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MRes Research Project

The cell mediated and humoral response to SARS-CoV-2 infection

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus that causes COVID-19 (coronavirus disease 2019), the respiratory illness responsible for the COVID-19 pandemic. We have worked with the University Hospitals Coventry & Warwickshire to identify patients hospitalized with SARS-CoV-2 to study their long-term cell mediated/humoral responses (i.e., immunological memory) to the infection. We aimed to identify the quality of their memory response in relation to the severity of their disease and other physiological differences. We isolated the patient's peripheral blood mononuclear cells (PBMCs) and stimulated them with peptides of SARS-CoV-2 from 2 months after their initial infection. The PBMCs were stimulated separately with the Spike protein, the Membrane protein and the Nucleocapsid protein of SARS-CoV-2. Subsequently, the samples stained to detect CD8 and CD4 T-cells and the cytokines (IL-2 and IFN-g) they expressed, and flow cytometry was then used to analyse the data. Alongside this we measured SARS-CoV2-specific antibody levels and conducted antibody neutralisation assays on the plasma of each patient to define the quality of the humoral immune response. We hope to develop an understanding of the lasting effects of SARS-Cov-2 immunity in those with and without an antibody response and observe the effects of severity of illness on these long-term immune responses.

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Introduction

1.1 Background to SARS-CoV-2

1.1.1. What is SARS-CoV-2

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the virus that is currently causing a global pandemic. The disease, COVID-19, referring to the year it was discovered, is a respiratory tract infection that can lead to severe symptoms such as acute respiratory distress syndrome, pneumonia and can be fatal. The mortality rate for SARS-CoV-2 is considerably lower at 5.6% compared to the other well-known coronaviruses; SARS-CoV-1 and MERS which have 13% and 35% mortality rates, respectively (Pormohammad *et al*, 2020). Though SARS-CoV-2 has greatly surpassed both these strains in infection rates.

Clinically few options aside from supportive and palliative care have been found to work successfully. Trials have attempted to screen pre-existing drug treatments for other diseases, such as cancer, to search for an effective treatment. Drugs to treat SARS-CoV-2 have centred around either drugs that ease the symptoms or reduce viral replication. There has been lots of attention on drugs that initially showed promising results in clinical trials such as hydroxychloroquine, but since then the trials have not found differences in those treated with or without this drug (Geleris *et al*, 2020). Examples, such as hydroxychloroquine, demonstrate the importance of multiple large trials carried out before widespread use. Drugs that are already known to be safe have also been undergoing clinical trials, such as nafamostat, camostat, lopinavir and ritonavir, which are types of protease inhibitors that would hopefully inhibit SARS -CoV-2s entry into the cell (Shaffer, 2020). While a selection of drugs to treat coronaviruses over the years have been trialled and found to have some benefit, a cure has not been found(Dyall *et al*, 2014). Multiple factors have been found to make individuals more vulnerable to the disease in including age: particularly the over 50s, sex: being female offers some protection, ethnicity: with BAME groups being more vulnerable, as well as those who are immunocompromised. Factors such as viral load upon infection have also been found to cause more severe disease, from studies on healthcare

workers and front-line workers (Chou *et al*, 2020). While treatment options have been explored, understanding the cell mediated immune response to SARS-CoV2 remains to be fully elucidated.

1.1.2. Structure of SARS -CoV-2

SARS -CoV-2 is an enveloped virus that consists of four structural proteins: spike, envelope, nucleocapsid and membrane proteins, as shown in Figure 1 (Kim *et al*, 2020) . The spike protein is an outer surface protein, composed of 1273 amino acids, and cleaves into the S1 and S2 subunit whose roles are receptor binding and membrane fusion, respectively (Xia, 2021). The membrane protein is the most prevalent structural protein in SARS -CoV-2 and has a role in coordinating viral shape, assembly, and in generating mature viral envelopes in coronaviruses in general (Siu *et al*, 2008). The spike protein, membrane protein and envelope protein are embedded in the viral envelope, and the nucleocapsid protein is within the virion protecting the viral RNA (as demonstrated by the schematic of SARS -CoV-2 in Figure 1).

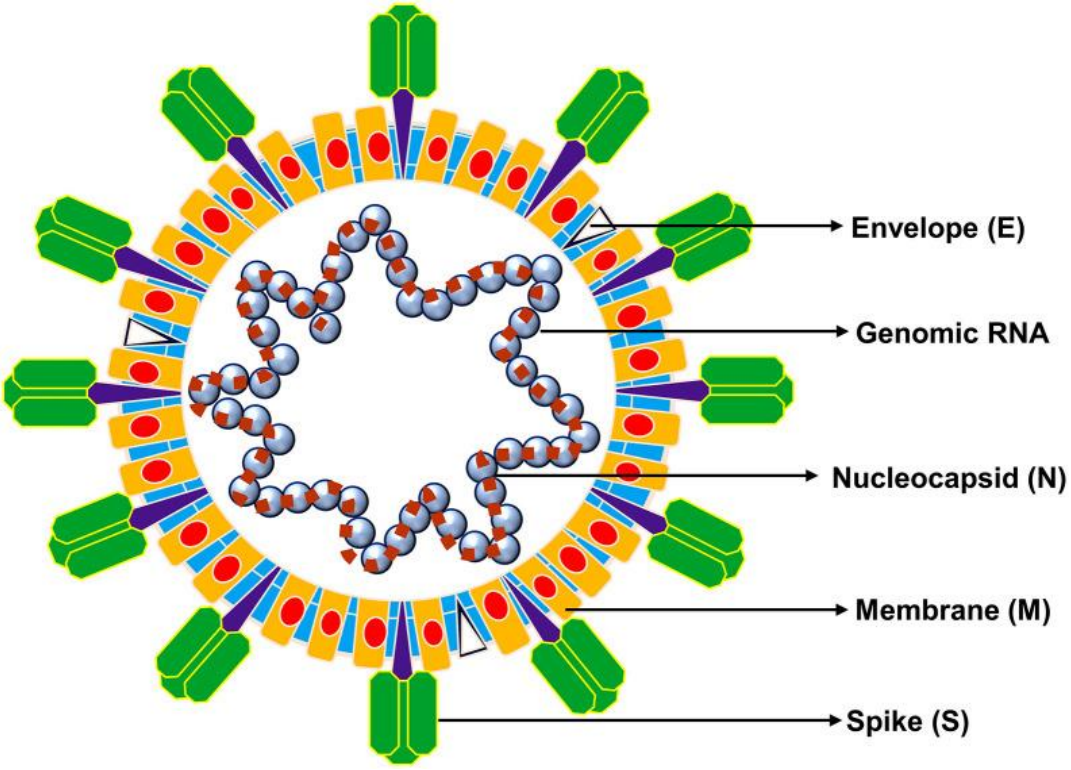


Figure 1 A schematic of the structure of SARS **-CoV-2.** Showing the structural proteins; spike, membrane, nucleocapsid and envelope proteins. The image was sourced from (Kirtipal *et al*, 2020)

To infect a cell, SARS -CoV-2 enters through binding to the receptor angiotensin-converting enzyme 2 (ACE2), using spike S1 and S2. The spike S1 region of the protein binds to ACE2 and spike is cleaved by a host enzyme (TMPRSS2) on the cell surface exposing S2 which can then fuse the viral membrane with the host cell membrane, allowing viral RNA to infiltrate the host cell and hijack its organelles (Scudellari, 2021). The critical role spike plays in infecting cells demonstrates the importance of understanding its recognition by the immune system and what neutralises it.

In this project peptide sequences that contain the full lengths of the three structural proteins that are most often recognised by the immune system, the spike, membrane and nucleocapsid proteins will be used. In the case of spike, multiple peptides which cover the full length of the protein will be used (see methods table 2.1 for details of peptide lengths). These peptides will be used to stimulate the SARS -CoV-2- specific cell-mediated response in convalescent patients. Full length spike and nucleocapsid protein peptides are used to assess antibody reactivity and pseudo particles of Lineage A (Wuhan) B.1 (D614G) B.1.351 (South African) P.1 (Brazil) spike are used to assess the neutralising capacity of antibodies in convalescent patients' sera.

1.1.3. Long term immunity to SARS -CoV2

Current research efforts into long-term immunity to SARS-CoV-2 has been focused on testing antibody levels, especially neutralising antibodies as the standard to determine immunity. These antibody responses to SARS-CoV-2 are displayed 10-14 days after infection and peak around three weeks after infection then last for at least six months (Figueiredo-Campos *et al*, 2020), though occasionally they aren't detected at all. On the other hand, the T-cell or cell-mediated response in similar coronaviruses such as MERS and SARS have been found to last years and appear in most cases (Sekine *et al*, 2020). SARS-CoV-2 cell-mediated memory is similarly looking to be long term, though evidence differs on whether the T-cell memory has a pathogenic or beneficial effect in severe cases due the "cytokine storm"

elicited (Kalfaoglu *et al*, 2020). Despite the robust antibody and T-cell memory recorded, reinfections have been documented. The risk of reinfection was determined to be low, and 8 months after their first infection, but highlights the importance of understanding the long-term immunity for SARS-CoV-2 (Breathnach *et al*, 2021). Vaccination has also been proven to boost both antibody and T-cell response to spike (Zollner *et al*, 2021).

1.2 Antibody responses to SARS-CoV-2 infection

1.2.1. Antibody responses to SARS-CoV2

The antibodies produced changes throughout SARS-CoV-2 infection and clearance. Initially, IgM and IgA respond to areas such as the receptor binding domain of spike, but these responses decline in as little as two and a half months after illness. The most persistent antibody is IgG, this response has been detected beyond 90 days after infection and correlates with neutralising antibody titre experiments. The antibody response has also been shown to be stronger in those with more severe infection, with seroreversion occurring faster in those with asymptomatic or mild disease (Iyer *et al*, 2020). Studies have found that convalescent patients who had more severe disease had a greater antibody magnitude, possibly because the spike and nucleocapsid antigen is present in higher quantities for longer in the lymph nodes in severe cases (Guthmiller *et al*, 2021). Despite the greater antibody response, these cases present with severe symptoms, and this has been linked to a loss of germinal centres and antibodies with a lower affinity for viral antigens (Woodruff *et al*, 2020; Kaneko *et al*, 2020). Antibodies have been found to be largely specific to the spike and nucleocapsid proteins, specifically the receptor binding domain of spike and the RNA binding-domain of the nucleocapsid. The suggested reason for these proteins' immunodominance is that spike is the main surface glycoprotein and the nucleocapsid encapsulates the whole genome of the virus so there is a high antigen burden for these proteins (Guthmiller *et al*, 2021).

1.2.2. Functional effects of antibodies

SARS-CoV-2 antibodies that do not neutralise the virus are important in the immune response because of their role in stimulating the T-cell response, though neutralising antibodies are more powerful in that they can prevent the virus from entering cells and spreading. The spike receptor binding domain (RBD) and the N-terminal domain (NTD) have been found to be the main targets for functional neutralising antibodies to SARS-CoV-2(Liu *et al*, 2020). Antibodies specific to epitopes on the RBD neutralise the virus because they prevent binding between the RBD and ACE2, and this interference means that the virus cannot enter the cell. In contrast to this mechanism, NTD-specific antibodies neutralise the virus through antibody binding resulting in steric hinderance on the spike protein, preventing its interaction with ACE2. The RBD has been found to have many non-overlapping sites of vulnerability to antibodies whereas the NTD may only have one single site of vulnerability. Neutralising antibodies for the NTD may also be less beneficial against different variants of SARS-CoV-2 as variants B.1.1.7 and B.1.351 have been found to have mutations in this domain(Cerutti *et al*, 2021).

1.2.3. Longevity of antibodies responses and immunological memory

As previously mentioned, the longevity of the antibody response has raised questions about whether immunity to SARS-CoV-2 is long-term, because these responses have been reported to decline over time. Even so, studies have found evidence of antibody responses that are neutralising and durable enough to suggest they are produced by long-lived plasma cells, indicating adequate immunity. Studies looking at the seroprevalence of antibodies specific to domains of spike as well as the nucleocapsid protein have shown that neutralising antibodies specific to spike (RBD and S2) were stable and persisted for at least 7 months following infection. Although, antibodies specific to the nucleocapsid protein were demonstrated to decline to undetectable levels 2-3 months after infection (Ripperger *et al*, 2020). Other studies went further, looking at the longevity of the response in addition to identifying variances in

seroconversion overtime in different disease severities. As mentioned previously, in cases of severe disease, the magnitude of antibody response has been measured to be higher, although (Wajnberg *et al*, 2020) found that in mild cases, seroconversion of spike-specific antibodies takes longer to rise as titres on day 30 were lower than on day 82, and these then dropped to their day 30 level after 148 days. Theorising that as IgG has a half-life of ~21 days, the antibodies observed later in the study are likely produced by long-lived plasma cells.

1.3 Cell mediated responses to SARS-CoV-2 infection

1.3.1. T-cell responses to SARS-CoV2

T-cells are produced as part of the adaptive immune response and is mounted a couple days following infection. This project focuses on CD4 and CD8 T-cells, which have a role in amplifying the immune response and killing infected cells, respectively. Their specific response to the structural proteins of SARS-CoV-2 has often been investigated in literature. Grifoni et al, 2020 showed that the spike, membrane and nucleocapsid proteins were all recognised by CD4 T-cells in their sample of mild non-hospitalised convalescent COVID-19 patients, while CD8 T-cell response was stimulated equally by spike and the membrane, though less by the nucleocapsid protein. Other studies have shown that T-cell IFN-γ responses were more varied and higher in severe cases of COVID-19 compared to mild cases, and flow cytometry analysis revealed a greater proportion of CD8 T-cells in mild disease compared with severe disease (Peng *et al*, 2020b). Like antibody responses, T-cell responses in severe cases have been found to have a greater breadth and magnitude compared to mild cases. T-cell memory specific to SARS-CoV-2 has shown to be extensive, such that T-cells have been observed to bind to epitopes on the spike, membrane and nucleocapsid protein. The T-cells of mild and severe convalescent patients produced at least one of the following cytokines: IFN-γ, TNF and IL-2, sometimes multiple in combination. T-cells that produce more than one cytokine are known as multi-functional. One difference between mild and severe disease, highlighted by (Peng *et al*, 2020b), was that more multi-functional CD8 T-cells were found to be specific to membrane or nucleocapsid than the spike protein in cases of mild SARS-CoV-2.

1.3.2. T-cell memory

Following infection/vaccination the initial response from CD8 and CD4 T-cells adequately clears/recognises the virus, T-cell levels specific to SARS-CoV-2 then reduce in number but long-living memory T-cells remain. This is the establishment of T cell memory/immunity. These memory T-cells remain in circulation within the body and would rapidly respond and clear the virus more quickly upon infection/re-infection. Although T-cells memory can exist for decades, it is not known if T-cells specific to SARS-CoV-2 from natural infection or vaccinations do. Different disease severities in SARS-CoV-2 infection may have implications for T-cell memory establishment, and classical and effective T-cell memory is not always achieved. For example, T-cells can enter an exhausted state if the immune response cannot clear the infection and a chronic infection occurs, or immune response can be immunopathological, such as in a cytokine storm. Through quantitatively observing T-cells and their cytokine responses after natural infection both with and without vaccination will lead to more insight on T-cell memory (Jarjour *et al*, 2021). Studies on similar coronaviruses such as SARS used peptides of the structural SARS proteins to stimulate peripheral blood mononuclear cells, a technique employed in this project, have demonstrated that CD8 and CD4 T-cell memory persists four years after infection (Fan *et al*, 2009).

T-cells differentiate through the following stages: Naïve → Central memory → Effector memory 1 (EM1) → Effector memory 2 (EM2) → pre-Effector 1 (pE1) → pre-Effector 2 (pE2) → Effector memory 4 (EM4) → Effector memory 3 (EM3) → (E) end-stage non-proliferative effector cells(Romero *et al*, 2007). Terminally differentiated effector memory (TEMRA) cells include pE1, pE2, and E cells. Effector memory includes EM1, EM2, EM3 and EM4. Following this differentiation sequence the higher percentages of cells in the later stages of differentiation suggests more exhausted cells, especially the TEMRA cells. CD8 T-cells have been found to be more affected by age than CD4 cells (Koch *et al*, 2008)

Through extracellular staining and flow cytometry gating, the level of differentiation of each patients T-cells can be assessed. CD45 is one crucial marker for distinguishing T-cell memory populations, of which there are different isoforms, namely CD45-RA and CD45-RO. CD45-RA is stained for in this experiment and is usually expressed on naïve cells that have not encountered antigen. Though it is re-expressed on TEMRA cells, these cells can be distinguished from the naïve population because they are negative for the "homing" receptor CCR7 which mediates the return of lymphocytes to the secondary lymphoid organs (Campbell *et al*, 2001). The difference in expression that is utilised in the cell gating strategy also demonstrates the functional difference between the T-cell memory populations. Naïve and central memory T-cell populations express CCR7 so can return to the secondary lymphoid organs after activation to be re-activated upon infection, whereas the effector and TEMRA populations do not, though only TEMRA cells re-express CD45-RA (Sallusto *et al*, 2004). Within both the TEMRA and effector memory populations, the more differentiated and more exhausted populations do not express CD28 or CD27 (EM3 and E cells, the two most differentiated populations), as demonstrated in Figure 2.3.

T-cells become exhausted as they differentiate further and have accumulative losses in their abilities, such as: their ability to produce cytokines such as IL-2, they are functionally less/non-responsive to antigens and have lower/no proliferative ability upon stimulation (Larbi & Fulop, 2014). Due to the loss of functionality in more exhausted cells, exploring patients' levels of these cell populations may provide insight into the strength of their cell-mediated immunity to SARS-CoV-2.

1.3.3. Methodologies for studying T cell mediated responses

There are multiple methods for studying T-cell mediated responses, such as Elispots and Flurospots. These work through taking individual monochromatic images at the right optimisation for each fluorochrome, so that cells secreting variable amounts of different cytokines can be detected (Megyesi *et al*, 2018). Another widely used method is flow cytometry, as

used in this project. Flow cytometry detects and measures different cell characteristics, through staining, T-cells can be distinguished from other cell populations and T-cells expressing different cytokines can be identified. This project centres on a 15-colour flow cytometry panel (16 antibodies as CD14 and CD19 were stained the same colour). The justification for the targets selected for both the cells and cytokines are shown in table 1.1.

Antibody Target	What the cell marker relates to
CD3	A T-cell co-receptor in both CD8 and CD4 cells.
CD4	A glycoprotein that serves as a co-receptor for the T-cell receptor, is what identifies a T-cell as a CD4 T-cell.
CD8	A transmembrane glycoprotein that serves as a co-receptor for the T-cell receptor and identifies the cell as a CD8 T-cell.
CD56	A transmembrane glycoprotein expressed on both natural killer and natural kill T-cells.
CD14	A human protein made by macrophages.
CD19	A transmembrane protein expressed in B lineage cells in humans.
CD45-RA	A receptor-linked protein tyrosine phosphatase that is an isoform of CD45 that is generally expressed on Naïve T-cells and TEMRA T-cells.
CCR7	CCR7, is expressed on all naive T cells, some memory T cells, B cells, and mature dendritic cells and plays a central role in lymphocyte trafficking and homing to lymph nodes.

