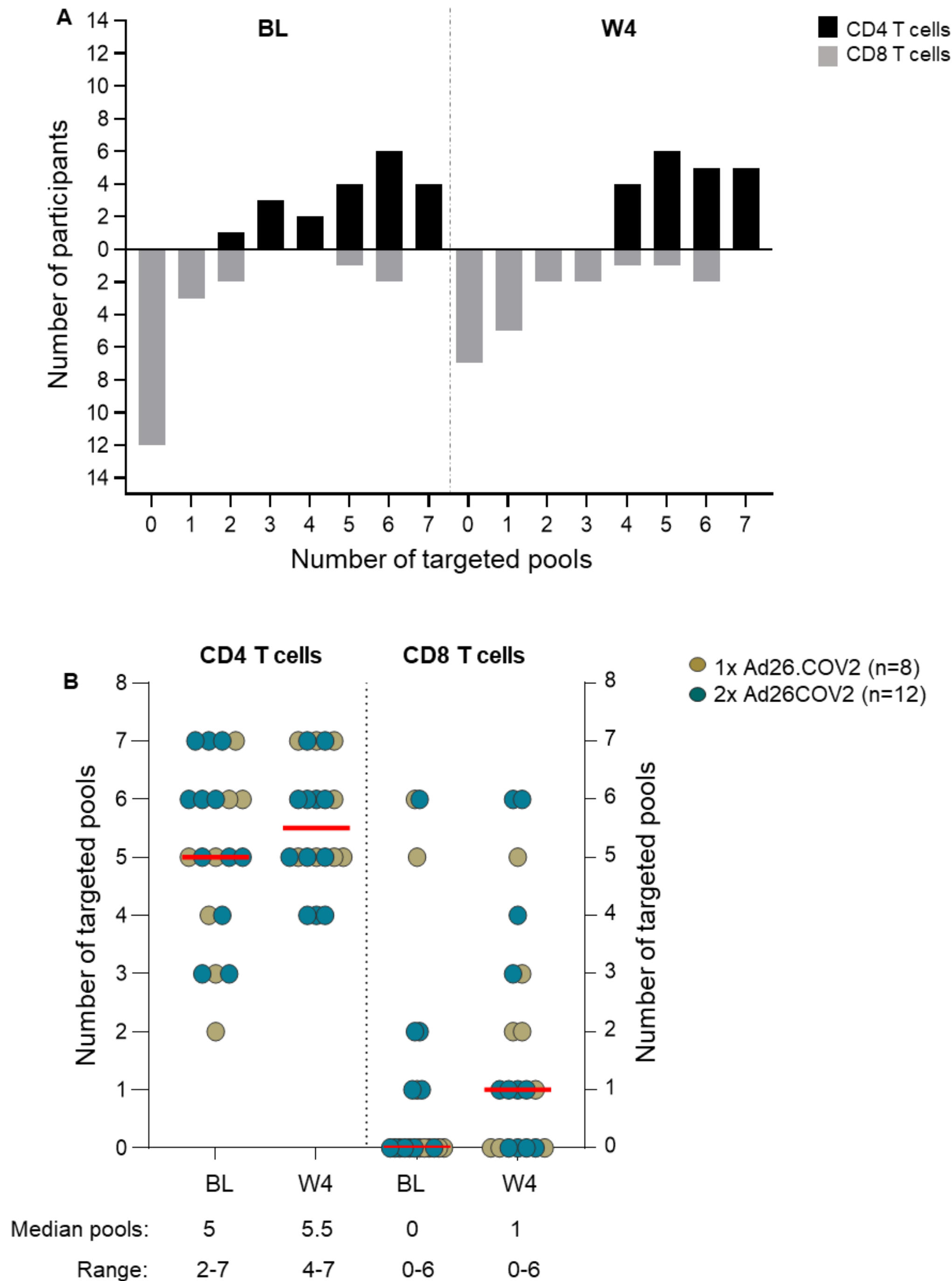


Figure 3.6: Breadth of spike peptide pool targeting. (A) Total number of pools targeted by the CD4 (black) and CD8 T cells (grey) by participants at BL (left panel) or W4 (right panel). (B) Total number of pools targeted by the CD4 (left panel) and CD8 T cells (right panel) by participants at BL and W4 following one (beige; n=8) or two (blue; n=8) prior Ad26.COV2.S vaccine doses. Median and range of targeted pools are indicated at the bottom. No statistically significant differences were observed after using the Wilcoxon matched-pairs signed rank test.

becoming the most targeted regions, each by 6/20 (30%) participants. RBD2, HR, and CT were targeted by 5/20 (25%) participants. Unlike CD4 responses, the FP domain exhibited the least targeting by CD8 cells at W4.

Overall, both CD4 and CD8 responders exhibited broad reactivity, mounting responses across the length of the spike protein.

Thereafter, I sought to compare the magnitude of the T cell response against the different pools at the BL and W4 timepoints (**Figure 3.8**). The S1 and S2 regions of the spike each had two pools that were highly targeted and that induced robust CD4 responses, which were subsequently higher at W4 (**Figure 3.8A)**. These high median response magnitudes were observed for pool 2 (0.011% to 0.025%), pool 5 (0.009% to 0.023%), pool 6 (0.007% to 0.019%) and pool 3 (0.008% to 0.015%). Despite having some of the highest number of CD4 responders (at least 15/20; 75%), pool 1 induced low CD4 responses (median 0.005%) at both timepoints. As expected, pool 4 and pool 7, which had the lowest proportion of responders also had the lowest magnitude of response at both timepoints (median: 0.003% to 0.004% and 0.002% to 0.004%, respectively). For CD8 responses, there were no differences in the median response magnitudes against the individual spike pools, as most participants had responses against a single pool at both timepoints.

