

Investigating SARS-CoV-2 immune responses in children

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LIST OF ABBREVIATIONS

°C	Degree Celsius
ACE2	Angiotensin-converting enzyme 2
ADCA	Antibody-dependent complement activation
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AIM assay	Activation-induced marker assay
AM	Alveolar macrophages
ANOVA	Analysis of variance
APC	Antigen presenting cells
APC-Cy7	Allophycocyanin-Cy7
APN	Aminopeptidase N
ARDS	Acute respiratory distress syndrome
ARI	Acute respiratory infection
BCV	Bovine coronavirus
BFA	Brefeldin-A
BL	Baseline
BV	Brilliant violet
CD	Cluster of differentiation
CDC	Center of Disease Control
CoVs	Coronaviruses
CO₂	Carbon dioxide
COVID-19	Coronavirus disease 2019
COVID-NET	COVID-19-Associated Hospitalization Surveillance
CMV	Cytomegalovirus
CRP	C- reactive protein
D614G	Aspartic acid to Glycine in position 614
DC	Dendritic cells
DCHS	Drakenstein Child Health Study
DENV	Dengue virus
DMSO	Dimethylsulphoxide
DMV	Double membrane vesicles
DPP4	Dipeptidyl peptidase 4
E protein	Envelope protein
ED	Early differentiated
EDTA	Ethylenediaminetetraacetic acid
Eff	Effector

ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent Spot
ER	Endoplasmic reticulum
Exon	Exoribonuclease
FBS	Fecal bovine serum
Fc	Fragment crystallizable
FCS	Forward scatter
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
GI	Gastrointestinal
GrB	Granzyme B
GSH	Groote Schuur hospital
HAdV	Human adenovirus
HC	Healthy SARS-CoV-2 seropositive children
HCl	Hydrochloric acid
HCoV	Human coronavirus
HCV	Hepatitis C virus
HCW	Healthcare workers
HE	Hemagglutinin-esterase
HEK	Human Embryonic Kidney
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen-DR
HPIV	Human parainfluenza virus
HREC	Human Research Ethics Committee
HSV	Herpes simplex virus
IBV	Infectious bronchitis virus
ICS	Intracellular cytokine staining
ICU	Intensive care unit
IFN	Interferon
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IP	Interferon gamma-induced protein
IQR	Interquartile range

IVIG	Intravenous immunoglobulins
Kb	Kilobases
kDa	Kilodalton
KD	Kawasaki disease
LD	Late differentiated
LRT	Lower respiratory tract
LRTI	Lower respiratory tract infection
M	Molar concentration
M protein	Membrane protein
MCP	Monocyte chemoattractant protein
MDA5	Melanoma differentiation-associated antigen 5
MERS	Middle East respiratory syndrome
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MHV	Murine coronavirus
MIP	Macrophage inflammatory protein
MIS-A	Multisystem inflammatory syndrome in Adults
MIS-C	Multisystem inflammatory syndrome in children
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
N protein	Nucleocapsid protein
NICD	National Institute for Communicable Diseases
NK	Natural Killer cells
NRF	National Research Foundation
nsp	Non-structural proteins
OAS	Oligoadenylate synthetase
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE-Cy	Phycoerythrin-Cy
PEDV	Porcine enteric diarrhoea virus
PD-1	Programmed cell death protein-1
PHA	Phytohemagglutinin

PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
RBD	Receptor binding domain
RC	Red Cross War Memorial Children's Hospital
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I like receptors
RLU	Relative light units
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSV	Respiratory syncytial virus
RV	Rhinovirus
RT	Room temperature
RTC	Replicase-transcriptase complex
S protein	Spike protein
S1	Spike Subunit 1
SA	South Africa
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory syndrome coronavirus
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
sgRNA	Subgenomic RNA
SSC	Side scatter
TCR	T cell receptor
TCM	Central memory T cell
TCRVβ	T cell receptor β chain variable region
TD	Terminally differentiated
TEM	Effector memory T cell
TEMRA	Terminally differentiated effector memory T cell
Th	T helper
TLR	Toll-like receptor
TMB	3',5,5'-Tetramethylbenzidine
TMPRSS2	Transmembrane protease serine 2
TNF	Tumor necrosis factor
Trm	Tissue-resident memory cells
TSCM	Stem cell memory T cell
TSS	Toxic shock syndrome
UCT	University of Cape Town