CD28	A protein on T cells that provide co-stimulatory signals required for T cell activation and survival, can signify memory CD8 T-cells.
CD27	CD27 is expressed on both naïve and activated effector T cells as well as NK cells and activated B cells.
CD95	CD95 is a member of the tumour necrosis factor receptor family and is predominantly expressed in activated T lymphocytes and natural killer cells.
CD38	In humans, it is expressed at high levels on plasma cells and activated T and B cells, natural killer (NK) lymphocytes, myeloblasts, and erythroblasts.
HLA-DR	It is expressed on B cells, activated T cells, monocytes/macrophages, dendritic cells, and other non-professional APCs
Dead Cells	Dead cells within the population
IL-2	IL-2 -producing cells within mixed cell populations.
IFN-γ	IFN-γ producing cells within mixed cell populations.

Table 1.1 of the cells and cytokines relating to the antibody target.
Antibody targets listed alongside information on what types of cells the targets indicate.

1.4 Aims and hypothesis

This study hopes to develop an understanding of the lasting effects of SARS-CoV-2 cell-mediated and humoral immunity and observe the effects of severity of illness on these long-term immune responses. The cell-mediated response will be explored through stimulation and staining of PBMCs followed by flow cytometry of convalescent and vaccinated SARS-CoV-2 patients. The humoral response will be assessed through antibody

reactivity ELISAs and neutralisation assays. We expect that vaccination and disease severity will increase the magnitude of both the T-cell and antibody responses. T-cell memory phenotypes of the patients in the study will be used to explore the differentiation sequence of the CD8 and CD4 T-cells, to observe links between levels of cellular exhaustion and the cell-mediated response. We would expect that the more severe the disease individuals developed the more cellular exhaustion would be seen.

2.1 Materials

2.1.1 Chemicals for cell culture

Phosphate buffered saline (PBS), RPMI-1640 Medium, Histopaque-1077, human AB serum (HS), Glutamine, Penicillin/Streptomycin and HEPES were all purchased from Sigma Aldrich, UK. Complete RPMI refers to RPMI-1640 Medium with 1% human AB serum, 1% of 200mM Glutamine, 1% Penicillin/Streptomycin (10,000 units penicillin and 10 mg streptomycin per mL) and 1% of 1M HEPES. SepMate™-50(IVD) tubes were purchased from Stem Cell Technologies. EDTA VACUETTE® (EDTA tubes) were used to hold and transport patients' blood. Dulbecco's modified eagle medium and the 10% Fetal Calf Serum (FCS) it was supplemented by was from Invitrogen and the 0.1mM nonessential amino acids from GIBCO, in 2.2.6 this is referred to as complete media. The OptiMEM and polyethylenimine were purchased from GIBCO and Polysciences respectively. The phCMV-5349 MLV Gag/Pol and pTG126 luciferase were kind gifts from François-Loïc Cosset. Spike constructs were a kind gift from Josh Duncan.

2.1.2 Peptides for cell stimulation

Peptides specific for SARS-CoV2 spike, membrane and nucleocapsid were used in this study. As a positive control a cytomegalovirus, Epstein-Barr virus, and influenza virus peptide pool (CEF) was also used (table 2.1).

Name of Peptides/ Cell stimulants	Domain	Company
Peptivator SARS-CoV-2 Prot S	aa 304-338, 421-475, 492-519, 683-707, 741-770, 785-802, and 885 – 1273 (sequence end)	Miltenyi Biotec
Peptivator SARS-CoV-2 Prot S+	aa 689–895	Miltenyi Biotec
Peptivator SARS-CoV-2 Prot S1	aa1–692	Miltenyi Biotec
Peptivator SARS-CoV-2 Prot M	complete sequence of the membrane glycoprotein ("M") of SARS-Coronavirus 2	Miltenyi Biotec
Peptivator SARS-CoV-2 Prot N	complete sequence of the nucleocapsid phosphoprotein ("N")	Miltenyi Biotec

Peptivator CEF MHC Class I Plus	32 MHC-1– specific peptides of 8–12 aa	Miltenyi Biotec

Table 2.1 Peptides. The table outlines the different peptides used to stimulate T-cells, where S, S+ and S1 = spike protein sequences, M = membrane protein, N = nucleocapsid). Peptivator CEF MHC Class I Plus was used as a positive T-cell stimulation control, where CEF = cytomegalovirus Epstein-Barr Virus and Influenza virus.

A second positive control was also used in the study, namely Phorbol 12-myristate 13-acetate (PMA) (50ng/mL) and Ionomycin (0.5µg/mL).

2.1.3 Antibodies

2.1.3.1 Extracellular Antibodies

All antibodies were used at 2 µL per sample, except anti-CD3 (1 µL) and Zombie Aqua for dead cells (0.5 µL).

Target	Fluorophore	Isotype	Clone	Company
CD3	PerCP-Cy5.5	Mouse IgG1, κ	SK7	Biolegend
CD4	APC-Fire750	Mouse IgG1, κ	SK3	Biolegend
CD8	Alexa Fluor 700	Mouse IgG1, κ	RPA-T8	Biolegend
CD56	Brilliant Violet 785	Mouse IgG1, κ	5.1H11	Biolegend
CD14	Brilliant Violet 605	Mouse IgG1, κ	63D3	Biolegend
CD19	Brilliant Violet 605	Mouse IgG1, κ	HIB19	Biolegend
CD45-RA	Alexa Fluor® 488	Mouse IgG2b, κ	HI100	Biolegend
CCR7	Brilliant Violet 421	Mouse IgG2a, κ	G043H7	Biolegend
CD28	PE/Dazzle 594	Mouse IgG1, κ	CD28.2	Biolegend
CD27	PE-Cy7	Mouse IgG1, κ	O323	Biolegend
CD95	BUV 395	Mouse C3H	DX2	BD Pharmingen™
CD38	Brilliant Violet 650	Mouse IgG1, κ	HB-7	Biolegend
HLA-DR	Brilliant Violet 711	Mouse IgG2a, κ	L243	Biolegend
Dead Cells	Zombie Aqua	N/A	N/A	Biolegend

Table 2.2 Extracellular antibodies. Extracellular antibodies and their fluorophore, isotype, clone, and company are shown.

2.1.3.2 Intracellular antibodies

For intracellular staining of the cells 5 µL anti-IL-2 and 2 µL anti-IFN-γ was used per sample.

Target	Fluorophore	Isotype	Clone	Company
IL-2	PE	Rat IgG2b, κ	JES6-5H4	Biolegend
IFN-γ	APC	Mouse IgG1, κ	B27	BD Pharmingen™

Table 2.3 Intracellular antibodies Intracellular antibodies and their fluorophore, isotype, clone, and company are shown.

2.1.4 Cell Lines

HEK293T cells were seeded at $1.2x10^6$ into 10cm diameter Primaria coated dishes in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with non-essential amino acids and heat inactivated FCS and left overnight at 37°C and 5%CO_2. VeroE6 (ECACC) cells were seeded at $2x10^4$ per well into a 96-well white plate in 100µL of complete media and left overnight at 37°C and 5% CO_2.

2.2 Methods

2.2.1 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood on a Histopaque density gradient with SepMate™ tubes. The reagents, PBS and RPMI were warmed to 37°C, Histopaque to room temperature (RT). The samples were always handled in sterile conditions, in the MSC and in closed containers in the centrifuge. In brief, ~10mL of whole blood per patient was diluted 1:1 in 37°C PBS and mixed gently by inversion, then layered over 15mL of Histopaque in Sepmate tubes. Then these were centrifuged with the brake on, at 1,200 x g for 10 minutes. Subsequently, 2mL samples of plasma was taken and centrifuged at full speed and acceleration and deceleration in a Fixed Angle Rotor centrifuge for 5 minutes. The plasma supernatant was taken and stored at -70°C. The mononuclear layer was then removed and washed twice, centrifuged at 300 x g for 8 minutes. The resultant PBMCs resuspended in 37°C complete RPMI at 1 x 10^6 cells/ mL and either incubated overnight 37°C, 5% CO_2 or at diluted to 1 x 10^7 cells/mL in Fetal Calf Serum (FCS) + 10% DMSO and frozen (as shown below).

2.2.2 PBMCs storage and removal from liquid nitrogen

After isolating the PBMCs, the cells were resuspended in Fetal Calf Serum (FCS) + 10% DMSO at 1 x 10^7 cells/mL, placed in a 'Mr Frosty' and frozen down to -80°C overnight then moved to liquid nitrogen the next day. After removal from liquid nitrogen, the cells were defrosted in a water bath at 37°C. The cells were then gently dripped into 10mL of 37 °C complete RPMI and centrifuged at 300 x g for 8 minutes and resuspended in 10mL to count. The cells were then resuspended in 37°C complete RPMI at 1x10^6 cells/mL with 1.25 units/µL benzonase and rested for an hour at 37°C, 5% CO2. The cells were then washed and stimulated and stained as normal.

2.2.3 Cell culture and stimulation of PBMCs

The peptides listed in table 2.1 were made up according to the manufacturers guidelines and aliquoted to reduce freeze thawing, then stored at -80°C until use. The aliquots were then placed at RT before use to defrost.

The PBMCs left overnight or rested in benzonase were resuspended, counted to measure viable cells, and pelleted at 300 x g at full acceleration and deceleration. The sample's supernatants were then discarded and the PBMCs were resuspended in RPMI (5% human AB serum) at 1 x 10^7 cells/mL. The cells were stimulated with peptides specific to SARS-CoV-2 in 6 different wells for 6 conditions in 96-well U bottom plates at 1.5x10^6 PBMCs per well in complete RPMI. The three Spike peptides were added to one well (~1µg/mL) and the membrane (1µg/mL) and nucleocapsid (1µg/mL) peptides to two different ones. An unstimulated well was used as a negative control and two stimulated wells with combined Phorbol 12-myristate 13-acetate (PMA) (50ng/mL) and Ionomycin (0.5µg/mL) and one cytomegalovirus, Epstein-Barr virus, and influenza virus peptides (CEF) well (1µg/mL) were included as positive controls. The cells were incubated for 6 hours at 37°C, 5% CO_2, Protein transport inhibitor cocktail (500x) (Invitrogen) diluted 1:10 in complete RPMI containing 10.6µM Brefeldin A and 2µm Monensin was added 2 hours into the stimulation. Cells were then stained via an antibody pool of the extracellular antibodies listed in table 2.2 for 30 minutes at 4°C in the dark. Then cells were washed with PBA and fixed with fixation buffer (Biolegend) and kept at 4°C overnight.

The following day the cells were washed and resuspended in diluted permeabilisation buffer (10-fold in de-ionised water) (Biolegend) then centrifuged at 500g for 5 minutes. The cells were then intracellularly stained for 30 minutes at RT in the dark using an antibody pool containing antibodies in table 2.3 and the diluted permeabilisation buffer. The cells were then washed and fixed in fixation buffer (Biolegend) and stored at 4°C for up to 24 hours before analysis on the Astrios flow cytometer or the Spectral flow cytometer (Sony ID7000).

2.2.4 Flow cytometry analysis

The cells were first gated to isolate the living singlet T cells: Zombie Aqua-, CD14-/CD19-, CD56-/CD3+ was used to identify the CD8 T-cells (CD8+/CD4-) and the CD4 T-cells (CD8-/CD4). The gates were applied for the identification of IFNg+, IL-2+, and Cyt+ CD8 and CD4 T-cells. In addition, CCR7, CD45, CD28, CD27 and CD95 were used to identify T-cell Memory phenotypes, namely effector memory, TEMRA, central memory, naïve cells, and memory stem cells. The gating strategies used is shown in Figure 2.1, Figure 2.2, and Figure 2.3.

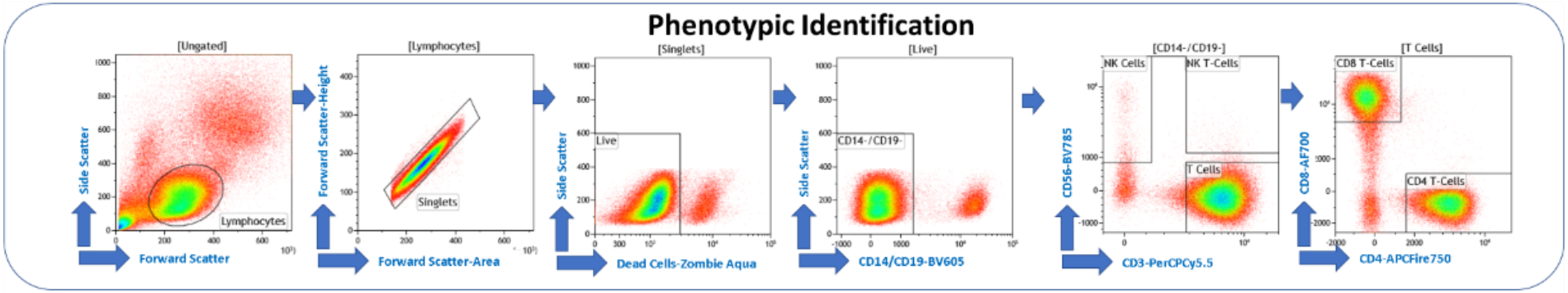


Figure 2.1 Gating strategy for cell identification. Gating with Kaluza was used to identify the live CD8 and CD4 cells. In each table from left to right is identifying the lymphocytes from debris and monocytes, then the singlets from doublets etc. The live cells were differentiated from dead cells that were stained with zombie aqua. Then CD14/CD19 antibodies were used to discern between CD14-/CD19- cells of interest from macrophages and other monocytes. Then CD56/CD3 antibodies were used to separate NK cells (CD56+/CD3-), NK T cells (CD56+/CD3+) and T cells (CD56-/CD3+) from each other. The CD3+ cells are T-cells then CD8 and CD4 antibodies were used to distinguish CD8 and CD4 T-cells.

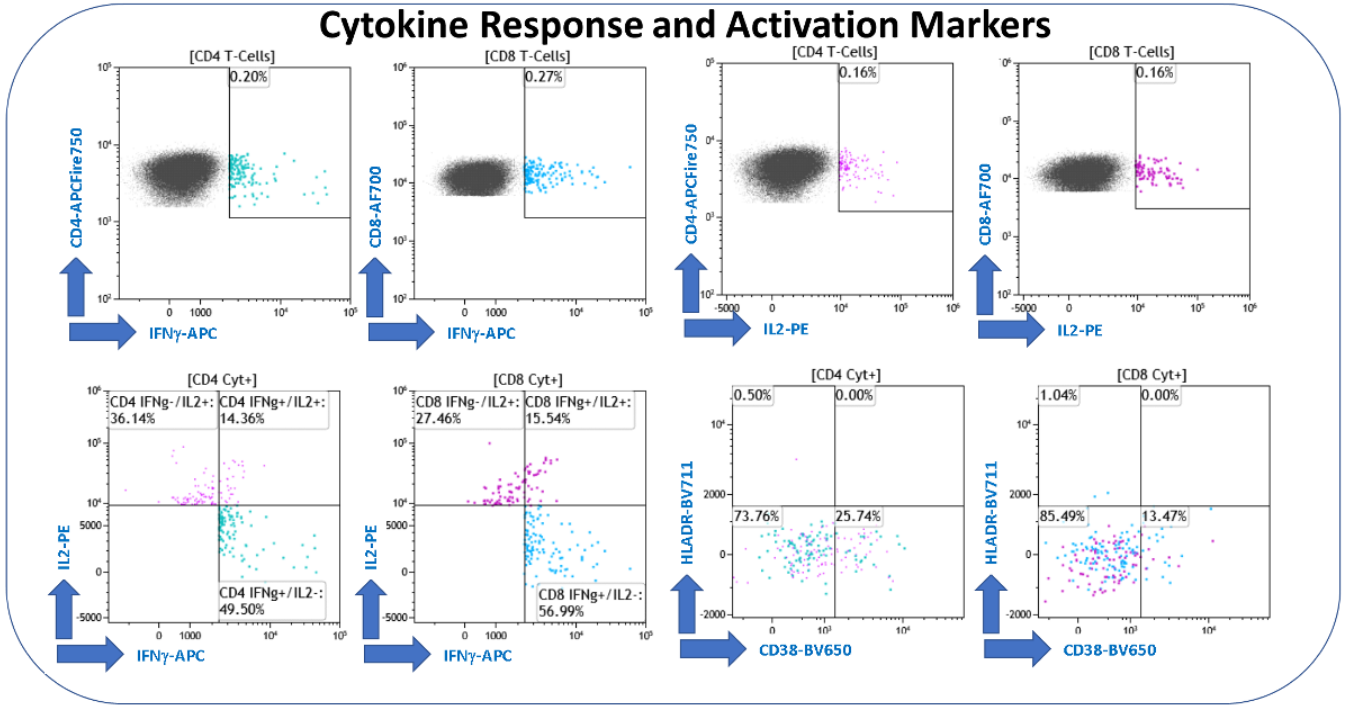


Figure 2.2 Gating strategy for cytokine positive cell identification. Gating within Kaluza was used to identify the percentages of CD4 and CD8 T-cells that were expressing IL-2 or IFN-γ and the subsets of cytokine positive T-cells. CD4 T-cells previously gated in phenotypic identification were plotted on CD4/IFN-γ antibody plots to identify the percentage of CD4+/IFN-γ cells. CD8 T-cells previously gated in phenotypic identification were plotted on CD8/IFN-γ antibody plots to identify the percentage of CD8+/IFN-γ cells. CD4 T-cells previously gated in phenotypic identification were plotted on CD4/IL-2 antibody plots to identify the percentage of CD4+/IL-2 cells. CD8 T-cells previously gated in phenotypic identification were plotted on CD8/IL-2 antibody plots to identify the percentage of CD8+/IL-2 cells. The CD4 cytokine (Cyt+) cells were identified as the CD4+/IFN-γ+ and CD4+/IL-2+ and the percentages of IFN-γ-/IL-2+, IFN-γ+/IL-2- and IFN-γ+/IL-2+ were found plotting CD4 Cyt+ cells on IL-2 /IFN-γ plots. The CD8 cytokine (Cyt+) cells were identified as the CD8+/IFN-γ+ and CD8+/IL-2+ and the percentages of IFN-γ-/IL-2+, IFN-γ+/IL-2- and IFN-γ+/IL-2+ were found plotting CD8 Cyt+ cells on IL-2 /IFN-γ plots. The CD4 Cyt+ and CD8 Cyt+ cells were plotted separately on HLA-DR/CD38 to identify the percentages of these cells that were activated (HLA-DR+ and or CD38+).