Finally, I compared the breadth of the T cell responses amongst the participants. **Figure 3.9** illustrates the frequencies of CD4 and CD8 T cells targeting various regions of the spike at BL and W4 for all the participants (PID#1-PID#20). Initially, all participants displayed diverse CD4 responses targeting both S1 and S2 regions of the spike (**Figure 3.9A**). The administration of the booster vaccine further expanded CD4 responses for some participants and increased the magnitude of previously recognised pools for most. Interestingly, five participants (PID#8, 11, 13, 14 and 18) experienced a loss of CD4 responses to one or two pools after initially targeting between five to seven pools, specifically pool 2, pool 3, pool 4, or pool 7.

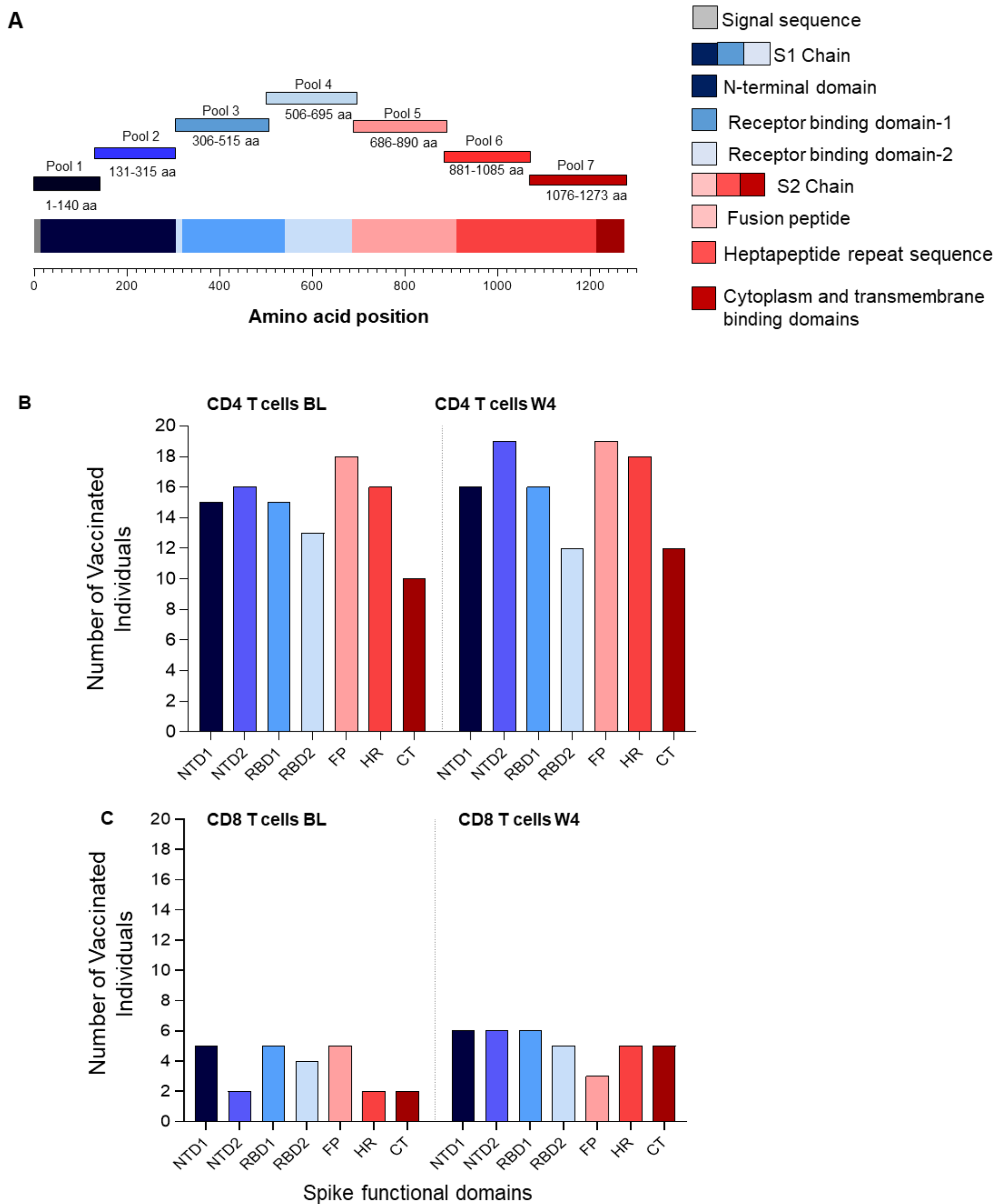


Figure 3.7: Specificity of T cell responses across the SARS-CoV-2 spike protein. (A) Schematic representation of the location of the seven spike peptide pools consisting of 15-mer overlapping peptides spanning the entire spike protein. Pools 1-4 represent the S1 region and pools 5-7 represent the S2 region. (B and C) Specificity of the CD4 (B) and CD8 T cell (C) responses at BL (left panels) and W4 (right panels).

These analyses further emphasised that most CD8 responses were narrowly targeted toward the S1 region, with all responding participants targeting one of its pools at BL

(**Figure 3.9B**). This persisted at W4, with the majority still targeting the S1 region. Similar to CD4 responses, there was an increase in the magnitude of previously targeted regions for most participants. Notably, seven participants (PID#1, 5, 11, 15, 17, 18 and 20) experienced a partial or complete loss of their CD8 response against specific pools across spike. There were no striking differences in the cumulative CD4 and CD8 responses between the one-dose and two-dose groups at BL or W4 (**Supplementary Figure S3.2**).