UK	United Kingdom
UNICEF	United Nations International Children’s Emergency Fund
URT	Upper respiratory tract
UTR	Untranslated region
USA	United State of America
µg	Microgram
µL	Microliter
VOI	Variants of interest
VOC	Variants of concern
WBA	Whole blood assay
WHO	World Health Organization

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causes a highly transmissible respiratory disease, coronavirus disease 2019 (COVID-19), with variable disease outcomes. Children infected with SARS-CoV-2 are more likely to exhibit asymptomatic or mild illness compared to severe clinical outcome, often observed in adults. A severe manifestation of SARS-CoV-2 infection in children is multisystem inflammatory syndrome (MIS-C), a delayed hyperinflammatory disease that occurs after exposure to SARS-CoV-2 and shares clinical features with Kawasaki disease (KD). MIS-C develops in a small proportion of children who have been previously exposed to SARS-CoV-2. Potential contributors to the reduced COVID-19 clinical outcome in children compared to adults, includes less comorbidities, differences in the expression of viral entry factors, robust innate immunity, age-associated differences in humoral and cellular immunity, and pre-existing immunity against endemic human coronaviruses (HCoVs). Notably, a critical role for T cells in controlling COVID-19 severity, has been well documented. The immune mechanisms for the differences in disease progression between children and adults remain to be fully understood and studies evaluating SARS-CoV-2 immune responses of paediatric populations in Africa are still rare. This thesis aims to investigate why children were initially spared from severe COVID-19 outcomes, whether T cell responses are maintained in children as seen in adults, and why a small fraction of children develop MIS-C after SARS-CoV-2 infection.

Overall, this thesis focused on characterizing SARS-CoV-2-specific immune responses in pediatric cohorts, determining the durability of T cell responses over a 16-month period, and finally assessing cross-reactive immunity to SARS-CoV-2 variants of concern (VOC) in children. In Chapter 3, we aimed to investigate the SARS-CoV-2-specific T cell responses in unvaccinated asymptomatic children (median age: 7 years) who were seropositive (n=41) or seronegative (n=30) for SARS-CoV-2. Our results showed that the magnitude of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses was comparable between SARS-CoV-2 seropositive and seronegative children. However, the functional profiles of SARS-CoV-2-specific CD4⁺ T cells were distinct, with seropositive children displaying a higher proportion of polyfunctional T cells, whereas seronegative children had predominantly monofunctional T cells. Additionally, the frequency of

SARS-CoV-2-specific CD4⁺ T cells in seronegative children was moderately associated with endemic HCoV-HKU1 antibody responses. Suggesting that SARS-CoV-2-reactive T cells in seronegative children may be due to pre-existing immunity from prior infection with endemic HCoVs. We also demonstrated that seropositive children had lower frequency of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses, in comparison to COVID-19 convalescent (n=30, median age: 38 years) adults. A limitation of this cohort was that the children did not have an exact time of SARS-CoV-2 infection by PCR confirmation, whereas the adults had PCR-confirmed SARS-CoV-2 infection. Therefore, the lower magnitude of responses in children might be due to more distant infection compared to the adults. Taken together, these findings provide insight into SARS-CoV-2 immunity in children.

In Chapter 4, we aimed to extend previous findings with another larger cohort of 73 matched household mother (median age: 33 years)-child (median age: 7 years) pairs, to address the limitation of the potential differing time of SARS-CoV-2 infection between children and adults in the previous cohort in Chapter 3. A small proportion (36%) of children living in the same household as their SARS-CoV-2 seropositive mother, were seronegative for SARS-CoV-2, despite the likelihood of shared SARS-CoV-2 exposure. The apparent resistance of infection in these children may be due to three reasons, namely 1) cross-reactive immunity to endemic HCoVs, 2) early aborted SARS-CoV-2 infection in children and/or 3) possibly early rapid viral clearance by innate immunity. Despite the serostatus, children in comparison to matched mothers displayed lower frequency of SARS-CoV-2-specific T cell responses. The overall functional profile of SARS-CoV-2-specific T cells were comparable between children and mothers, suggesting although lower, the quality of the T cell response is equivalent during SARS-CoV-2 infection. When comparing the memory differentiation profile of SARS-CoV-2-specific T cells, we found that children and mothers had early differentiated (ED) memory profile as the dominant memory subset of SARS-CoV-2-specific T cells, which is associated with long-term immunity. The reduced SARS-CoV-2-specific T cell responses found in children compared to adults raised the question of whether these responses would be durable and cross reactive to SARS-CoV-2 VOCs. We therefore evaluated of the maintenance of SARS-CoV-2-specific T cell responses over 16-month period since