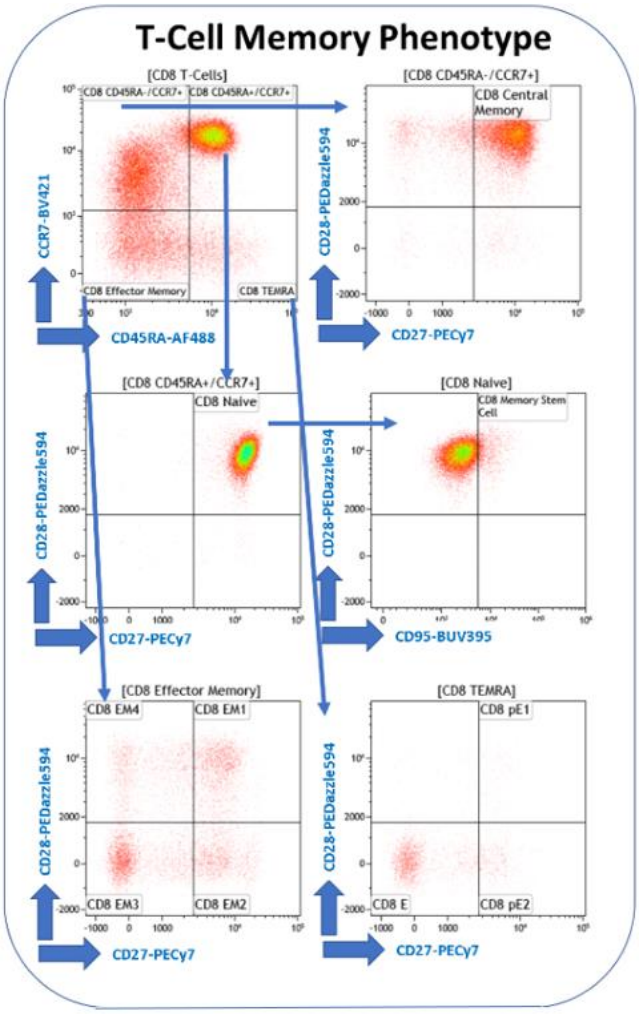


Figure 2.3 Gating strategy for memory T cell populations. Further gating within Kaluza was used to differentiate and identify the memory T-cell populations. CD45RA/CCR7 plots were used to differentiate CD8 cells into four memory subset, CD45RA-/CCR7+, CD45RA+/CCR7+, CD8 Effector memory (CD45RA-/CCR7-) and CD8 TEMRA (CD45RA+/CCR7-). The cells in each of these subsets were then plotted against CD28/CD27. The CD45RA-/CCR7+ cells that were CD28+/CD27+ were identified as CD8 central memory cells. The CD45RA+/CCR7+ cells that were CD28+/CD27+ were identified as CD8 Naïve cells and then these CD28+/CD27+ cells were plotted against CD95/CD28 and those that were CD95+/CD28+ were identified as CD8 memory stem cells. The Effector memory CD8 cells segregated into CD8 EM4 cells (CD27-/CD28+), CD8 EM1 cells (CD27+/CD28+), CD8 EM2 (CD27+/CD28-) and CD8 EM3 (CD27-/CD28-). The TEMRA CD8 cells were discerned into CD8 pE1 (CD27+/CD28+), CD8 pE2 (CD27+/CD28-) and CD8 E (CD27-/CD28-). The CD4 T-cells were gated via the same strategy though are not shown.

2.2.6 Production and use of Coronavirus Pseudo types/Neutralisation assay

2.2.6.1 SARS-CoV-2 pseudo type particles

To generate pseudo type particles 2ug phCMV-5349 MLV Gag/Pol packaging construct plasmid, 2ug pTG126 Luciferase encoding reporter plasmid and 2ug of SARS-CoV-2 spike plasmid was mixed with 300uL of OptiMEM. We generated 4 different spike variants: Lineage A (Wuhan), B.1 (D614G), B.1.351 (South African) and P.1 (Brazil). To each plasmid mix, 24uL polyethyleneimine (PEI) diluted in 276uL OptiMEM was added and left for 30 minutes at RT. The negative control virus lacking S was created through omitting the addition of SARS-CoV-2 spike plasmid.

The 600µL plasmid: PEI mixes were then added in a dropwise fashion to each respective dish of HEK293T cells and the constructs were left to incubate at 37°C, 5% CO_2 for 6 hours. The media on the cells was then removed and replaced with 10mL complete media. After 72 hours incubation the supernatant was harvested and passed through a 0.45µM pore-size syringe and stored at 4°C or used immediately on the VeroE6 cells. The pseudo typed spike was not titrated after production as the neutralisation curves were normalised to a 100% (no inhibition) and 0% (background) for each run.

2.2.6.2 Neutralisation Assays

To measure neutralising antibody activity in serum samples a single point dilution of 1:200 was made of sera in against spike variants was made in V-bottomed 96-well plates. A single point dilution was used as a subjective marker of level of protection. A single point 1:200 dilution was selected as opposed to a sera dilution series because previous SARS-CoV-2 challenge studies in non-human primates have shown that serum samples yielding an in vitro IC50 neutralization titer of at least 1:200 are protective (Vogel *et al*, 2021). Each well had 270µL of SARS-Cov-2 pseudotype added for each sample as well as one extra as a control, in addition to 30µL of heat-inactivated diluted sera or 30µL PBS accordingly. The diluted antibody plate was then mixed and left for an hour at RT. After, this mix was

transferred into the previously seeded 96-well white plates with VeroE6 cells 100µL of the pseudoparticles and 100µL of each diluted antibody sample was added in triplicate in addition to the control "antibody" mix. The plate was then incubated for 4 hours at 37°C, 5% CO_2. The wells were then each topped up with 200µL of complete media and left to incubate for 72 h at 37°C, 5% CO_2. The cell media was discarded and 50µL of cell lysis buffer (Promega, made up according to manufacturer's protocol) was added to each well and rocked for 15 minutes at RT. The luminescence was detected using a luminometer (BMG Labtech FluoStar Omega plate reader) at PMT gain 3600 programmed to inject 50µL of luciferase substrate (Promega). The plate was shaken for 2 seconds then read for its luminescence after 1 second. Neutralisation curves were calculated using the delta E as 0% and uninhibited control as 100%.

2.2.7 Anti-S1 and anti-nucleocapsid ELISA

Spike and nucleocapsid specific antibody levels in the serum samples collected was measured in an ELISA using the Opentons OT-2. The Spike used was recombinant full-length SARS-CoV-2 spike (Lineage A (Wuhan)) glycoprotein, manufactured in Chinese hamster ovary (from the Native Antigen Company) and the Baculovirus-expressed Nucleocapsid were produced by SinoBiologicals (Stratech Scientific UK). Assay plates (384 well Maxisorp (NUNC)) were coated in 20 µL per well of 0.5 µg.mL−1 Spike or Nucleocapsid in carbonate-bicarbonate buffer (CBC; Merck). Plates were sealed and incubated overnight at 4 °C. A ThermoFisher Wellwash Versa plate washing robot was used to wash the plates 3 times with PBS with 0.05% Tween 20 (PBS-T) each time. Plates were then blocked overnight at 4 °C with 100 µL of 3% skimmed milk powder (w/v) in PBS and 0.05% sodium azide (PBS-MA). Following 3 more washes the plates were filled, in duplicate wells, with 20µL of diluted sera, initial patient sera was diluted to 1:218 in 3% skimmed milk powder in PBS containing 0.05% Tween 20 and 0.05% sodium azide, as were the randomly selected negative control serum samples (samples 20/B770-35 and 20/B770-36 from NIBSC). To detect the Spike and nucleocapsid specific IgG the plates were incubated for an hour, washed, and 20 µL of gamma chain-specific anti-human IgG-HRP conjugate (Sigma A0170–1ML)

at 1:30,000 dilution was added to the plates. After incubating for one hour at 21 °C the plate was washed three times and 40 µL of One-step Ultra-3,3’,5,5’-Tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific) was added to each well. The plates were incubated for 20 minutes at RT, then 40 µL of 2N H_2SO_4 was added to each well and to read the results the GlowMax Explorer microplate reader (Promega) was used at 450nm Absorbance.

2.2.7 Optimisation of cell staining and assay development for flow cytometry analyses

For the successful staining of cells specific to the SARS-CoV-2 peptides for flow cytometry, it was necessary to optimize various parts of the PBMC stimulation as well as both the extracellular and intracellular staining of these cells. Samples HC01-04 were used for these optimisations.

The efficiency of the positive controls was tested by measuring cellular activation responses using flow cytometry staining of effector cytokines IFN-γ and IL-2 (A cell stimulation was deemed positive if the percentage of cells in the cytokine gate was above that of the unstimulated sample, the unstimulated response was then subtracted from the total.) Initially, Peptivator EBV BMLF1 (Miltenyi Biotech, UK) was used as the positive control, this consists of peptides that cover the complete sequence of the Epstein-Barr Virus BMLF-1 protein. This peptide stimulation causes the secretion of effector cytokines, which then allow the detection and isolation of antigen-specific T cells. The effectiveness of this positive control was assessed against the PepTivator® CEF MHC Class I Plus, human (Miltenyi Biotech, UK) that consists of MHC-1– specific peptides derived from cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and influenza virus and have been shown to elicit IFN-γ release from CD8+ T cells in most individuals. In addition to using PepTivator® CEF MHC Class I Plus, a positive control combining PMA and Ionomycin were included, together these components can induce T cell activation and proliferation. All the tested controls produced a cytokine response indicating the stimulation worked, although the extent of the responses varied, suggesting that the CEF and PMA + Ionomycin stimulations indicated more positive T-cell activation, specifically the proportion of IFN- γ and IL-2 positive cells. The

antibodies in Table 2.2 and Table 2.3 were optimised by titrating the antibodies through staining single colour controls either by myself or had been previously titrated within Professor Lucy Fairclough's Lab group.

2.2.5 Statistical analysis

Data and statistical analyses were done in GraphPad Prism 9 unless otherwise stated. Graphs presenting the data were made using the percentages of cells expressing positive Spike, Membrane or Nucleocapsid results provided they were 10% higher than the corresponding results from unstimulated samples.

3. Results

3.1 Patient demographics

This study utilised samples from 26 individuals that had attended NHS Coventry and Warwickshire NHS Trust. The samples were categorised according to their disease severity and in the case of spike-specific results they were further divided according to their vaccination status. The demographics of the patients from which the samples were taken from are shown in Table 3.1. Patients deemed as having a "mild" disease severity were not hospitalised due to their COVID-19 infection whereas both those in moderate and severe were, which is why the categories were combined for analysis.

Clinical and Vaccination Status	Size	Ethnicity	Gender	Age at sample collection (Years)	Time between disease and sample collection (days)	Time between 1st vaccine and sample collection (days)	Time between second vaccine and sample collection (days)
Negative – 2nd vaccine	1	Other British	Female	49	N/A	N/A	20
Suspected – 1st vaccine	1	Other white	Male	41	N/A	8	N/A
Suspected – 2nd vaccine	1	Pakistani	Male	48	N/A	N/A	20
Asymptomatic	1	White British	Female	31	N/A	N/A	N/A
Mild - not vaccinated	6	2= White British 1= Afro-Caribbean 3= Pakistani	3 Males and 3 Females	Avg. 37 (Range 27-51)	Avg. 119 (Range 30-288)	N/A	N/A
Mild – 1st vaccine	5	3=White British 2=Pakistani	1 Male and 4 Females	Avg. 36 (Range 28-59)	Avg. 255 (Range 118-303)	Avg. 43 (Range 28-63)	N/A
Mild – 2nd vaccine	2	2= White British	2 Females	Avg. 53 (Range 51-55)	Avg. 244 (Range 153-335)	N/A	Avg. 65 (Range 40-90)
M/S – not vaccinated	5	4= White British 1= Other White	3 Males and 2 Females	Avg. 59 (Range 53-73)	Avg. 185 (Range 72- 321)	N/A	N/A
M/S – 1st vaccine	3	3= White British	2 Males and 1 Female	Avg. 66 (Range 59-78)	Avg. 247 (Range 128-348)	Avg. 22 (Range 10-35)	N/A

Table 3.1 detailing the demographics of the patients in our sample set as well as their times since disease and vaccination. Demographic data was collected by NHS Coventry and Warwickshire NHS Trust upon attendance or admission to the hospital following SARS-Cov-2 infection. N/A is used when data not collected or not appropriate. In some cases, time between disease and sample collection, and between disease and vaccination were only given in months, therefore an estimated conversion to days was included for numerical continuity. Patients named "suspected" were named so because they had symptoms of SARS-CoV-2 though could not be PCR tested for the virus at the time or have their antibodies tested/ it came back negative

3.2 Cell mediated response to SARS-CoV-2 infection in convalescent patients

This immunoassay was developed to quantitively investigate spike-specific, membrane-specific, and nucleocapsid-specific cytokine producing CD8 and CD4 T-cells, specifically those producing IFN- γ and IL-2. Figure 3.1 demonstrates spike-specific cellular responses classified by their vaccination status whereas Figure 3.2 shows these responses according only to disease severity. Only the spike-specific results were further divided into vaccination status as the vaccines used, Pfizer-BioNTech BTN162b2 and AstraZeneca AZD1222 (ChAdOx1), both use full-length spikes, so the spike-specific response would be expected to change after vaccination unlike the membrane and nucleocapsid-specific response. The sample size was likely too small to infer any statistically significant differences, but the data can be used to draw some possible conclusions.

The data in Figure 3.1 in the moderate/severe group with one vaccination compared to no vaccinations show in general, in every graph excluding Figure 3.1 (I) "only IFN-γ producing CD8 T-cells", that the vaccinated group has a higher percentage of cytokine producing cells or remain at about the same level. Patients of mild disease severity with 1 or 2 vaccinations show more varied results. The Figure 3.1 (G) showing IFN-γ and IL-2 producing CD8 T-cells, clearly demonstrates the average response increases after patients were vaccinated once, and again after patient's 2nd vaccination. More often, those with mild disease who were unvaccinated have a higher T-cell response than those with one or two vaccinations, as shown in Figure 3.1 (A, B, E, F, H and K), or there is not a clear pattern/ low results such as in 3.1 (C, D, I, J and L). T-cell response would be expected to boost following a 2nd vaccination. This result may be due to the small sample size. Another factor could be the age difference in the groups, for example both mild-2nd vaccinated individuals are over 50 years old whereas those in the mild-1st vaccinated category are under 33 years old (with exception to one 59-year-old). Comparing each of these individuals' pre-vaccine as well as after each vaccine would have led to greater insight. Table 3.1 indicates a large variety in days between sample collection and disease, those in the mild-unvaccinated group have a lower

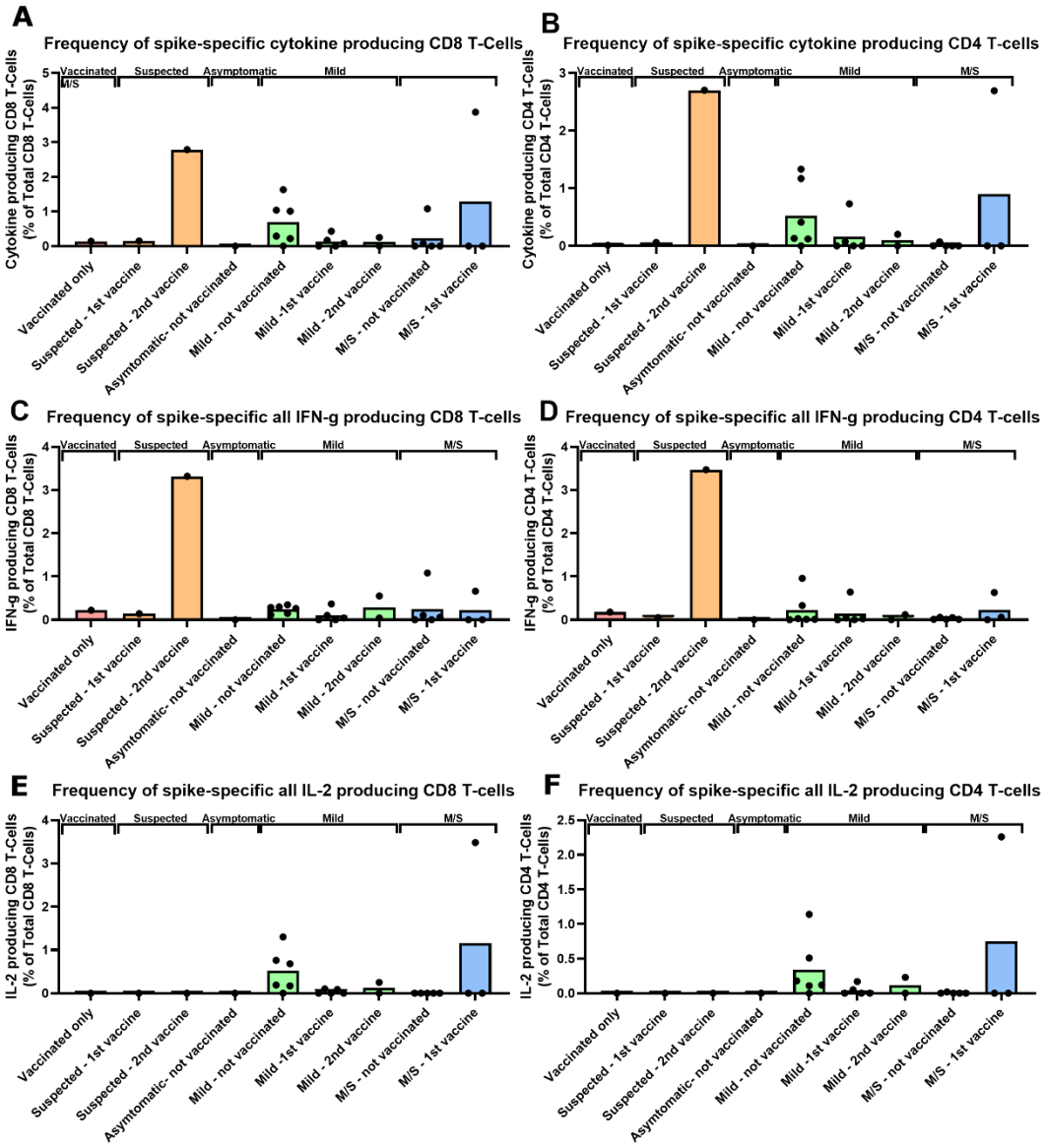
average number of days between their sample and collection to the other to mild disease categories which may have affected their T-cell response.