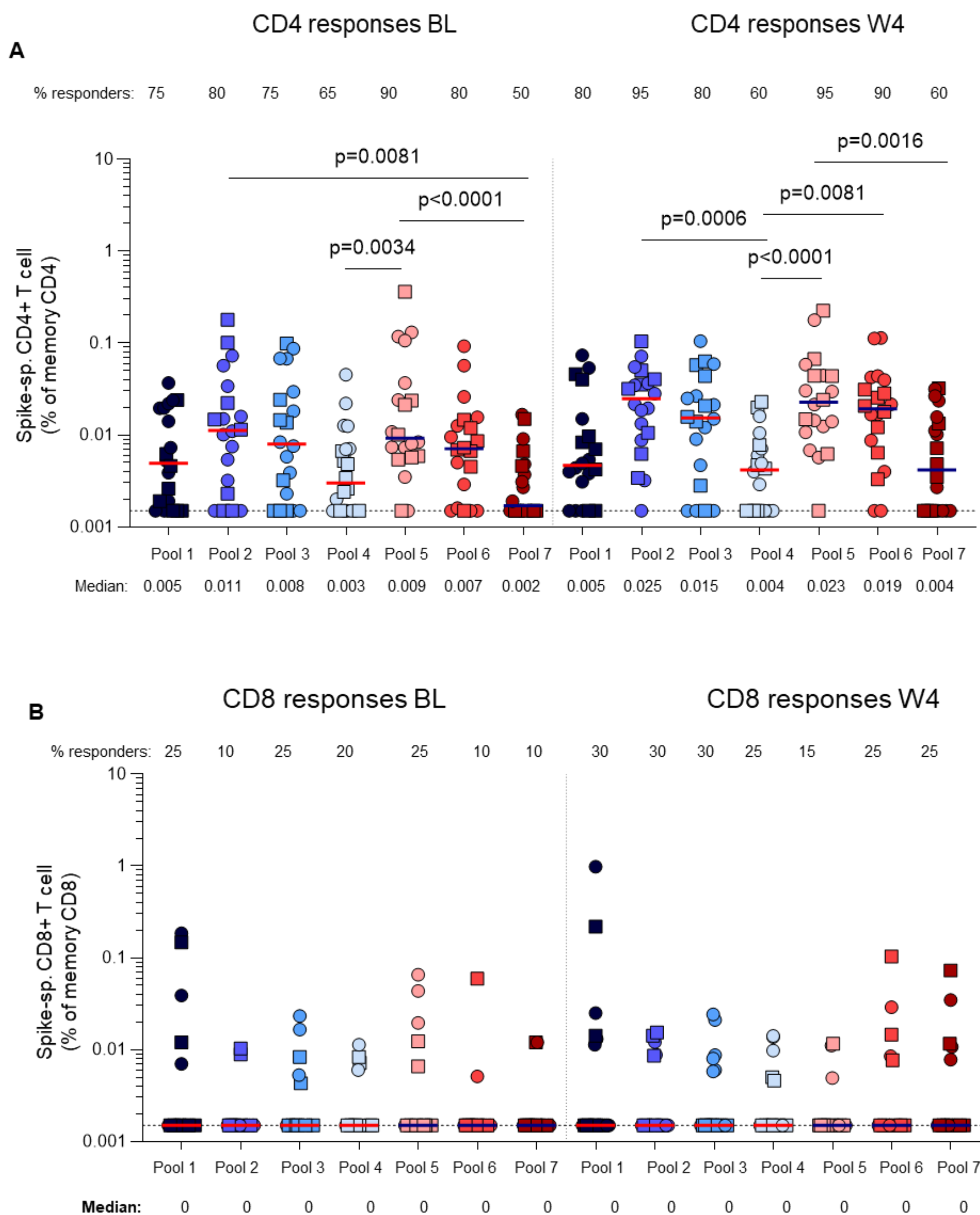
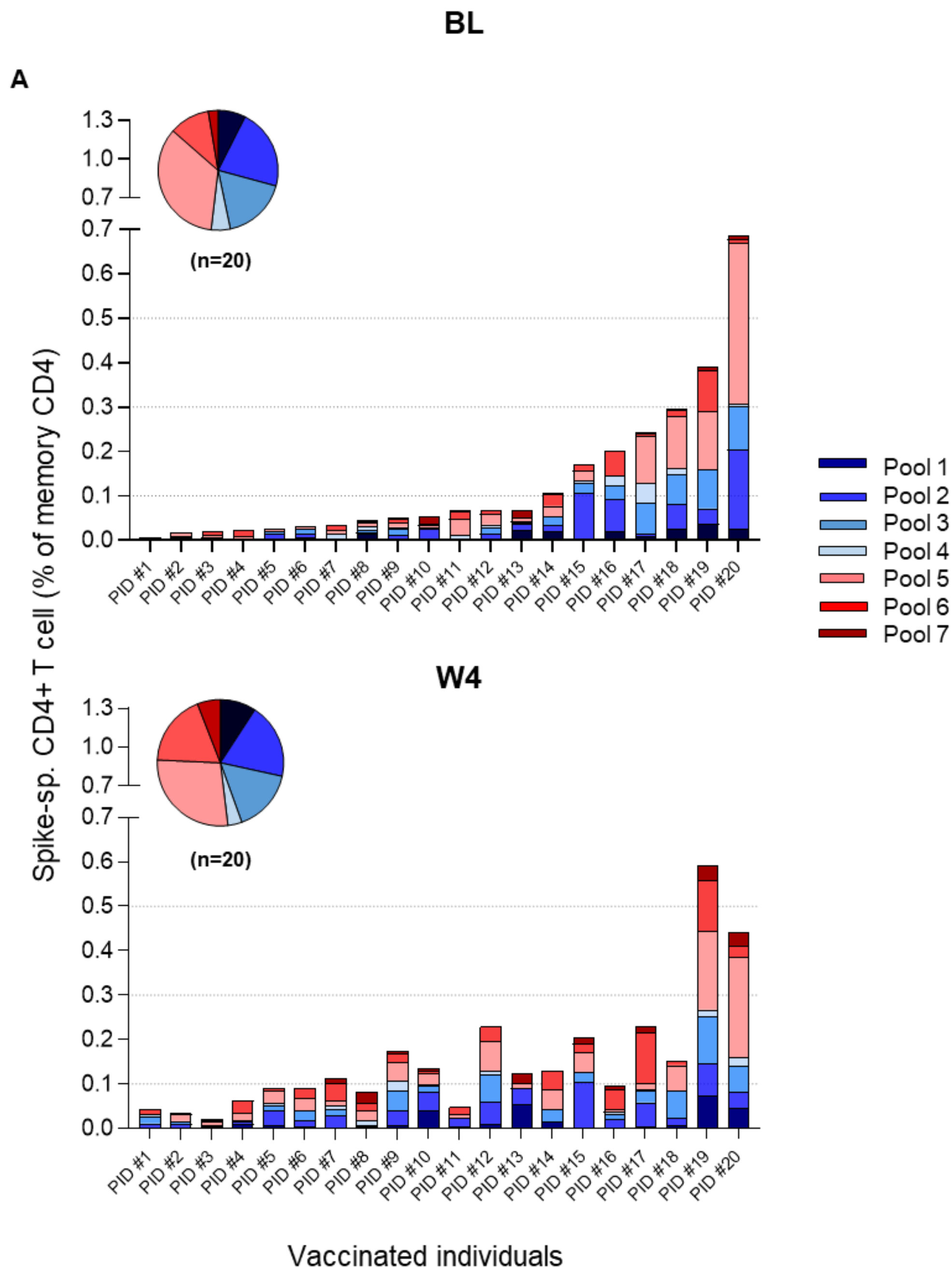