The spike-specific CD8 IFN-γ only producing cells, Figure 3.1 (I) show more variability than all other categories. Additionally, more patients, of differing disease severities, have a higher percentage of CD8 cells expressing only IFN-γ, than other cells producing cytokines. Nine patients have more than 10% of their cytokine producing T-cells being CD8 cells that only express IFN-γ. The equivalent graphs for membrane-specific and nucleocapsid-specific results show slightly different findings. The membrane-specific only IFN-γ producing CD8 T-cells Figure 3.3 (I) have a much lower percentage response, with most patients displaying less than 10%. Although all three of the stimulations show a higher response in the M/S group on average than the mild group, the spike-specific table separated by vaccination status shows that the M/S groups IFN-γ response declines after vaccination. The nucleocapsid-specific responses in Figure 3.4 (I) demonstrate a higher percentage of cells than those that are membrane-specific, which may highlight that there is more memory of nucleocapsid protein than that of the membrane protein.

There are some notably high percentages of cytokine producing T-cells, such as one patient in the "mild-not vaccinated" category has over 15% of their cytokine producing T-cells being both IFN-γ and IL-2 producing CD4 T-cells (Figure 3.1 (H)), whereas every other patient sample shows to have less than 5%. This exaggerated difference also doesn't extend to CD8 cells producing both cytokines stained ((Figure 3.1 (G)).

As there is only one patient in each group in the vaccinated, suspected 1st vaccine and suspected 2nd vaccine groups, we can't see overall trends in the groups. Although in all 3 groups there is an exaggerated difference in the Figure 3.1 (I, J, K and L) graphs, in both IFN-γ graphs, whether it be CD8 T-cells or CD4 T-cells, there is a percentage of positive cells that drops to zero in the IL-2 only graphs. The stark difference in IFN-γ response after stimulation compared to IL-2 may suggest a difference in

memory to SARS-CoV-2 in patients who in the vaccinated patient's case didn't have the disease and in the suspected cases that may have had a very mild infection. This difference is only apparent in the spike-specific response, the membrane and nucleocapsid response tends to be low, though comparatively higher in the membrane-specific graphs. The antibody reactivity to the nucleocapsid protein (Figure 3.8 (C)) is similarly low, though the spike reactivity increased after the second vaccination (Figure 3.8 (A)).



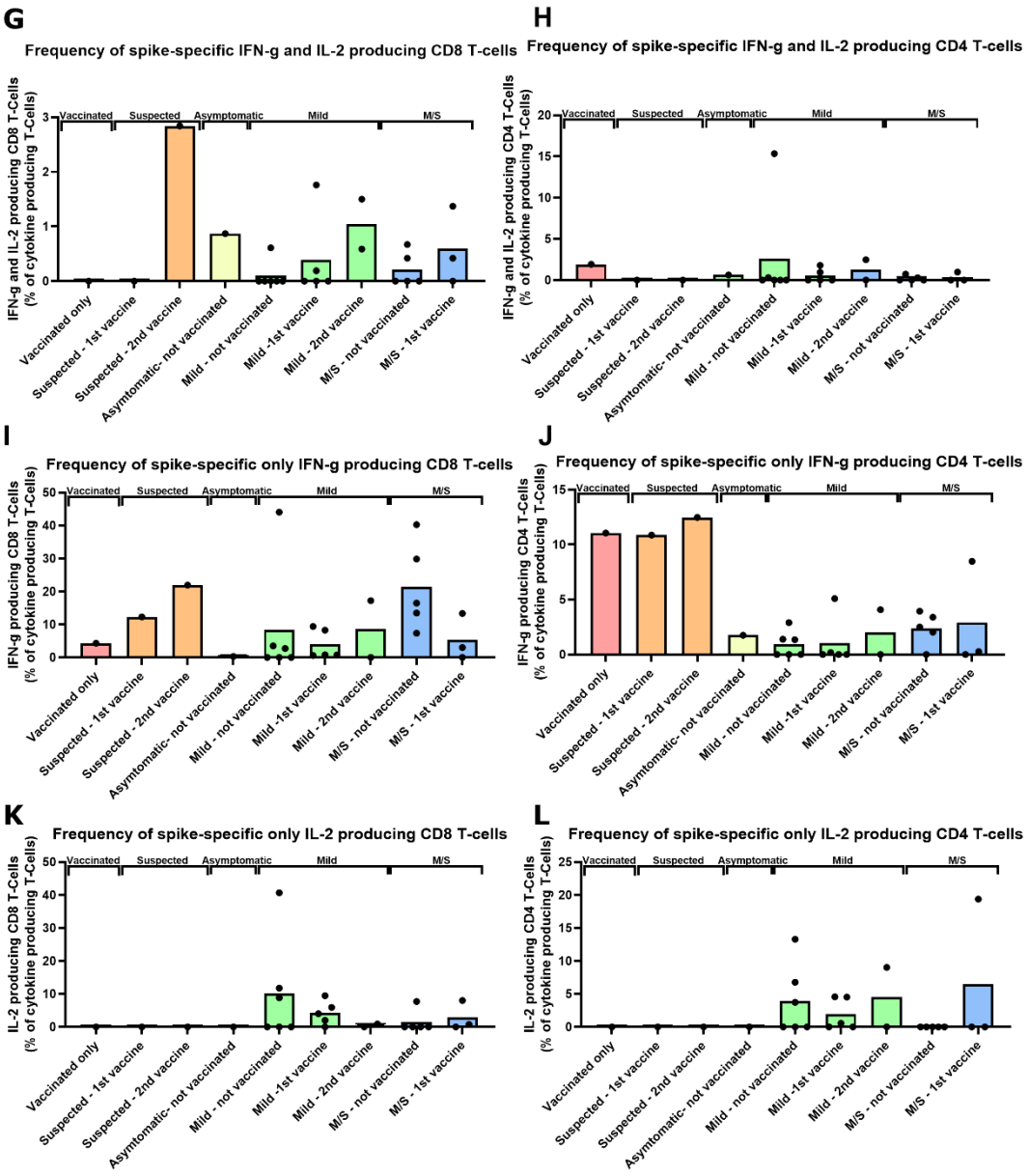
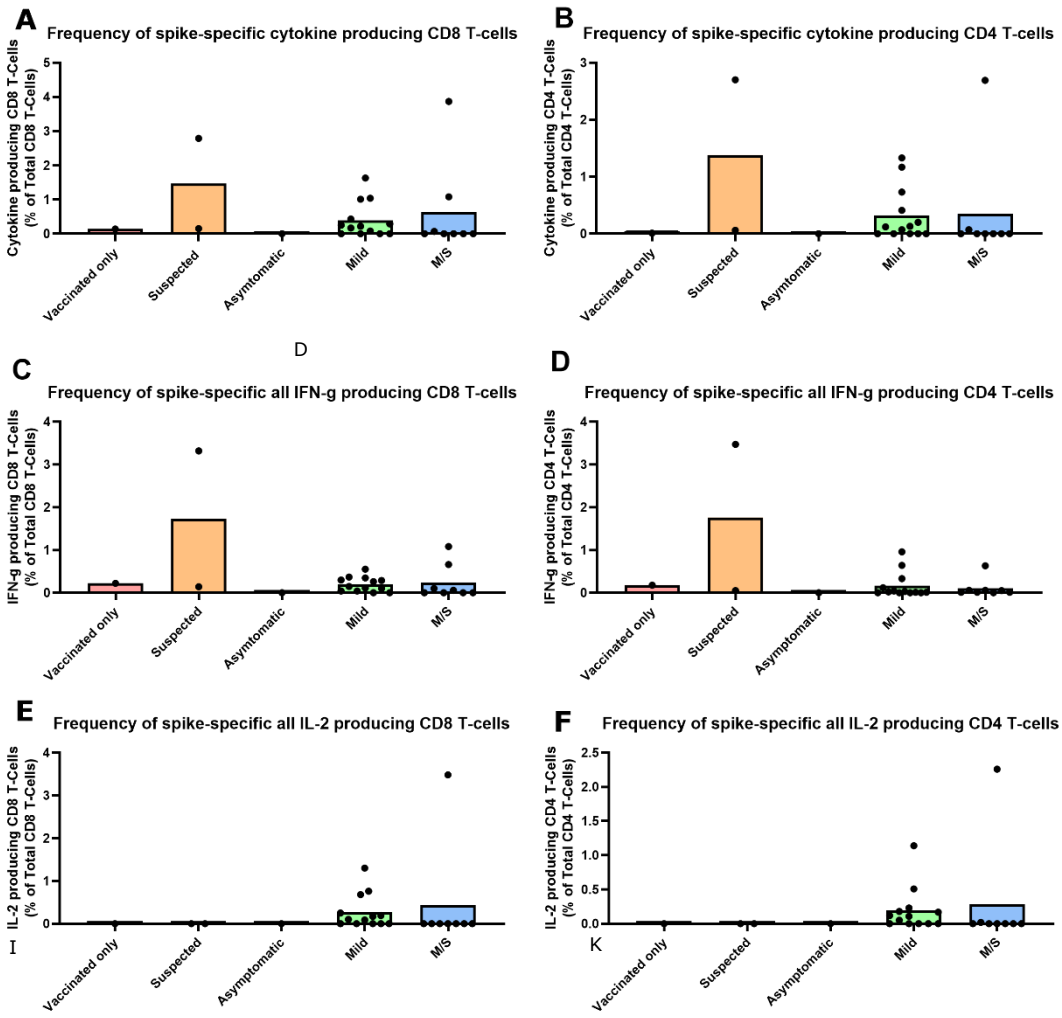


Figure 3.1 Cytokine response in Spike-specific CD8 and CD4 T-cells in convalescent COVID-19 patients. Flow cytometry was used to gate cells into their cytokine and CD8 or CD4 groups. In all graphs M/S refers to moderate/severe disease severity. A "positive" result was determined by subtracting the unstimulated patient sample from the result of the spike stimulation, if this value was above zero it was included if not it was listed as zero. Each line refers to the mean result. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant.

Figure 3.2 showing the spike-specific cellular responses without the vaccination data shows that some of the m/s patients results are similar. In 3.2 (H) this is shown as all the patients CD4 T-cells producing IFN-γ and IL-2 are close to zero, as the mild category also shows a very low response but slightly higher than m/s. There could also be a link with the age range of patients who suffered a higher severity of the disease also being older so their immune response may be lower. In Figure 3.2 (B, D, E, F and L) all except one in the m/s disease severity have close to zero percent. Interestingly, the Figure 3.1 graphs show that it is one of the patients who had been vaccinated who had the larger response, though the other vaccinated patients do not show the same jump in response after vaccination. The data for each patient shows that it is P025 in all the previously mentioned graphs, they are the youngest patient in the m/s-1st vaccine category at 59, male and had COVID-19 the most recently with a positive test 128 days before their blood sample. The other two in the category had a positive PCR test 266 and 348 days previously. These differences may have made an impact, comparing all three patients' pre-vaccine results and taking blood samples at set points after infection and vaccination may have illustrated whether the patient's response was distinct. Though as the sample size is just 3 patients in the same category it would be difficult to suggest the reason for the large difference, through increasing our sample size average it would become clearer and more reliable.


A
Frequency of spike-specific cytokine producing CD8 T-cells
Cytokine producing CD8 T-Cells (% of Total CD8 T-Cells)
Vaccinated only
Suspected
Asymtomatic
Mild
M/S
B
Frequency of spike-specific cytokine producing CD4 T-cells
Cytokine producing CD4 T-Cells (% of Total CD4 T-Cells)
Vaccinated only
Suspected
Asymtomatic
Mild
M/S
D
C
Frequency of spike-specific all IFN-g producing CD8 T-cells
IFN-g producing CD8 T-Cells (% of Total CD8 T-Cells)
Vaccinated only
Suspected
Asymtomatic
Mild
M/S
D
Frequency of spike-specific all IFN-g producing CD4 T-cells
IFN-g producing CD4 T-Cells (% of Total CD4 T-Cells)
Vaccinated only
Suspected
Asymptomatic
Mild
M/S
E
Frequency of spike-specific all IL-2 producing CD8 T-cells
IL-2 producing CD8 T-Cells (% of Total CD8 T-Cells)
I
Vaccinated only
Suspected
Asymptomatic
Mild
M/S
F
Frequency of spike-specific all IL-2 producing CD4 T-cells
IL-2 producing CD4 T-Cells (% of Total CD4 T-Cells)
K
Vaccinated only
Suspected
Asymptomatic
Mild
M/S

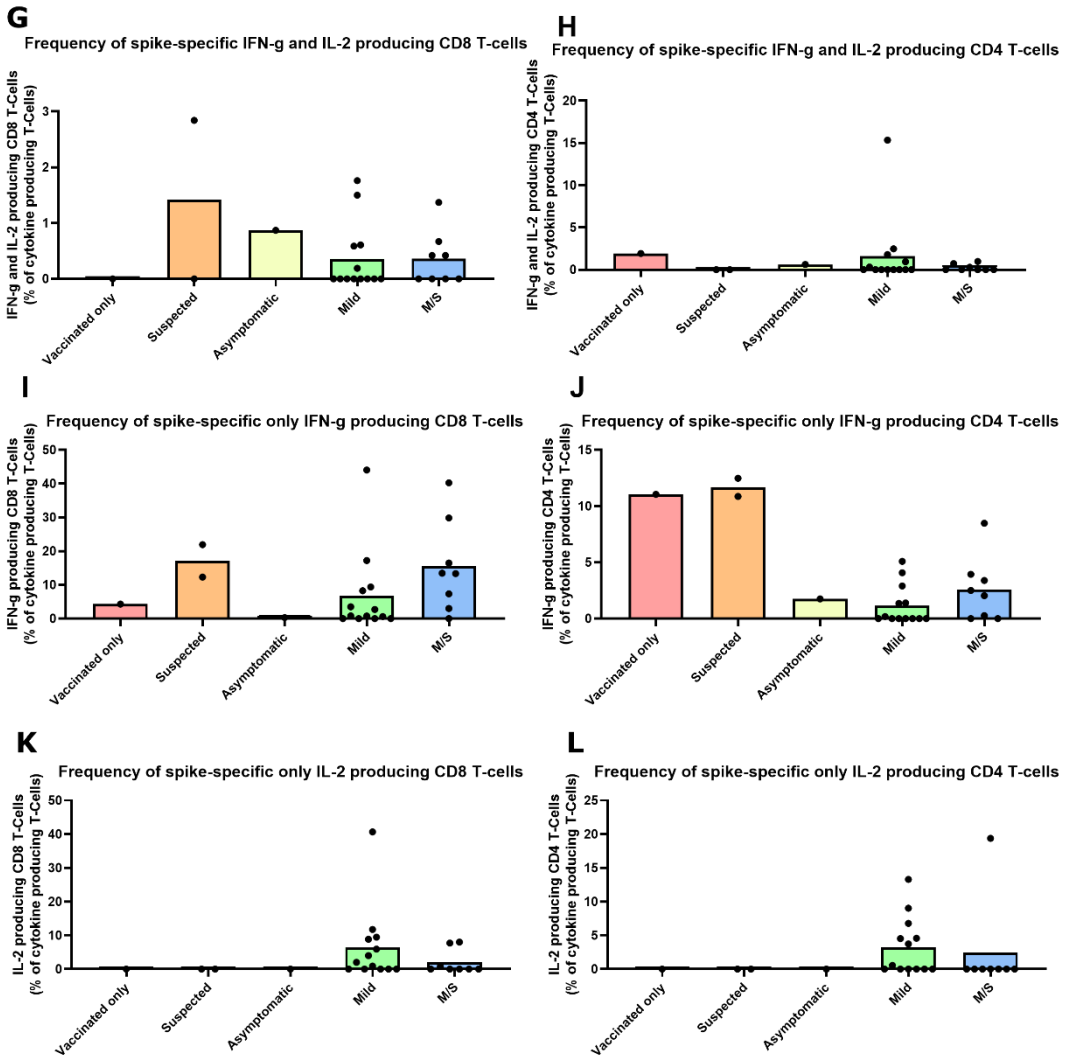
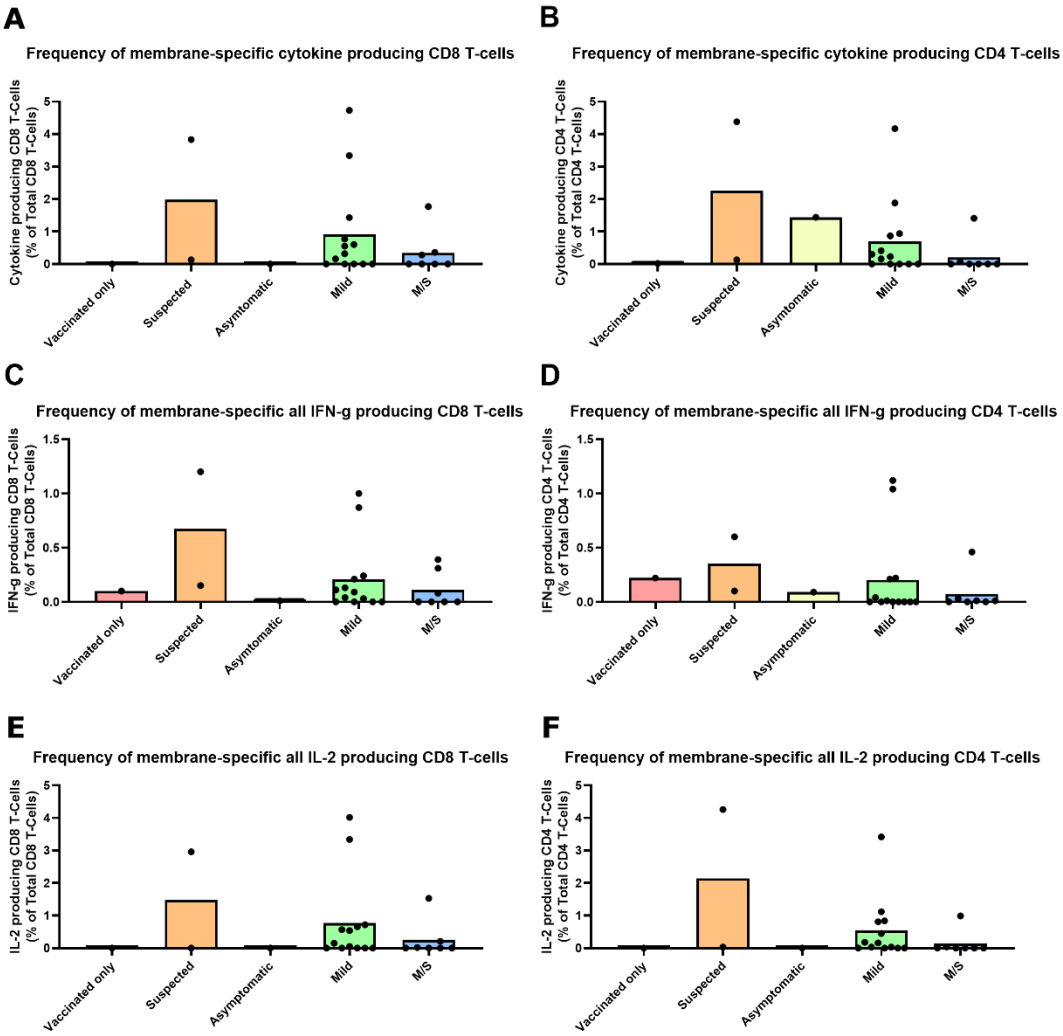


Figure 3.2 Cytokine response in Spike-specific CD8 and CD4 T-cells in convalescent COVID-19 patients regardless of vaccination status. Flow cytometry was used to gate cells into their cytokine and CD8 or CD4 groups. In all graphs M/S refers to moderate/severe disease severity. A "positive" result was determined by subtracting the unstimulated patient sample from the result of the spike stimulation, if this value was above zero it was included if not it was listed as zero. Each line refers to the mean result. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant.

Seasonal coronavirus infections are thought to affect memory response, particularly regarding the membrane and nucleocapsid proteins as these proteins in SARS-CoV-2 share more similarity to those in seasonal coronaviruses. Here we show membrane and nucleocapsid-specific

cytokine producing T cells (Figure 3.3 and Figure 3.4 respectively) that the individual who has only been vaccinated (and the vaccine only offers memory to spike) has some positive responses in their membrane-specific and nucleocapsid-specific response (specifically IFN-γ producing CD4 T cells), possibly indicating they have previously been infected with similar structured seasonal coronaviruses.

Many patient's responses to the membrane and nucleocapsid protein, within both mild and m/s disease categories, are low and close to zero. The percentages of patients with mild disease severity responding to the membrane protein are generally low (Figure 3.3) though are higher than that of those in the m/s disease severity patients.


A
Frequency of membrane-specific cytokine producing CD8 T-cells
Cytokine producing CD8 T-Cells (% of Total CD8 T-Cells)
B
Frequency of membrane-specific cytokine producing CD4 T-cells
Cytokine producing CD4 T-Cells (% of Total CD4 T-Cells)
C
Frequency of membrane-specific all IFN-g producing CD8 T-cells
IFN-g producing CD8 T-Cells (% of Total CD8 T-Cells)
D
Frequency of membrane-specific all IFN-g producing CD4 T-cells
IFN-g producing CD4 T-Cells (% of Total CD4 T-Cells)
E
Frequency of membrane-specific all IL-2 producing CD8 T-cells
IL-2 producing CD8 T-Cells (% of Total CD8 T-Cells)
F
Frequency of membrane-specific all IL-2 producing CD4 T-cells
IL-2 producing CD4 T-Cells (% of Total CD4 T-Cells)
Vaccinated only
Suspected
Asymtomatic
Asymptomatic
Mild
M/S

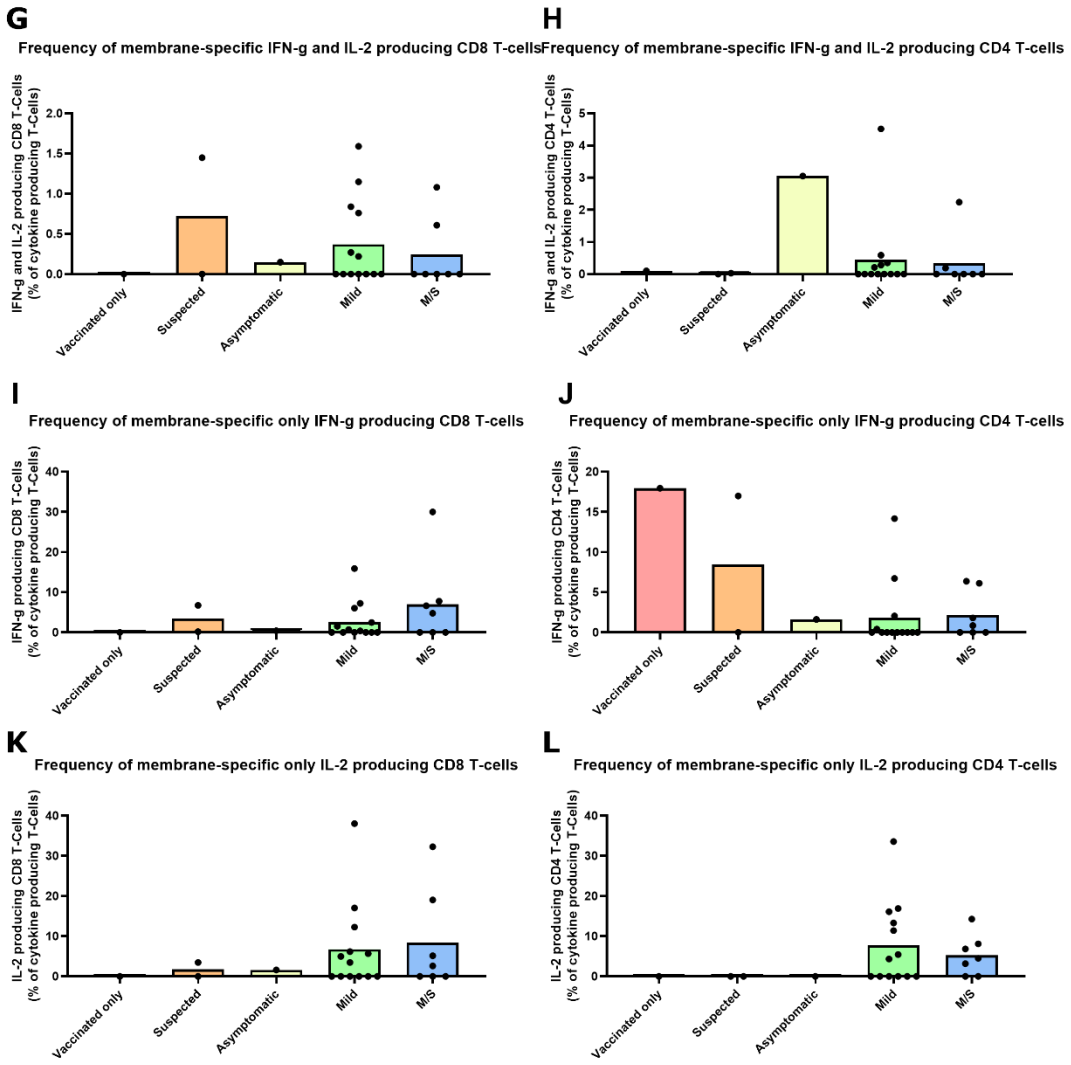
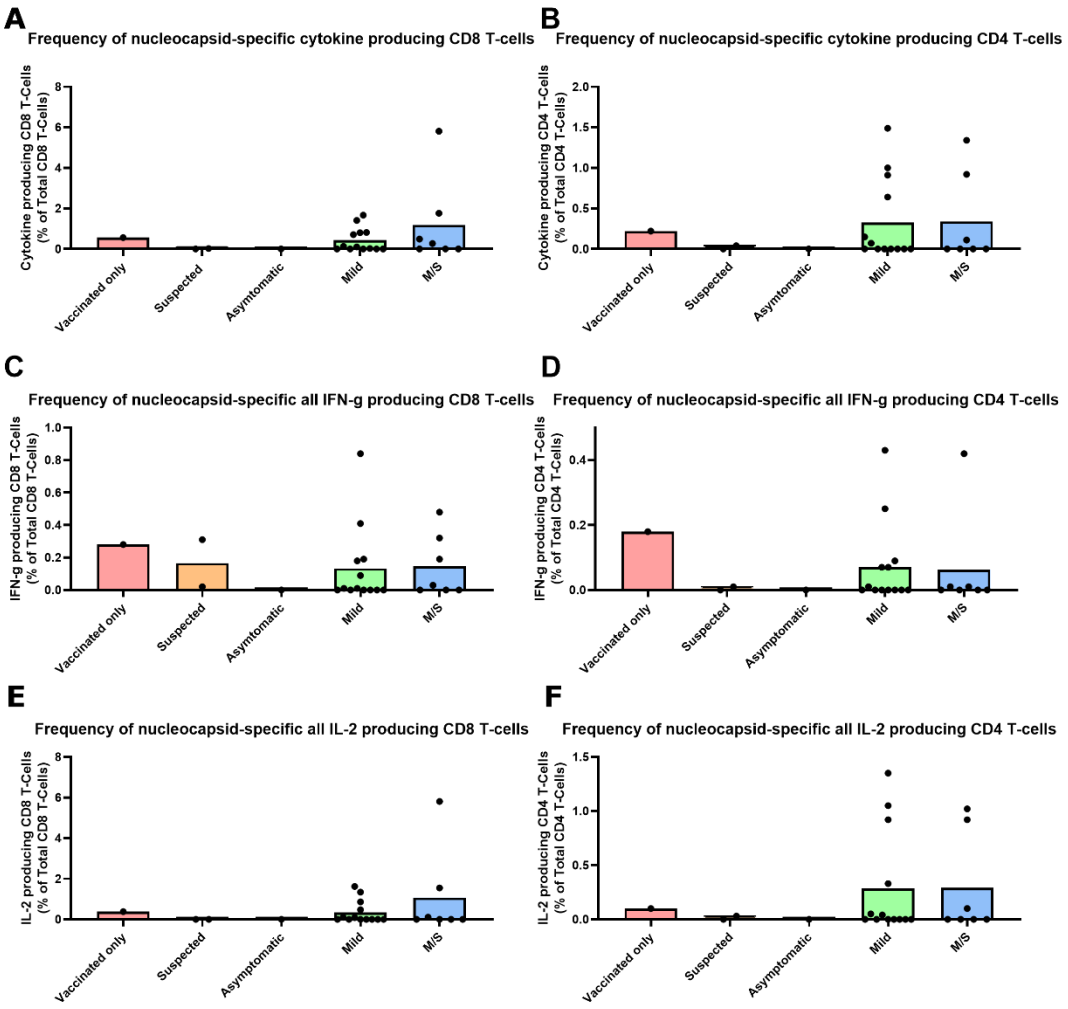


Figure 3.3 Cytokine response in Membrane-specific CD8 and CD4 T-cells in convalescent COVID-19 patients. Flow cytometry was used to gate cells into their cytokine and CD8 or CD4 groups. In all graphs M/S refers to moderate/severe disease severity. A "positive" result was determined by subtracting the unstimulated patient sample from the result of the spike stimulation, if this value was above zero it was included if not it was listed as zero. Each line refers to the mean result. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant.

The nucleocapsid response, Figure 3.4, differs to the membrane response in that the average response of the m/s is higher than mild more often than shown in the membrane-specific data. As shown in graphs 3.4 (A, D, I and J), these increases are small and could possibly be linked to those with m/s disease being exposed to the virus for longer so are exposed

more to the nucleocapsid protein than those who suffered a milder infection. Additionally, the results in Figure 3.4 (I, J, K and L) show that the responses in a few patients are vastly higher than the majority at zero in both the mild and m/s disease severity patients. The percentages of cell-mediated responses in these cases varies massively. The high percentages are not correlated with disease severity, or time since disease data.


A
Frequency of nucleocapsid-specific cytokine producing CD8 T-cells
Cytokine producing CD8 T-Cells (% of Total CD8 T-Cells)
B
Frequency of nucleocapsid-specific cytokine producing CD4 T-cells
Cytokine producing CD4 T-Cells (% of Total CD4 T-Cells)
C
Frequency of nucleocapsid-specific all IFN-g producing CD8 T-cells
IFN-g producing CD8 T-Cells (% of Total CD8 T-Cells)
D
Frequency of nucleocapsid-specific all IFN-g producing CD4 T-cells
IFN-g producing CD4 T-Cells (% of Total CD4 T-Cells)
E
Frequency of nucleocapsid-specific all IL-2 producing CD8 T-cells
IL-2 producing CD8 T-Cells (% of Total CD8 T-Cells)
F
Frequency of nucleocapsid-specific all IL-2 producing CD4 T-cells
IL-2 producing CD4 T-Cells (% of Total CD4 T-Cells)
Vaccinated only
Suspected
Asymtomatic
Asymptomatic
Mild
M/S

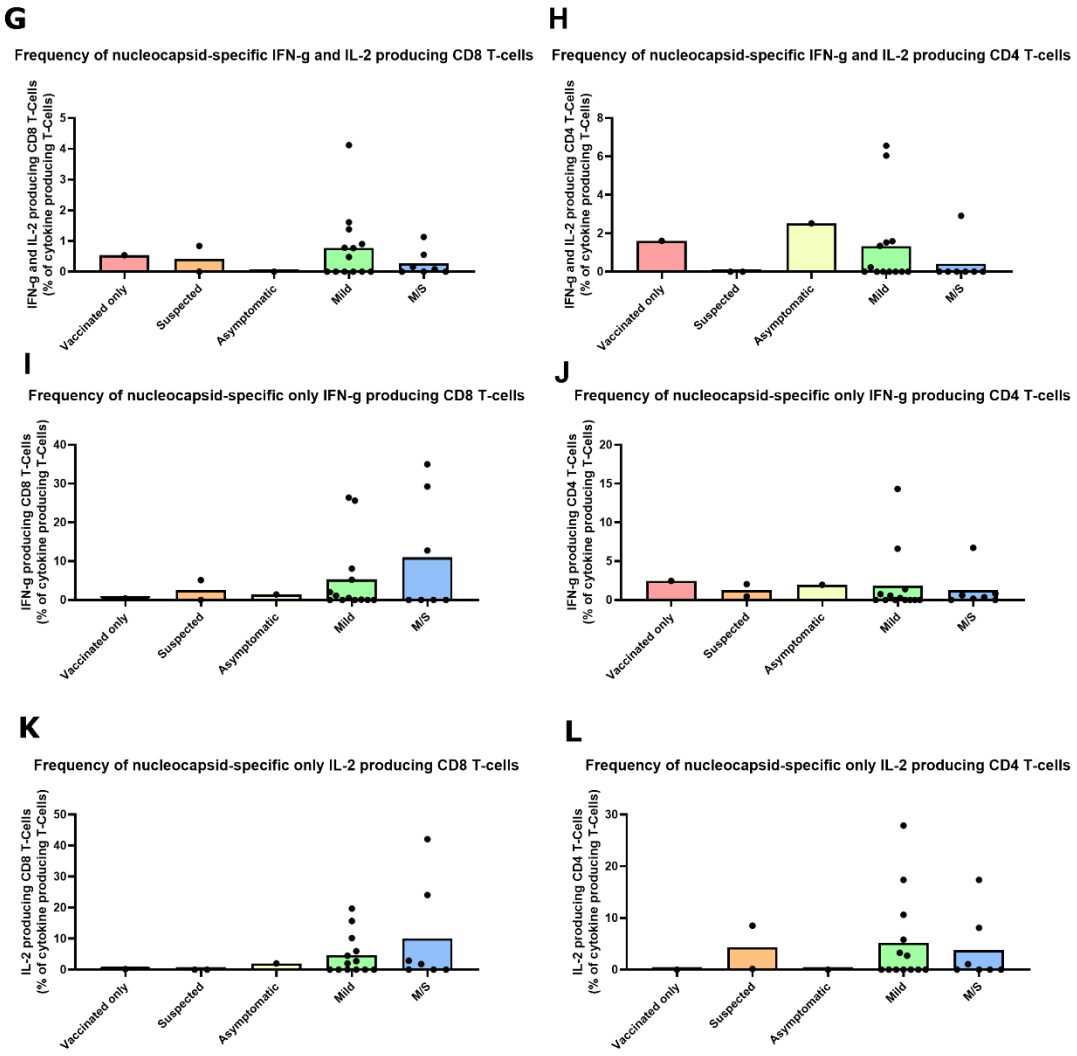
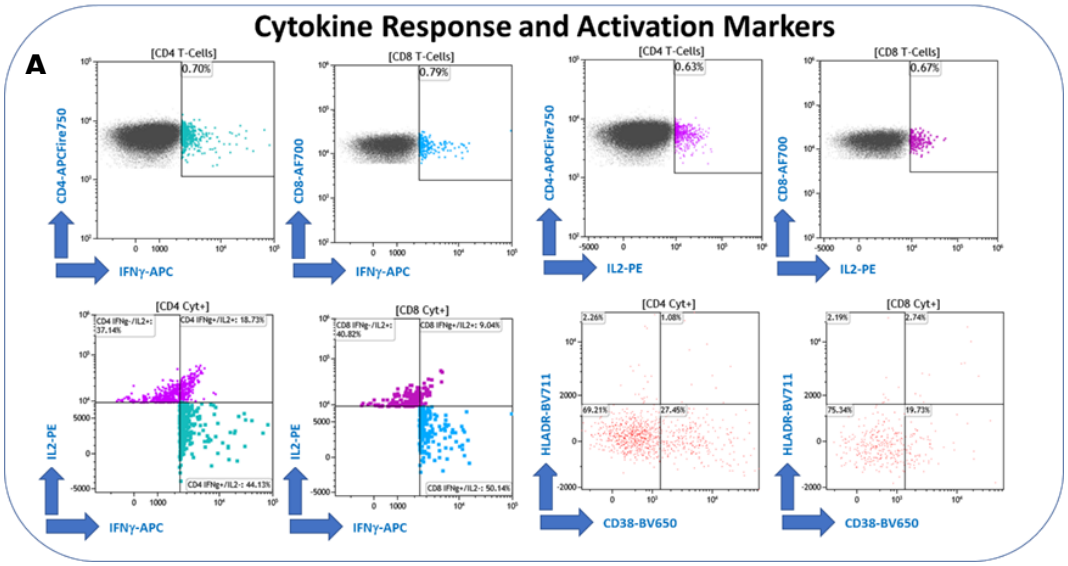
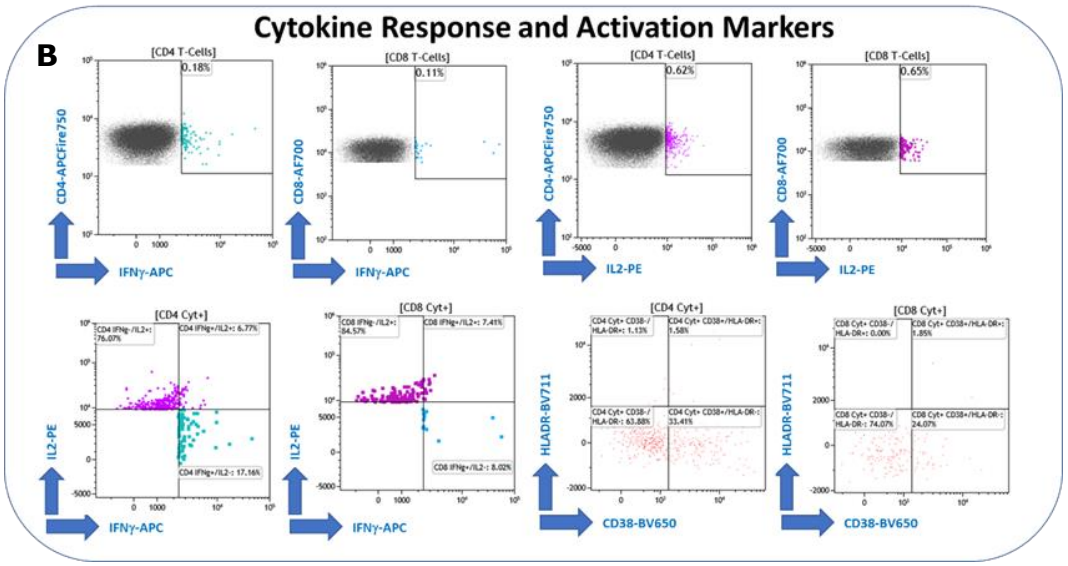


Figure 3.4 Cytokine response in Nucleocapsid-specific CD8 and CD4 T-cells in convalescent COVID-19 patients. Flow cytometry was used to gate cells into their cytokine and CD8 or CD4 groups. In all graphs M/S refers to moderate/severe disease severity. A "positive" result was determined by subtracting the unstimulated patient sample from the result of the spike stimulation, if this value was above zero it was included if not it was listed as zero. Each line refers to the mean result. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant.