Figure 3.8: T cell response magnitudes towards the seven individual spike peptide pools. (A and B) Frequency of the CD4 (A) and CD8 T cell (B) responses

against the seven peptide pools at BL (left panels) and W4 (right panels). The squares and circles represent the represent the one and two prior Ad26.CoV2.S dose groups, respectively. Proportion of responders are indicated on top of the graphs. Horizontal lines indicate medians and median values are indicated below the graphs. All statistical analysis were performed using Friedman test with Dunn's multiple comparisons tests. p values <0.05 were considered statistically significant.



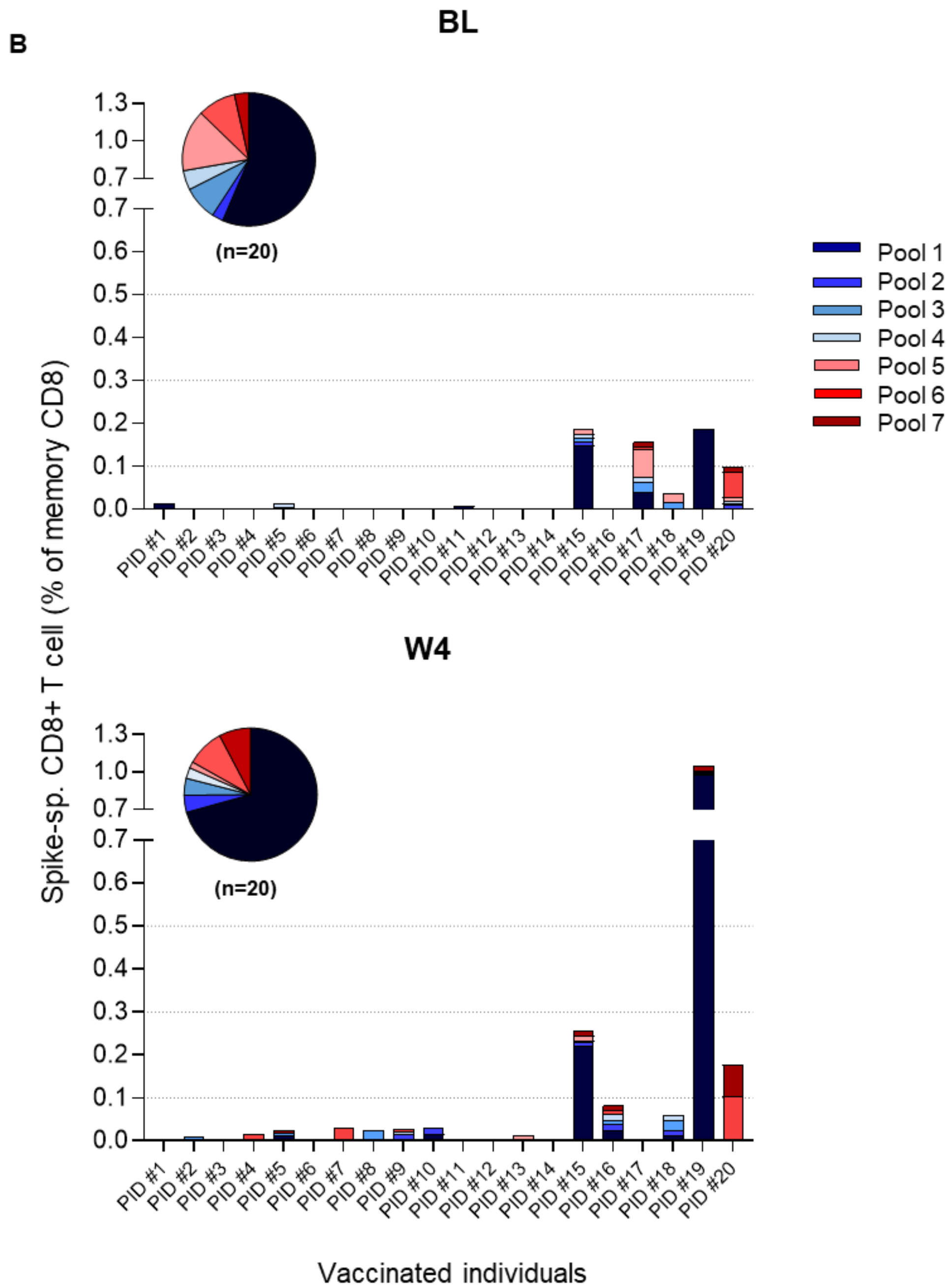


Figure 3.9: Cohort profile of cumulative spike-specific T cell responses. (A and B) Frequency of CD4 (A) and CD8 T cell (B) responses to the individual peptide pools (pool 1–7) in each vaccinee at BL and W4. Pie charts represent the percentage of responses to the individual pools.

Chapter 4: Discussion

For the majority of SARS-CoV-2 vaccines that have been in use, the spike protein serves as the immunogenic target, inducing antibody responses that may protect from infection but that viral variants may escape. Additionally, these vaccines elicit T cell responses associated with protection against severe COVID-19 (Bergamaschi et al., 2021; Braun et al., 2020; Gil-Etayo et al., 2020; Moderbacher et al., 2021; Notarbartolo et al., 2021; Roncati et al., 2020; Sekine et al., 2020). While significant efforts have been dedicated to assessing the magnitude of T cell responses to spike post-vaccination, fewer studies have characterised their breadth and specificity. Understanding the breadth and specificity of T cell responses is crucial for evaluating the preservation of T cell responses against future variants, in case mutations affect the targeted regions. In this study, I investigated the magnitude and crude breadth of SARS-CoV-2 spike-specific CD4 and CD8 T cell responses induced by a heterologous prime-boost vaccination strategy comprising Ad26.COV2.S (Johnson & Johnson/Janssen) and mRNA-1273 (Moderna/Spikevax) vaccines. PBMC were stimulated with a pool of peptide spanning the full spike pool as well as seven peptide pools spanning the length of spike but covering different regions, at baseline and four weeks after mRNA-1273 booster vaccination. CD4 and CD8 T cell responses were quantified using intracellular cytokine staining (IFN-γ or IL-2) and flow cytometry.

This study recruited participants from South Africa, with most participants being female. The extent to which this influenced the observed immune responses is unclear, as there is conflicting information regarding the impact of vaccination on different genders. Some studies report no difference between genders, while others note higher responses in either males or females (Bai et al., 2022; Cangemi et al., 2022; Ewer et al., 2021; Nam et al., 2022; Notarte et al., 2022). In the four years since the initial cases of SARS-CoV-2 were reported in March 2020, a considerable portion of the South African population has encountered the viral spike protein through infection, vaccination, or a combination of both (Bingham et al., 2022; Madhi et al., 2022). Considering the distinct influences of these exposures on the immune system, it is important to take infection history into account when assessing SARS-CoV-2-specific immune responses (Crotty, 2021). Ninety percent (18/20) of the participants

had detectable anti-N IgG responses. This observation is noteworthy as it shows that most of the participants had hybrid immunity, which has been demonstrated to induce more robust cellular and humoral responses against all viral proteins compared to infection or vaccination alone (Collier et al., 2022; Lim et al., 2022; Mantus et al., 2022; Naranbhai et al., 2022; Sedegah et al., 2022; Vespa et al., 2022). Two participants were seronegative, one of whom had previously tested positive by PCR test a year before the baseline samples were collected. This finding might be explained by other studies indicating that anti-N IgG responses may wane over time, with approximately 19% and 5% of convalescent individuals showing anti-N IgG responses a year and 21 months post-infection, respectively (Faas et al., 2022; Ortega et al., 2021; Terpos et al., 2021). Similarly, there was a significant decrease in the magnitude of anti-N IgG responses within our cohort between the two timepoints. The presence of anti-N IgG might, therefore, indicate a relatively recent infection among the participants (Faas et al., 2022). It is worth noting that some individuals mount antibody responses to proteins other than the nucleocapsid following infection (Tutukina et al., 2021; Van Rooyen et al., 2023). Consequently, these study results are generally applicable to individuals with hybrid immunity, which has become common in South Africa since the emergence of the Omicron variants (Bingham et al., 2022; Sun et al., 2022).

The heterologous booster vaccine in this study significantly increased the T cell responses following Ad26.COV2 vaccination. These results are in accordance with another study that has shown that Ad26.COV2 vaccination induces T cell responses in most participants, which significantly increase following the administration of heterologous mRNA-1273 vaccine booster compared to homologous Ad26.COV2 vaccination (Atmar et al., 2022). Other heterologous vaccine regimens, such as ChadOx1 nCov-19 combined with mRNA-1273 or BNT162b2 vaccines have also been shown to significantly increase the magnitude of humoral and cellular T cell immune responses compared to homologous ChadOx1 nCov-19 vaccine boosters (Barros-Martins et al., 2021; Liu et al., 2021; Schmidt et al., 2021; Pozzetto et al., 2021).

We found a positive correlation between the CD4 and CD8 responses at both pre- and post-vaccination. However, there were four CD8 spike non-responders, two of whom

lost their responses at the four week post boost time point. This is similar to what other studies have reported (Erdinc et al., 2021; Knudson et al., 2014; Kumar et al., 2022; Olea et al., 2022). While our assessments were confined to blood samples, and SARS-CoV-2 primarily affects the respiratory system, it is possible that spike-specific CD8 cells were present in the respiratory tract. Indeed, some studies have documented the presence of spike-specific CD8 cells in respiratory epithelium from individuals who did not exhibit corresponding CD8 responses in their bloodstream (Grau-Expósito et al., 2021; Poon et al., 2021). CD8 cells are also targeted against other viral proteins, thus it is possible that these individuals might have had responses to non-spike proteins, as has been described (Grifoni et al., 2021; Nesamari et al., 2024).

The breadth of the T cell responses was characterised using seven peptide pools spanning the length of the spike protein. This experimental approach was validated by comparing the combined magnitude of CD4 and CD8 responses against the seven pools to that of the full spike pool. Considering that each of the seven pools contained an average of 36 different peptides in contrast to the 253 peptides present in the full spike pool, it was expected that the combined magnitude of T cell responses in seven pools may be higher due to decreased epitope competition (Mahajan et al., 2021; Mateus et al., 2020; Zhang et al., 2012). Overall, the magnitude of the seven spike pools strongly correlated with the full spike pool for both CD4 and CD8 responses. Interestingly though, the magnitude of the CD4 T cell responses against the full spike was higher than the combined seven pool magnitude. A possible explanation for this finding may be that some epitopes within the seven peptide pools represented low magnitude responses, resulting in responses below the detection limit of the assay when the smaller pools were tested individually. However, the magnitude of the responses to the full spike pool represents the cumulative magnitude of all these lower frequency responses, which may have facilitated their detection and thus a slightly higher overall response. This finding reflects that of Mahajan et al. (2021), who also found that some participants had higher T cell responses towards the full spike pool compared to combined responses of the S1 and S2 subunits of the spike.