A
Cytokine Response and Activation Markers
[CD4 T-Cells]
0.70%
CD4-APCFire750
IFNγ-APC
[CD8 T-Cells]
0.79%
CD8-AF700
IFNγ-APC
[CD4 T-Cells]
0.63%
CD4-APCFire750
IL2-PE
[CD8 T-Cells]
0.67%
CD8-AF700
IL2-PE
[CD4 Cyt+]
CD4 IFNg+/IL2+: 18.73%
CD4 IFNg+/IL2-: 44.13%
IL2-PE
IFNγ-APC
[CD8 Cyt+]
CD8 IFNg+/IL2+: 9.04%
CD8 IFNg+/IL2-: 50.14%
IL2-PE
IFNγ-APC
[CD4 Cyt+]
2.26%
1.08%
69.21%
27.45%
HLADR-BV711
CD38-BV650
[CD8 Cyt+]
2.19%
2.74%
75.34%
19.73%
HLADR-BV711
CD38-BV650


B
Cytokine Response and Activation Markers
[CD4 T-Cells]
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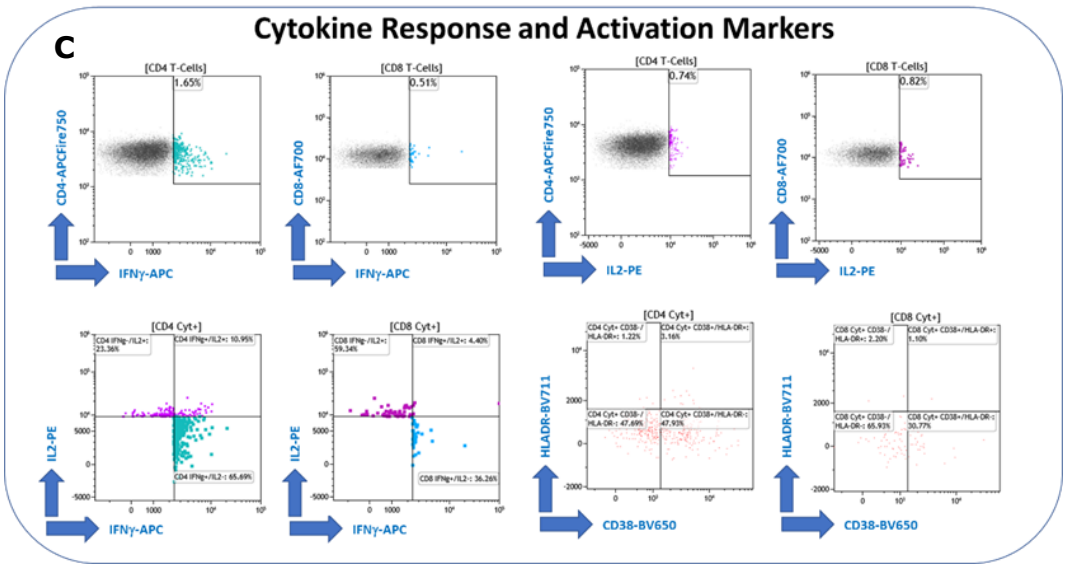
IFNγ-APC
[CD8 T-Cells]
0.11%
CD8-AF700
IFNγ-APC
[CD4 T-Cells]
0.62%
CD4-APCFire750
IL2-PE
[CD8 T-Cells]
0.65%
CD8-AF700
IL2-PE
[CD4 Cyt+]
CD4 IFNg+/IL2+: 6.77%
CD4 IFNg+/IL2-: 17.16%
IL2-PE
IFNγ-APC
[CD8 Cyt+]
CD8 IFNg+/IL2+: 7.41%
CD8 IFNg+/IL2-: 8.02%
IL2-PE
IFNγ-APC
[CD4 Cyt+]
CD4 Cyt+ CD38-/HLA-DR+: 1.13%
CD4 Cyt+ CD38+/HLA-DR+: 1.58%
CD4 Cyt+ CD38-/HLA-DR+: 83.88%
CD4 Cyt+ CD38+/HLA-DR+: 13.41%
HLADR-BV711
CD38-BV650
[CD8 Cyt+]
CD8 Cyt+ CD38-/HLA-DR+: 0.00%
CD8 Cyt+ CD38+/HLA-DR+: 1.85%
CD8 Cyt+ CD38-/HLA-DR+: 74.07%
CD8 Cyt+ CD38+/HLA-DR+: 24.07%
HLADR-BV711
CD38-BV650


C
Cytokine Response and Activation Markers
[CD4 T-Cells]
1.65%
CD4-APCFire750
IFNγ-APC
[CD8 T-Cells]
0.51%
CD8-AF700
IFNγ-APC
[CD4 T-Cells]
0.74%
CD4-APCFire750
IL2-PE
[CD8 T-Cells]
0.82%
CD8-AF700
IL2-PE
[CD4 Cyt+]
CD4 IFNg+/IL2+: 10.95%
CD4 IFNg+/IL2-: 65.69%
IL2-PE
IFNγ-APC
[CD8 Cyt+]
CD8 IFNg+/IL2+: 4.40%
CD8 IFNg+/IL2-: 36.26%
IL2-PE
IFNγ-APC
[CD4 Cyt+]
CD4 Cyt+ CD38-/HLA-DR+: 1.22%
CD4 Cyt+ CD38+/HLA-DR+: 3.16%
CD4 Cyt+ CD38-/HLA-DR+: 47.69%
CD4 Cyt+ CD38+/HLA-DR+: 47.93%
HLADR-BV711
CD38-BV650
[CD8 Cyt+]
CD8 Cyt+ CD38-/HLA-DR+: 2.20%
CD8 Cyt+ CD38+/HLA-DR+: 1.10%
CD8 Cyt+ CD38-/HLA-DR+: 65.93%
CD8 Cyt+ CD38+/HLA-DR+: 30.77%
HLADR-BV711
CD38-BV650

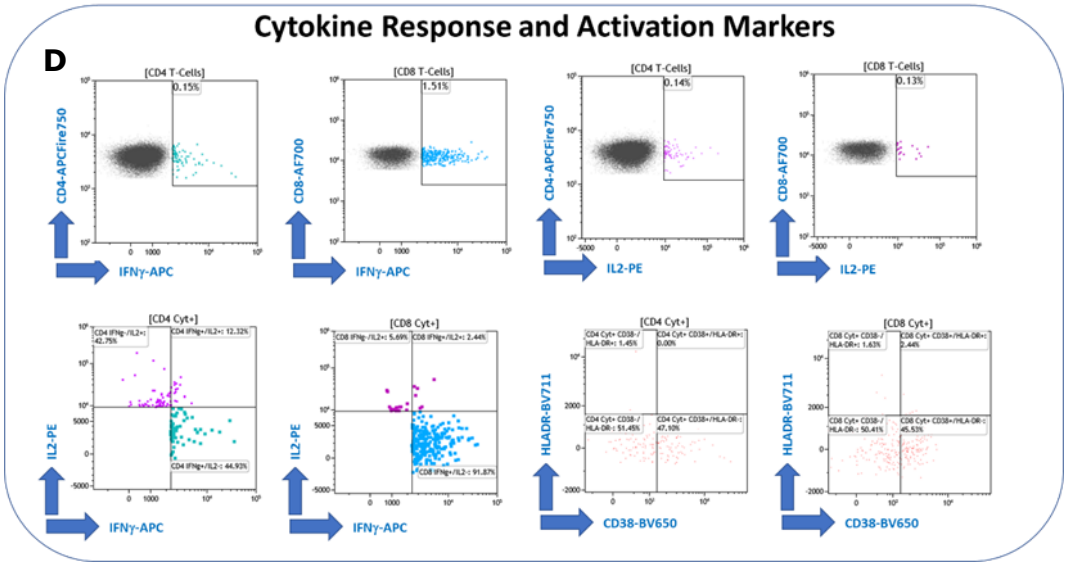


Figure 3.5 Representative specific spike cytokine response for different patient group. The figure shows the cytokine response and activation specific to spike stimulation in individuals from different patient groups. (A) Vaccinated. (B) Asymptomatic. (C)Mild-no vaccine. (D) M/S- no vaccine.

In addition to the overall analysis of cytokine responses between patient groups in Figures 3.1, Figure 3.2, Figure 3.3, and Figure 3.4, the flow cytometry analysis of individuals representing the vaccinated, asymptomatic, mild-no vaccine and moderate/severe no vaccine patients spike specific cytokine response is shown in Figure 3.5. In terms of the CD8 T-cell response the percentage of cells expressing both IL-2 and IFN-γ declines between the patients consecutively from A to D. The flow cytometry data in Figure 3.5 demonstrates the breadth of the results analysed.

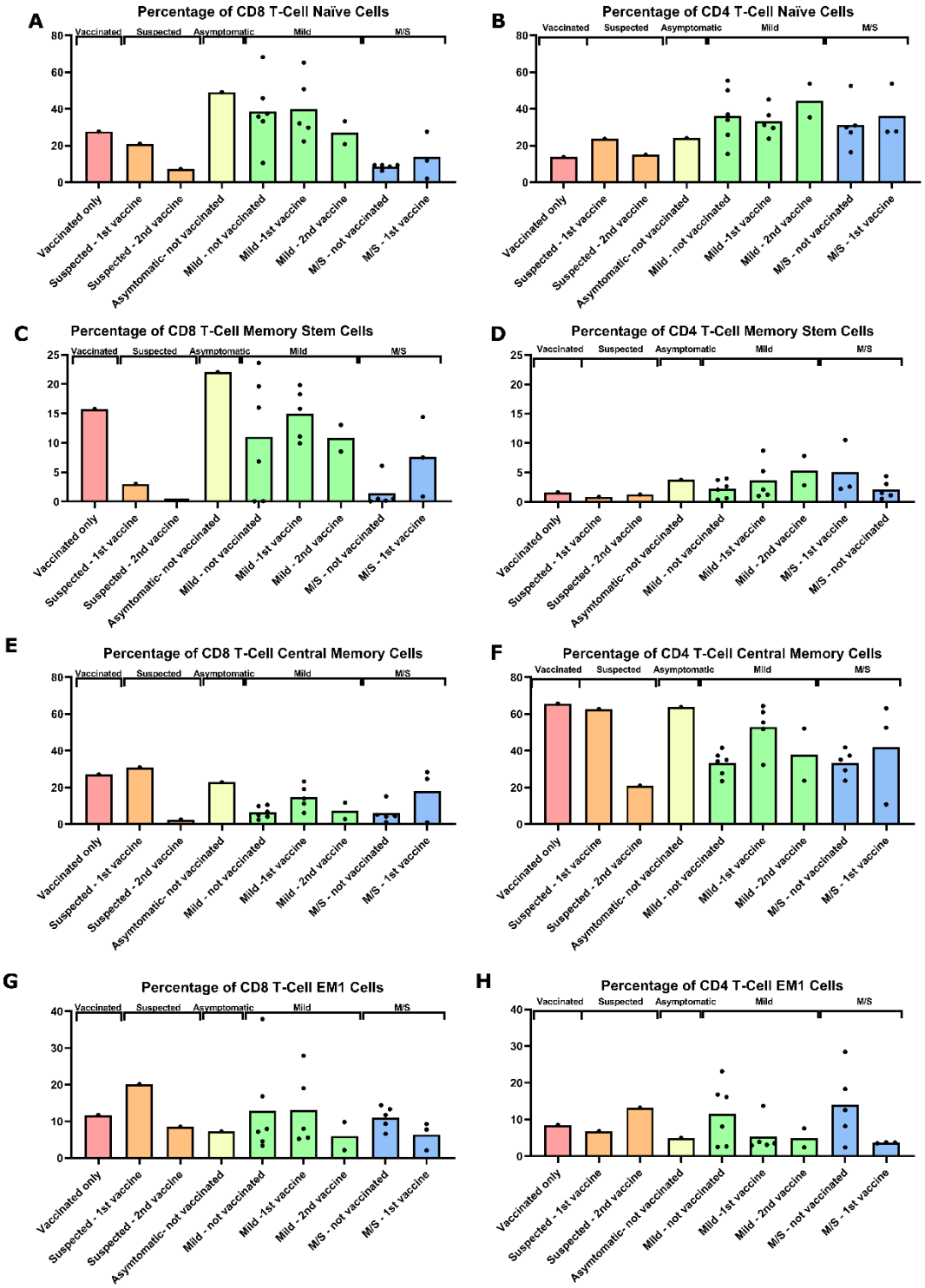
3.3 T-cell Memory phenotype after SARS-CoV-2 infection in convalescent patients

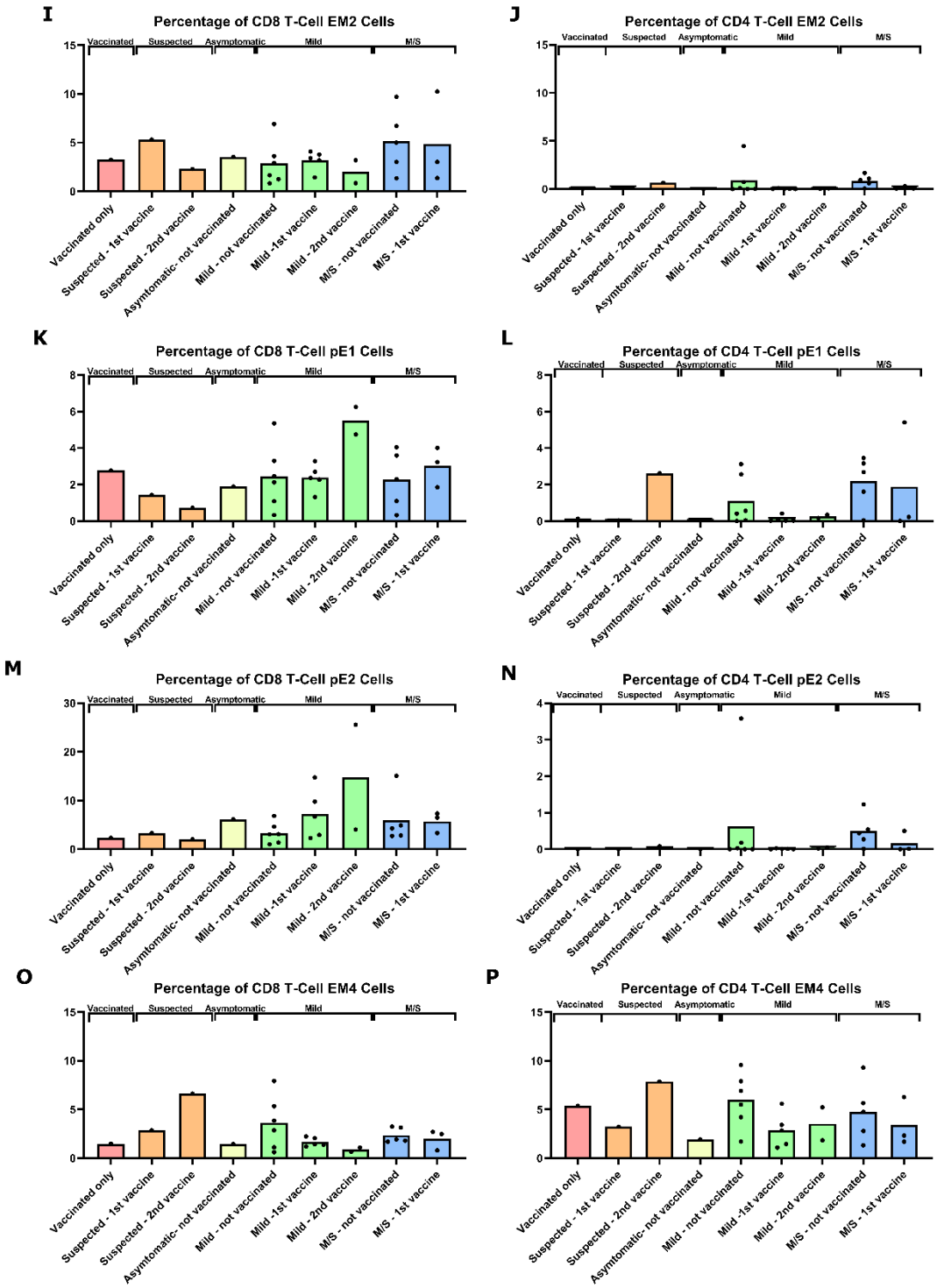
In addition to examining cytokine positive T cell responses, we also examined T cell memory phenotypes. Figure 3.6 demonstrates that the memory phenotype of CD8 and CD4 T-Cells are vastly different, particularly in their percentages of TEMRA cells. The CD4 TEMRA results of all, but two patients are lower than 15%, with the averages of all disease severities falling below 10% (excluding the suspected-2nd vaccination patient, at 12%). In contrast most of the averages of CD8 TEMRA cell percentages (not including suspected-1st vaccine and the asymptomatic patient) are above 20%, with averages of over 40% in all the mild-2nd vaccine and m/s patients. In Figure 3.6 (S and T) all patients have less than 10% CD4 E cells whereas one m/s-1st vaccine patient has above 60% of their cells differentiated into CD8 E cells, and the only patients with less than 10% CD8 cells E cells are the suspected-1st vaccine patient and asymptomatic patient. These results show that CD8 cells are proportionally more exhausted than CD4. The EM3 results of CD8 and CD4 cells, Figure 3.6 (Q and R) show a different pattern, the percentages of both types of cells are similarly low with exception in both graphs to the suspected-2nd vaccine patient, and the results of m/s-not vaccinated in the CD8 cell results. The m/s patients have higher percentages of both EM3 and E cells than the mild patients' cells, demonstrated in Figures 3.6 (Q, R, S and T) suggesting that the m/s patients are more exhausted.