One of the important aspects of the study is that we were able to compare the magnitude, breadth and specificity of the T cell responses following the mRNA-1273 vaccine booster in people who previously received one or two Ad26.COV2 vaccine doses. Notably, we did not observe any differences in the magnitude of responses between the two groups. However, there was an increase in the number of CD8 responders following the booster vaccine for both groups. This is consistent with our earlier findings where the increase in spike exposures through vaccination, infection or both increased the number of CD4 and CD8 responders (Keeton et al., 2021, 2022). Similarly, there was no difference in the breadth or specificity of regions targeted between the two groups, but there was an increase in the magnitude of responses against the individual pools at week four. This suggests that the significant increase we detected for the full spike pool may not be due to the targeting of the new epitopes but mostly due to the increase in responses against previously recognised epitopes, although this is an assumption as we did not map the specific epitopes targeted.

T cell activation depends on the recognition of specific epitopes bound to HLA molecules, so the occurrence of mutations can alter the epitope and prevent its processing or binding to HLA or enable presentation but prevent TCR binding and subsequent T cell activation. Interestingly, we found that CD4 responses were broadly targeted at an average of five and 5.5 pools of the seven spike pools. This is in stark contrast with the CD8 responses, which were targeted at a median of only one pool after boosting. These results are similar to those of Khoo et al. (2022) who also found T cell responses targeting between 0 to 7 pools following different vaccine regimens. However, they used an enzyme-linked immunosorbent spot (ELISpot) assay which did not allow for the differentiation of the CD4 and CD8 responses. Thus, our data serve to highlight this important difference in the breadth of CD4 responses compared to CD8 responses in spike. There may be significant implications when it comes to SARS-CoV-2 variants. The broad CD4 responses mean that even if there are variants that acquire mutations that knock out specific epitopes, cross-recognition can still occur for the remaining epitopes in conserved regions. This is supported by studies which have noted the overall preservation of the CD4 responses against the Beta, Delta and Omicron-lineage VOCs despite a decrease in the magnitude of responses compared to the wild-type strain (Riou et al., 2022; Keeton et al., 2021; Keeton et al.,

2022; Naranbhai et al., 2022; Nesamari et al., 2024). The narrow targeting by CD8 T cells means that they are potentially more vulnerable to losing their cross-reactivity should a knockout mutation occur in the targeted CD8 epitope. This has been reflected by some studies which reported a complete loss of the CD8 responses in some participants against variants such as the Delta and Omicron (Dolton et al., 2022; Keeton et al., 2021; Keeton et al., 2022; Naranbhai et al., 2022; Nesamari et al., 2024). This is concerning since CD8 responses are important for rapid viral clearance in both human and animal studies following reinfection, thus their loss may have pathologic impacts upon reinfection (Koutsakos et al., 2023; Moderbacher et al., 2020).

Studies have reported that following infection, most people mount CD4 and CD8 responses to at least 17 epitopes which are spread across the length of the spike (Grifoni et al., 2021; Karsten et al., 2022; Tarke et al., 2021). Consistent with this, we detected CD4 and CD8 responses in all seven pools. Furthermore, CD4 cells exhibited immunodominant responses for pool 2 (131-315 aa) which contains NTD2 and pool 3 (306-515 aa) which contains RBD, both in S1, as well as pool 5 (686-890 aa) which contains FP and pool 6 (881-1085 aa) which contains HR functional regions of the S2 region of spike. These findings are similar to that of Karsten et al. (2022) who reported immunodominant epitopes in regions covered by pool 2, pool 3 and pool 5. It is worth noting that others have also reported immunodominant T cell targeting in the S1 or S2 subunit or both (Khoo et al., 2022; Lim et al., 2022; Sedegah et al., 2022). However, these studies were limited by the use of ELISpot or Flourospot assays which do not allow for the differentiation of CD4 and CD8 T cells. Of the four immunodominant regions, RBD-containing pool 3 has the highest occurrence of mutations in VOCs. This may further explain the preservation of the CD4 responses (Dolton et al., 2022; Keeton et al., 2021; Keeton et al., 2022; Naranbhai et al., 2022; Nesamari et al., 2024). Interestingly, other studies have noted that aa sequence 816-830 is highly conserved among human coronaviruses and induces cross-reactive T cell responses (Low et al., 2021; Loyal et al., 2021; Palacios-Pedrero et al., 2022). The sequence is covered by pool 6 which was one of the most targeted, by over 80% (18/20) of the participants, which might also mean that the high magnitude of responses may be contributed to through cross-reactive responses with the other coronaviruses.

This study had several limitations. Firstly, the study was predominantly made of up females. This gender imbalance may have influenced overall immune responses observed, potentially limiting the generalizability of the findings. Therefore, caution should be exercised when extrapolating these results to other populations, such as males, children, or pregnant women with SARS-CoV-2 infection. Secondly, our sample size was small due to the limited availability of PBMC. While we measured T cell responses in the blood, these may not fully represent T cell responses at the site of infection. Additionally, our study did not assess the secretion of other cytokines by T cells, such as cytotoxic markers, leaving the possibility that other cytokines with potential immunological significance were not accounted for. Moreover, the generalisability of our findings is restricted to individuals with hybrid immunity. Further investigations are necessary to determine the specific targeted epitopes as well as the HLA alleles of the participants. Since we evaluated responses four weeks post-vaccination, longer-term studies may be required to ascertain any changes in breadth and specificity to the T cell memory response.

This study aimed to assess the magnitude and breadth of SARS-CoV-2 spike-specific CD4 and CD8 T cell responses following the administration of Ad26.COV2.S and mRNA-1273 vaccine prime-boosting strategy within the context of hybrid immunity. Overall, these data indicate that the mRNA-1273 vaccine booster significantly increased the magnitude of CD4 and CD8 spike responses. In addition, the booster expanded the breadth of CD4 and CD8 responses in some participants while enhancing the magnitude of previously targeted regions in others. Additionally, vaccination elicited broad CD4 responses targeting the entire length of the spike protein, suggesting potential preservation against future variants. In contrast, most individuals exhibited CD8 responses that were narrowly focused on specific regions of the spike, raising concerns about the potential loss of cross-reactive CD8 T cell responses in the event of mutations in the targeted regions that affect epitope processing, presentation or TCR recognition. These findings highlight the need for efforts to develop or introduce vaccine regimens that elicit more CD8 responses and induce broader CD8 responses to preserve cross-reactivity against future variants of concern and potentially mitigate severe COVID-19.

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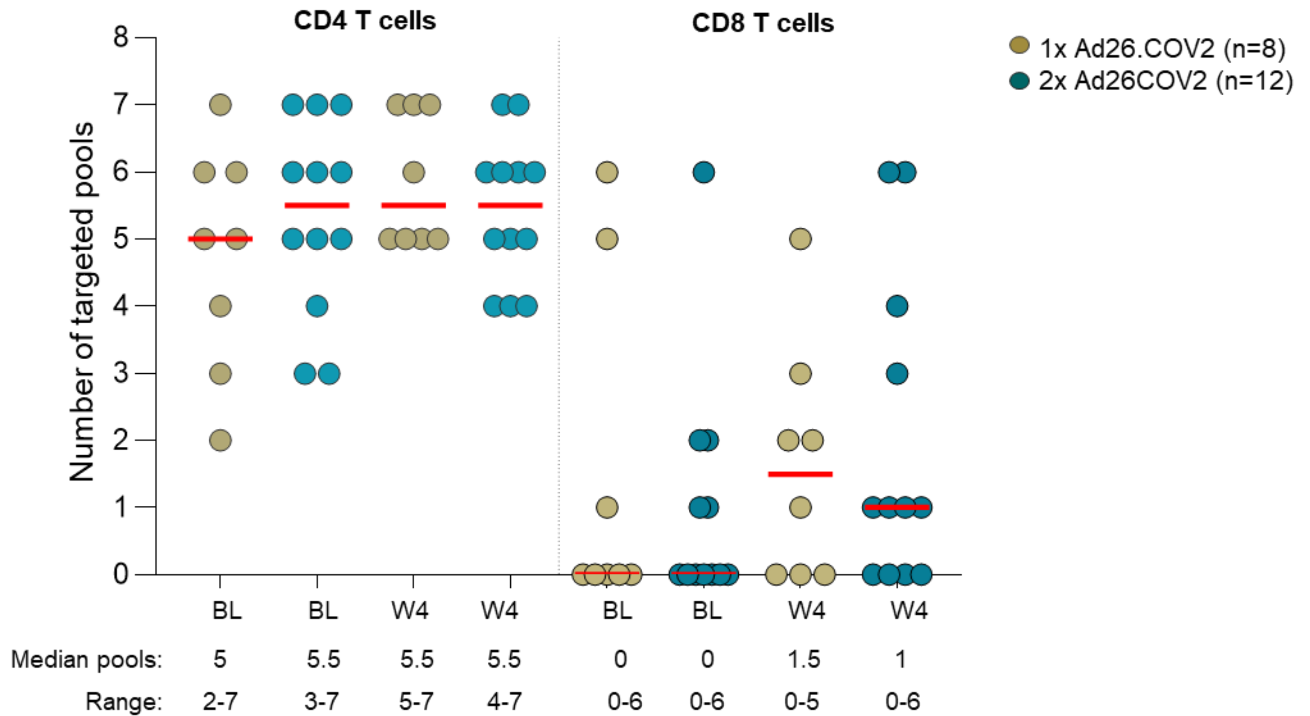
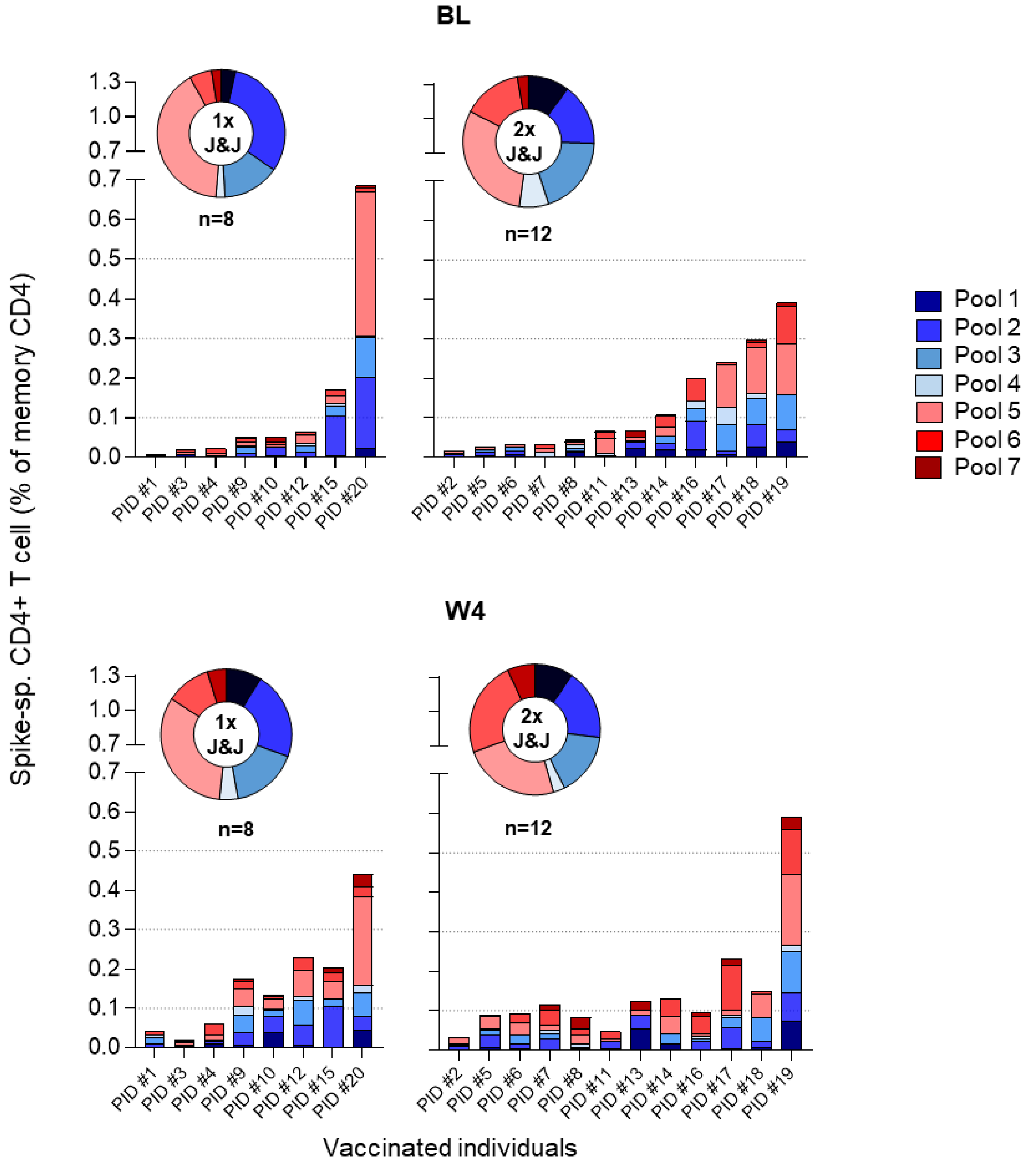
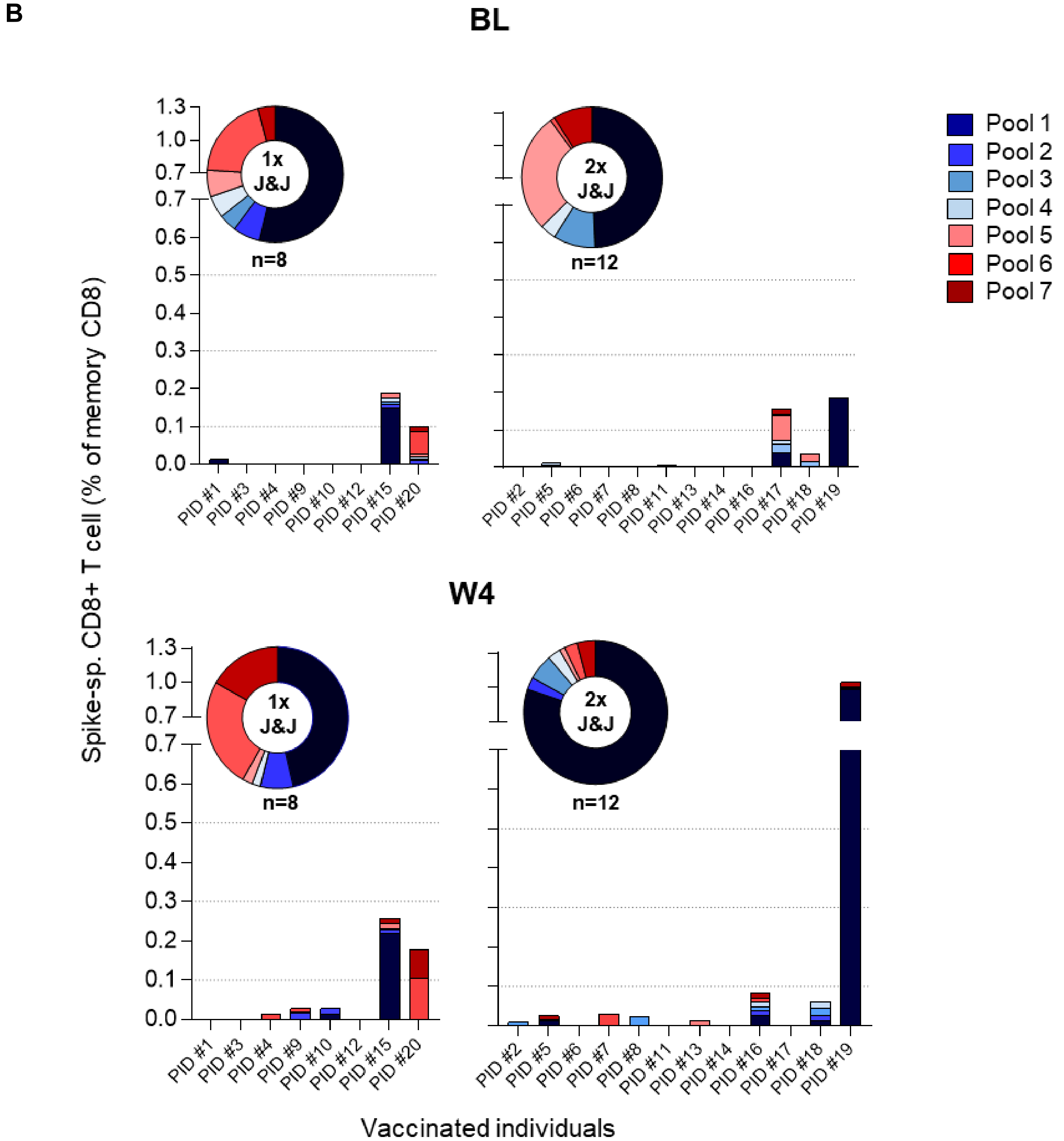
Supplementary Figures



Supplementary Figure S3.1. Number of spike pools targeted at BL and W4 following one or two Ad26.COV2.S vaccine doses. Total pools targeted by the CD4 (left panel) and CD8 T cells (right panel) by participants at BL and W4 following one (beige; n=8) or two (blue; n=8) prior Ad26.COV2.S vaccine doses. Median and range of targeted pools are indicated below the graph. No statistically significant differences were observed after using the Kruskal-Wallis test.

A





Supplementary Figure S3.2: Cohort profile of cumulative spike-specific T cell responses following one or two prior Ad26.COV2.S vaccine doses. (A and B) Frequency of CD4 (A) and CD8 T cell (B) responses to the individual peptide pools (pool 1–7) in groups that had one (n=8) or two (n=12) prior Ad26.COV2.S vaccine doses at BL and W4. Pie charts represent the percentage of responses to the individual pools.