In the CD8 TEMRA, pE1, pE2 and E graphs, Figures 3.6 (K, M, S and W), the patients in the mild group show an increase in percentages of cells on average in the 2nd vaccination group. However as previously mentioned the mild-2nd vaccination group has just two patients and both are over 50, the mild-not vaccinated group and the mild-1st vaccine groups are larger and have a more similar age range of 27-51 and 28-59 years-old respectively. The TEMRA percentages do not change massively between the mild-not vaccinated and mild-1st vaccine suggesting that the demographic differences in the mild group may play a role in the more

exhausted cells in the 2nd vaccine group rather than it be linked to vaccination.

The CD8 naïve, memory stem cell and central memory graphs, Figure 3.6 (A, C and E), show there is an increase in the percentages of these cells mild and m/s groups 1st vaccine group compared to their comparative unvaccinated group. The increase is not demonstrated in the mild 2nd vaccine group, possibly because of the higher age range mentioned previously. The increase in these cell types would imply a greater CD8 T-Cell memory after vaccination. The CD8 effector cells, Figure 3.6 (U) graph, however, shows that in both mild and m/s 1st vaccine groups have a lower percentage of effector cells that their respective not vaccinated groups. The mild 2nd vaccine group has a lower percentage than the mild-1st vaccine group.


A
Percentage of CD8 T-Cell Naïve Cells
B
Percentage of CD4 T-Cell Naïve Cells
C
Percentage of CD8 T-Cell Memory Stem Cells
D
Percentage of CD4 T-Cell Memory Stem Cells
E
Percentage of CD8 T-Cell Central Memory Cells
F
Percentage of CD4 T-Cell Central Memory Cells
G
Percentage of CD8 T-Cell EM1 Cells
H
Percentage of CD4 T-Cell EM1 Cells
Vaccinated
Suspected
Asymptomatic
Mild
M/S
Vaccinated only
Suspected - 1st vaccine
Suspected - 2nd vaccine
Asymtomatic- not vaccinated
Mild - not vaccinated
Mild -1st vaccine
Mild - 2nd vaccine
M/S - not vaccinated
M/S - 1st vaccine


I
Percentage of CD8 T-Cell EM2 Cells
J
Percentage of CD4 T-Cell EM2 Cells
K
Percentage of CD8 T-Cell pE1 Cells
L
Percentage of CD4 T-Cell pE1 Cells
M
Percentage of CD8 T-Cell pE2 Cells
N
Percentage of CD4 T-Cell pE2 Cells
O
Percentage of CD8 T-Cell EM4 Cells
P
Percentage of CD4 T-Cell EM4 Cells
Vaccinated
Suspected
Asymptomatic
Mild
M/S
Vaccinated only
Suspected - 1st vaccine
Suspected - 2nd vaccine
Asymtomatic- not vaccinated
Mild - not vaccinated
Mild -1st vaccine
Mild - 2nd vaccine
M/S - not vaccinated
M/S - 1st vaccine

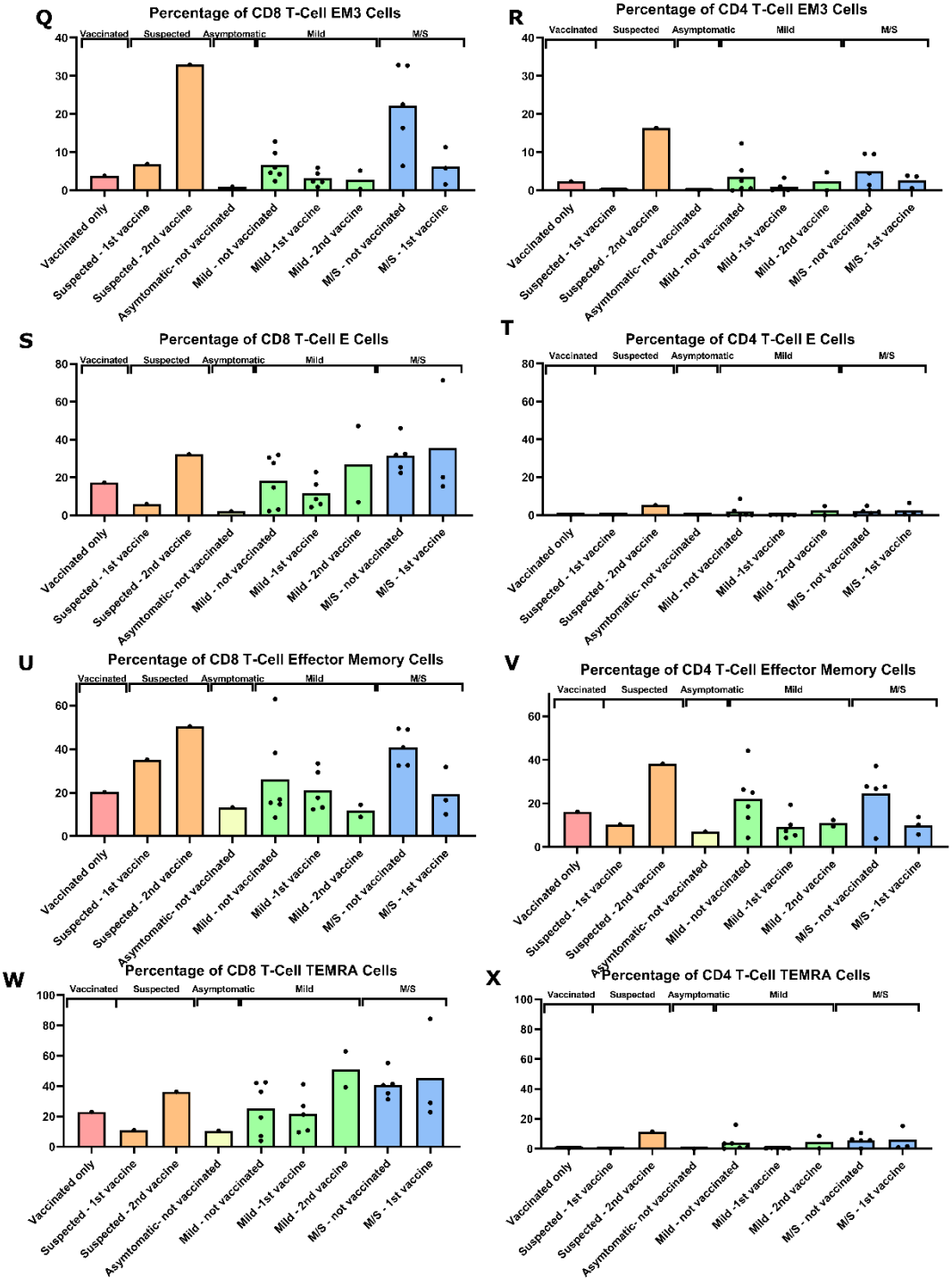
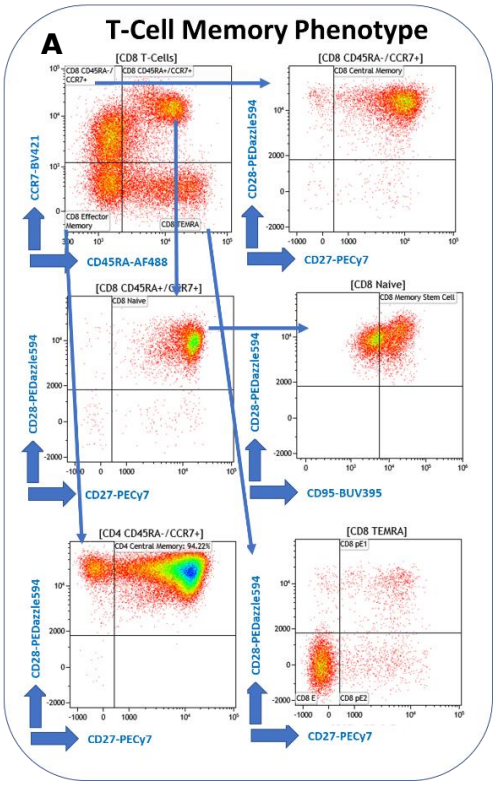
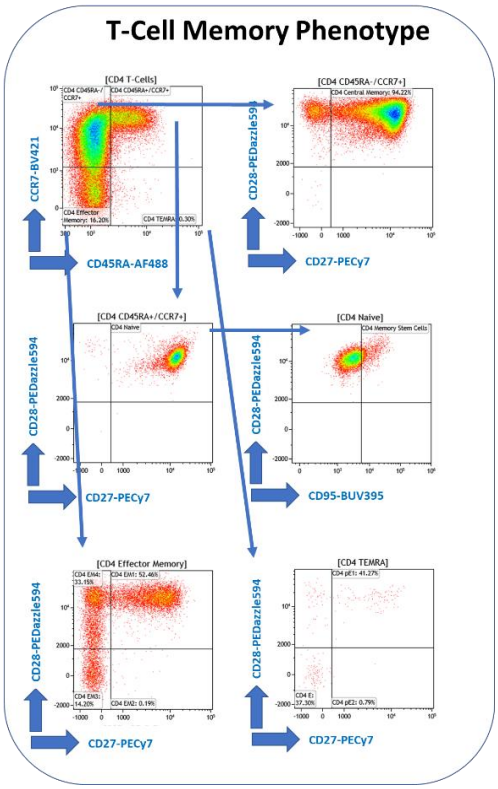
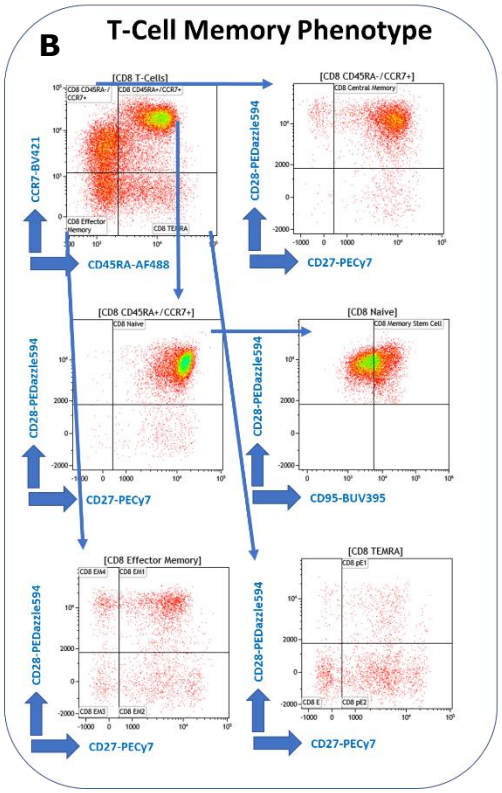


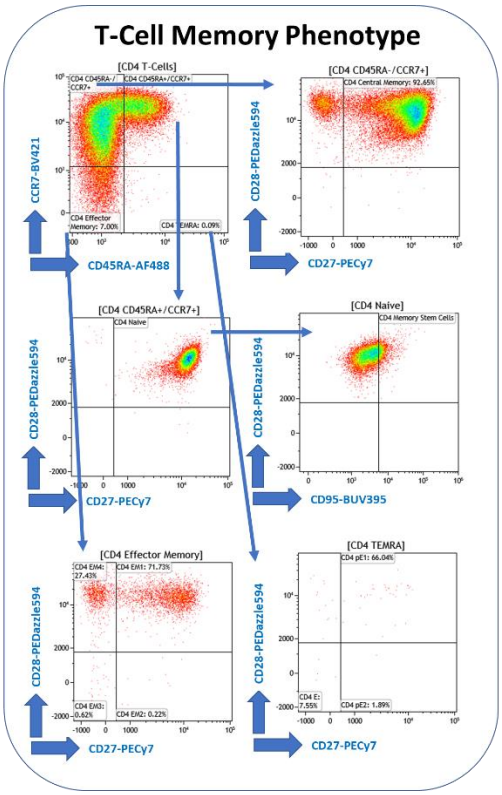
Figure 3.6 The Memory Phenotype of CD8 and CD4 T-cells in convalescent COVID-19 patients. Flow cytometry gating strategies were used to find the percentages of each cell type. Naïve= CD45RA+/CCR7+/CD28+/CD27+, Central Memory= CD45RA-/CCR7+/CD28+/CD27+, Memory Stem Cell= CD45RA+/CCR7+/CD28+/CD27+/CD95+, Effector Memory= CD45RA-/CCR7-, EM1= CD45RA-/CCR7-/CD28+/CD27+, EM2= CD45RA-/CCR7-/CD28-/CD27+,

EM4= CD45RA-/CCR7-/CD28+/CD27-, EM3 CD45RA-/CCR7-/CD28-/CD27-, EM4= CD45RA-/CCR7-/CD28+/CD27+, TEMRA= CD45RA+/CCR7-, pE1= CD45RA+/CCR7-/CD28+/CD27+, pE2= CD45RA+/CCR7-/CD28-/CD27+, E= CD45RA+/CCR7-/CD28-/CD27-.Each line refers to the mean result. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant.


A
T-Cell Memory Phenotype
[CD8 T-Cells]
[CD8 CD45RA-/CCR7+]
[CD8 CD45RA+/CCR7+]
[CD8 Naive]
[CD8 TEMRA]
CCR7-BV421
CD45RA-AF488
CD28-PEDazzle594
CD27-PECy7
CD95-BUV395


T-Cell Memory Phenotype
[CD4 T-Cells]
[CD4 CD45RA-/CCR7+]
[CD4 CD45RA+/CCR7+]
[CD4 Naive]
[CD4 Effector Memory]
[CD4 TEMRA]
CCR7-BV421
CD45RA-AF488
CD28-PEDazzle594
CD27-PECy7
CD95-BUV395


B
T-Cell Memory Phenotype
[CD8 T-Cells]
[CD8 CD45RA-/CCR7+]
[CD8 CD45RA+/CCR7+]
[CD8 Naive]
[CD8 Effector Memory]
[CD8 TEMRA]
CCR7-BV421
CD45RA-AF488
CD28-PEDazzle594
CD27-PECy7
CD95-BUV395


T-Cell Memory Phenotype
[CD4 T-Cells]
[CD4 CD45RA-/CCR7+]
[CD4 CD45RA+/CCR7+]
[CD4 Naive]
[CD4 Effector Memory]
[CD4 TEMRA]
CCR7-BV421
CD45RA-AF488
CD28-PEDazzle594
CD27-PECy7
CD95-BUV395

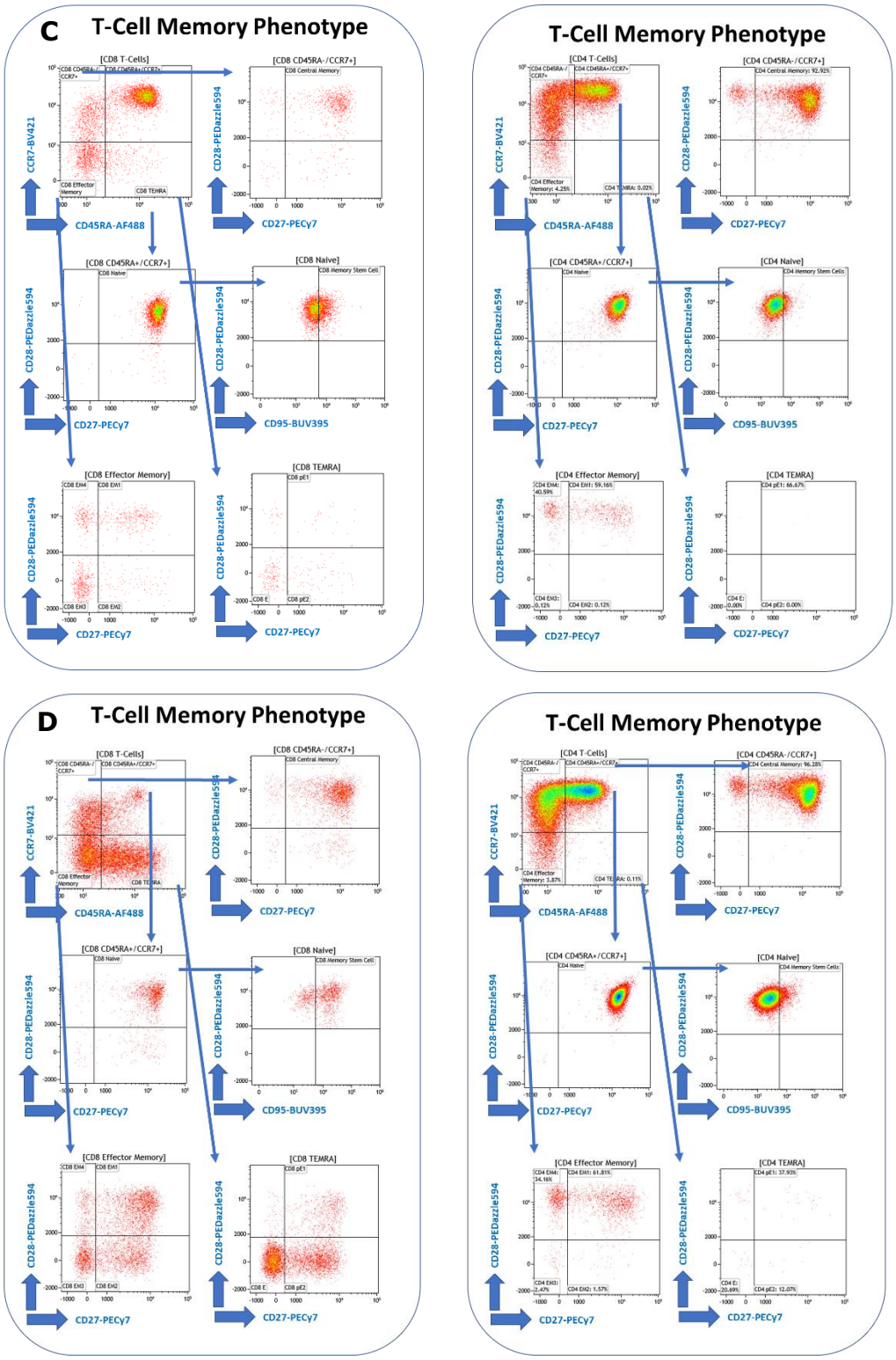


Figure 3.7 Representative flow gating strategy for moderate/severe infected unvaccinated patient group. The figures show the moderate/severe infected unvaccinated individual's T-cell memory phenotype for their CD8 T-cells on the left and CD4 T-cells on the right. These show their T-cell memory phenotype altogether not the phenotype specific to SARS-CoV-2. (A) Vaccinated. (B) Asymptomatic. (C)Mild-no vaccine. (D) M/S- no vaccine.

Figure 3.7 shows the flow cytometry data for individuals from vaccinated, asymptomatic, mild-no vaccine and moderate/severe-no vaccine patients. The data is only showing the results of the memory phenotype of T-cells for one patient in each of these groups and there is variation within the patients though it also demonstrates the finding that CD4 cell numbers are considerably less differentiated into TEMRA cells. The visual representation of the different T-cell subsets also conveys the richness of the data and how much information on T-cell populations can be gathered through this flow cytometry panel.

3.4 Humoral response to SARS-CoV-2 infection in convalescent patients

The antibody reactivity data, Figure 3.8, shows a clear difference in the mild and m/s plus 1st vaccine data, with higher antibody responses than those individuals not yet vaccinated in these two groups. Interestingly those participants with m/s disease have a higher antibody reactivity to nucleocapsid.

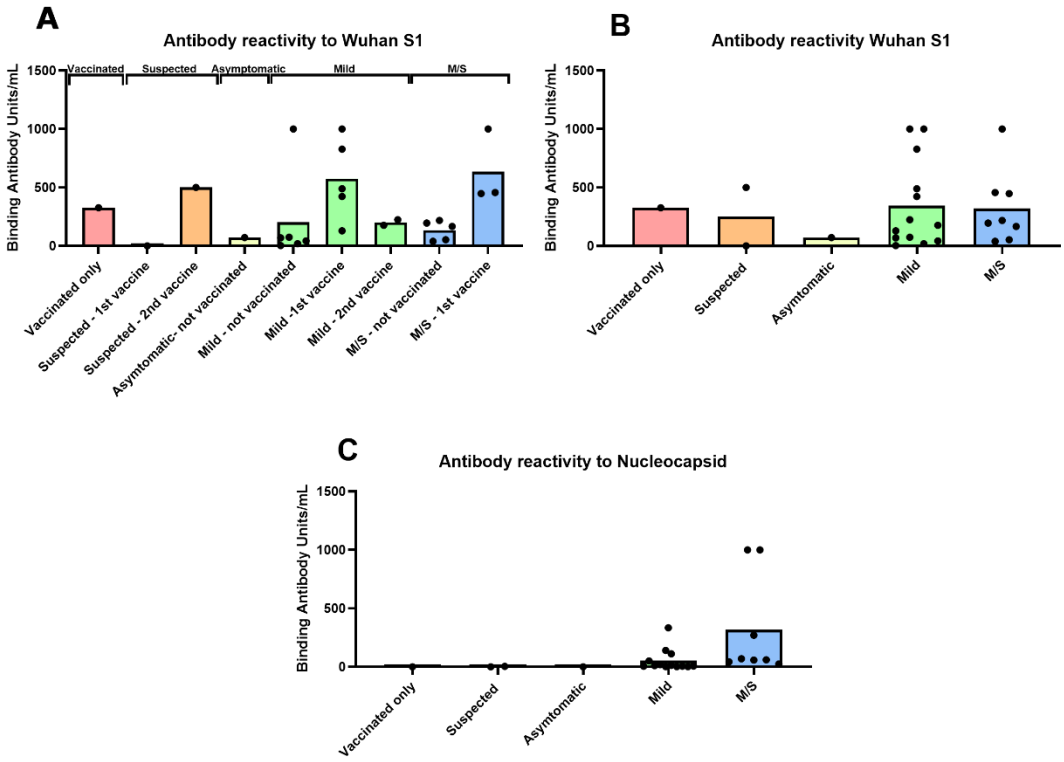


Figure 3.8 Antibody reactivity of serum sample from convalescent COVID-19 patients to lineage A Spike and the SARS-CoV-2 Nucleocapsid. Plasma serum was diluted 1:218 in in 3% skimmed milk powder in PBS containing 0.05% Tween 20 and 0.05% sodium azide. Full length spike lineage A was used. The assays upper limit is 1000 binding antibody units/mL. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant. Graphs A and B contain the same spike-specific data, but A presents the results divided by the vaccination status and disease severity of the patients whereas B just the disease severity. C shows the antibody reactivity specific to SARS-CoV-2 nucleocapsid.

The antibody neutralisation results (Figure 3.9) shows that the neutralising capacity differs depending on the strain of the SARS-CoV-2. The mean data demonstrates that on average the neutralising capacity is around 40%, though when separated by vaccination, the unvaccinated neutralising capacity drops, as would be expected.

The antibody neutralisation data like the spike antibody reactivity shows that vaccination affects the average result and that the neutralising capacity rises after the first vaccine and then falls after the second in the mild disease severity group.

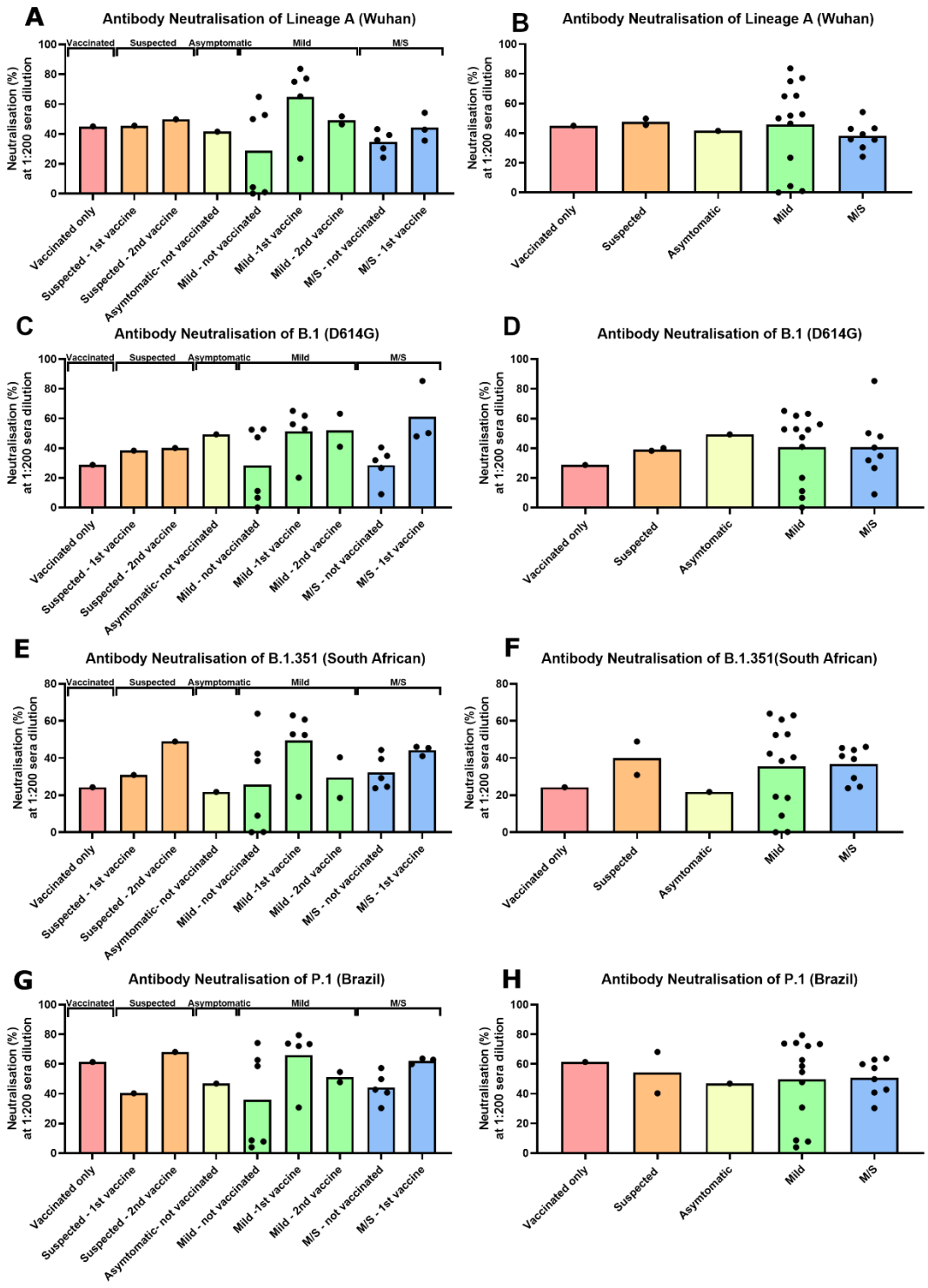


Figure 3.9 Percent neutralization of serum samples for Lineage A, B.1 (D614G), B.1.351, and P.1 pseudo viruses. A Kruskal-Wallis with Dunn's multiple comparison test revealed the differences in the data were not significant.

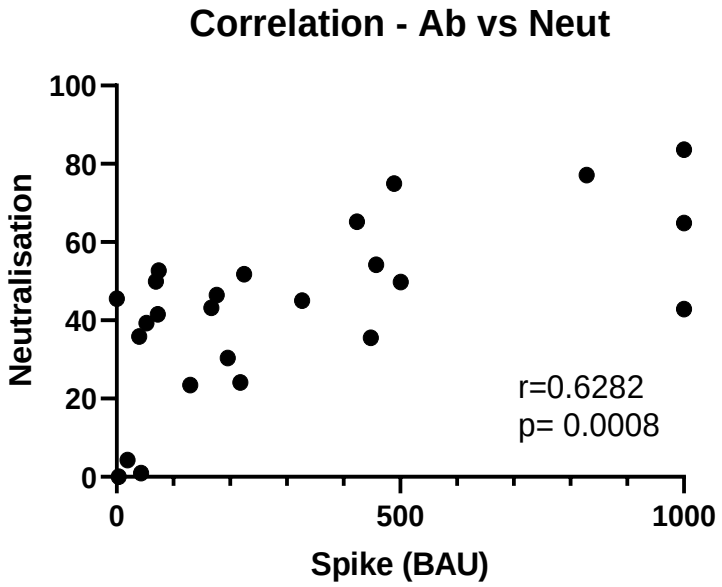


Figure 3.10 The correlation between the percentage of neutralization of the serum samples against their antibody reactivity. The percentage neutralising capacity that patient serum samples have against lineage A Wuhan spike protein was correlated against the antibody reactivity the patient's serum samples have against Wuhan spike (where BAU = binding antibody units/mL). The Spearman's rank correlation coefficient is shown as r=0.6282, the two tailed P-value is significant at p=0.0008.

The antibody and neutralization data, Figure 3.9 and Figure 3.10 respectively, shows that the patients of m/s disease severity do exhibit a larger neutralizing capacity and larger antibody reactivity after their first vaccination. This could possibly suggest that vaccination has a larger effect on antibody responses than T-cells and that infection is a bigger driver of T-cell response, as our one vaccinated only patient also displays low T-cell responses compared to their antibody reactivity and neutralization. The mild disease severity changes more after vaccination, occasionally increasing as mentioned in the IFN-γ and IL-2 CD8 T-cell graph, though is also often seen decreasing such as in Figure 3.1 (A) and (B), but it is worth noting that all these results are below 1% to begin with, so though there is a decrease it is small. Conversely in the mild disease severity in both the antibody reactivity and neutralizing results there is a fall in activity after the second vaccination, the first vaccination results were higher than that of the unvaccinated patients then the average drops after

the second vaccination. The sample size mild-second vaccination is smaller than those of mild unvaccinated and 1st vaccine which could have contributed to this, as well as the higher average age of those with mild disease and two vaccinations. That those who were of highest priority to be vaccinated were the most vulnerable may also play a part.

4. Discussion

This study demonstrates the vast variety in immune responses to SARS-CoV-2 infection and vaccination. The results display increases in particular cytokine producing T-cells and cell types after vaccination, as well as the decline in other cell types. Literature findings of differences between CD8 and CD4 T-cell expression were linked to their proportion of differentiation and exhaustion levels. Natural infection memory-specific responses to membrane and nucleocapsid protein was assessed in comparison to spike and the disease severity of patients also compared. Finally, humoral responses within the sample set, including antibody titre and neutralisation was examined.

The cell mediated response to SARS-CoV-2 displays consistent increase in T-cell responses in m/s disease patients following vaccination. Though often due to one patient's higher response singularly increasing the average response. The T-cell memory phenotype results reveal that cellular exhaustion in m/s patients CD8 cells is higher than other patients, with larger percentages of the highly differentiated EM3 and E cells, which may explain why in most of the vaccinated m/s patients', vaccination has little effect on the cell-mediated response. However, the mild disease patients do not show this, only CD8 multi-functional T-cells producing both IFN-γ and IL-2 illustrate a clear increase. The exception of this cell type may highlight its importance, research has often found larger CD8 in patients of lower disease severity as well as the benefits of multi-functional T-cells (Peng *et al*, 2020) (Grifoni et al, 2020). The T-cell memory phenotype results show that the less differentiated CD8 cells such as naïve, memory stem and central memory increase on average after first vaccination. This increase perhaps indicates vaccination provides an increase in the reserve of T-cells, able to rapidly differentiate to clear SARS-CoV-2 if reinfected. Possibly highlighting why, as well as looking at magnitude of the cell mediated response, looking at the phenotype of cells in future experiments could suggest the utility in examining the cell-mediated response. Zollner et al, 2021 found that both antibody and T-cell responses specific to spike boosted after vaccination. In our study it is

important to consider one of the limits of the study is the sample size of just 26 patients, with samples taken at random time-points after both natural infection and vaccination. This makes it difficult to draw any firm conclusions from this work. It is worth noting that in this study T-cell memory subsets were used to establish a picture of the levels of cellular exhaustion in patients' disease severity groups, and the more differentiated populations of cells were seen to indicate exhaustion, though there are other markers often used to establish cellular exhaustion such as PD-1 or CTLA-4. Using these markers could provide an alternative method for characterising T-cell exhaustion.

The size of the study likely affects some of the observations shown in the results, as well as that the average age of each group is often very different. From this it could be inferred that observations may be due to age rather than disease severity or vaccination status. For example, the fall in CD8 responses in mild -2nd vaccine compared to unvaccinated and 1st vaccine patients. Though, if it were possible to get pre-vaccine samples from the patients in the study as well as samples at select intervals after infection and vaccination, the effects of the small sample and average ages in the groups could be established more definitively. More samples at select time intervals may have also highlighted the typical timing of T-cell and antibody responses forming in the patients in this study. A larger study could also enable the magnitude of humoral and cell-mediated responses and the proportions of differentiated T-cells to be compared across disease severity and vaccination status in different demographics, including age, gender, or ethnicity. Additionally, the cell-mediated and humoral responses in just vaccinated or asymptomatic individuals could be characterised further compared to infected individuals. The one asymptomatic individual in the study shows signs of little cellular exhaustion, little antibody reactivity but a similar neutralising antibody capacity to mild patients and close to zero T-cell responses aside from the CD8 T-cell IFN-γ and IL-2 response. Whether these responses are typical of asymptomatic individuals could be established in a study with more of these patients. Establishing the T-cell memory subsets for the antigen

specific population for the spike, membrane and nucleocapsid peptides would show more insight into the T-cell memory that is specific to SARS-CoV-2, rather than the whole T-cell population. Though the volume of blood samples taken from each patient would have needed to be larger to gather enough antigen-specific cells to perform such analysis.

This study has consistently found differences between CD8 T-cell expression and CD4 T-cell expression. As literature has suggested and previously established, beneficial multi-functional CD8 T-cells are more highly expressed in more mild cases of SARS-CoV-2 (Peng *et al*, 2020) (Grifoni et al, 2020). The same was not found in CD4 cell expression. Higher levels of cellular exhaustion are seen in CD8 cells in more severe cases, CD4 T-cells however show little to no cellular exhaustion in the study. CD4 T-cells were found to mostly be differentiated into central memory cells, with comparatively lower percentages of central memory CD8 T-cells. CD4 T-cells are not seen to be highly exhausted like CD8 T-cells and are less differentiated as mostly central memory cells. There is likely an imbalance in the responses of both cells in more severe cases of SARS-CoV-2 which may lead to the pathogenic cytokine storm linked to CD4 in severe cases (Kalfaoglu *et al*, 2020). The cytotoxic CD8 T-cells may be exhausted whereas the CD4 T-cells are unaffected, meaning that they would continually stimulate pro-inflammatory cell-mediated responses.
The results reveal lower percentages of cytokine producing T-cells are specific to membrane and nucleocapsid proteins compared to spike, even in unvaccinated individuals. There are many patients in both the mild and m/s disease severity categories, after stimulation with the membrane and nucleocapsid proteins, who continually have close to zero percent of their cells showing a response. These low responses may indicate that these proteins are less responsible for stimulating a T-cell response that kills the virally infected cells. Alternatively, the membrane and nucleocapsid structural proteins may not be seen by the immune system, especially as the findings are similar in people who had mild illness. Mild patients showing low membrane and nucleocapsid responses demonstrates these patients' cell-mediated responses could clear the virus more quickly than

more severe patients without recognition of the proteins. In a larger sample group that could factor in age, gender, and time since infection the reasoning for why some do develop a more robust response to the membrane and nucleocapsid proteins may become clearer.

The antibody reactivity and neutralisation assay results demonstrated that moderate and severe patients had a slightly greater response than mild patients, though following vaccination these results raised to similar levels. Guthmiller et al, 2021 also found that more severe patients of COVID-19 had a greater response than more mild patients, proposing it was due to the prolonged time SARS-CoV-2 remained in the lymph nodes of the more severe patients. That vaccination led to the mild and m/s patients displaying more similar reactivity and neutralisation results suggest that vaccination led to more exposure to antigens on the spike protein within the vaccine. Spike-specific antibody responses were found to be higher than nucleocapsid specific antibody response. As previously mentioned, nucleocapsid-specific antibodies were found to seroconvert sooner after infection than spike-specific antibodies, in other studies. Ripperger et al, 2020 found nucleocapsid antibody levels to seroconvert after three months, compared to seven months for spike-specific antibodies. Nucleocapsid-specific reactivity is lower than the spike-specific reactivity in our results and it may be because only four patients within the study were infected within three months of their blood sample. However, unlike Ripperger et al, 2020 the nucleocapsid responses in m/s patients is still present more than three months after infection. These results may indicate that more severe disease severity leads to the nucleocapsid response seroconverting more slowly.

Overall improvements could be made to the study, such as: increasing the study size, including pre-vaccine and multiple post vaccine samples, ensuring the average ages of the groups are similar, classifying the results on more of the demographics of the patients and increasing the amounts of just vaccinated and asymptomatic individuals. But the results have emphasized further areas of interest, such as applying individuals' cellular

differentiation data for insight on cytokine production, possible changes in T-cell responses after 2nd vaccination and contrasting differences in asymptomatic and vaccinated data compared to infected individuals. The study has highlighted that there are differences in the cellular exhaustion levels based on disease severity and that patients with more severe illness do generate a greater cell-mediated and humoral response. The differences between the literature and the results of the study may be due to the study size but it also demonstrates that more research following the techniques used in this study could provide further insight in the basis of immunity to SARS-CoV-2.

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