the initial visit. This analysis was somewhat confounded by possible repeated exposures to SARS-CoV-2 during the Delta and/or Omicron BA.1/2, BA.4/5 waves preceding the second sampling visit. Nonetheless, the predominant ED memory subsets at the 16-month sampling does suggest potential durability albeit in the context of an ongoing pandemic. Lastly, we reported that, T cell responses were cross-reactive to SARS-CoV-2 Delta and BA.1 VOC, demonstrating T cells are preserved across variants of concern in South African unvaccinated children.

Finally, in Chapter 5, we investigated the underlying immune pathology of MIS-C, by quantifying SARS-CoV-2-specific T cell responses in children with MIS-C compared to SARS-CoV-2 seropositive children with clinically similar paediatric febrile diseases (non-MIS-C) and healthy SARS-CoV-2 seropositive children (HC). The magnitude of SARS-CoV-2-specific T cell responses was comparable in children with MIS-C, non-MIS-C and HC. However, healthy SARS-CoV-2 seropositive children had a higher proportion of polyfunctional SARS-CoV-2-specific CD4⁺ T cells compared to children with MIS-C and those with other inflammatory or infectious diagnoses, who both presented a largely monofunctional SARS-CoV-2-specific CD4⁺ T cell profile. Additionally, children with MIS-C displayed a higher frequency of TCR V β 21.3⁺ SARS-CoV-2-specific CD4⁺ T cells compared to HC but similar to non-MIS-C controls. The skewed T cell profile in children with MIS-C and non-MIS-C controls could be due to sustained systemic inflammation inducing T cell exhaustion and/or superantigen-like responses inducing polyclonal expansion of V β 21.3⁺ T cells to SARS-CoV-2 spike protein.

Overall, this thesis provides important relevant data for understanding the adaptive immunity of SARS-CoV-2 in children compared to adults and provides insights into the cross-reactive immunity that may provide protection against re-infections in children. Despite the end of the COVID-19 pandemic as a global health emergency and decrease in MIS-C incidence rates over the last 3 years, SARS-CoV-2 continues to circulate and may possibly evolve into endemic seasonal waves of infection. This exposes children, the vulnerable and least vaccinated population to increasing respiratory viral coinfections. Future work includes investigating systemic and local immune responses against respiratory syncytial virus (RSV) in the context of recent or concurrent SARS-

CoV-2 infection in children to better understand the immunological mechanisms contributing to the increased RSV disease severity observed after the COVID-19 pandemic.

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1.1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19), a global health concern for the past four years. The disease was first reported in Wuhan, China in 2019 and resulted in over 2 000 people infected with SARS-CoV-2 in China with 80 deaths, and 10 other countries by the end of January 2020 (P. Zhou et al., 2020). In November 2019 clusters of severe acute respiratory syndrome coronavirus (SARS-CoV) of the species *severe acute respiratory syndrome-related coronavirus* and the genus *Betacoronaviruses* (Gorbalenya et al., 2020) were reported in China. In March 2020, COVID-19 was declared a global pandemic by the World Health Organization (WHO) and by 24 October 2023, over 701 million cases and 6.9 million deaths had been reported (WHO, 2023a). South Africa (SA) is the most severely affected country on the African continent, with over 4.07 million confirmed cases and at least 102 000 deaths as of October 2023 (WHO, 2023a). By the end of 2020, COVID-19 vaccination strategies were implemented in high-income countries. By early February 2021, SA's national vaccination program for highly exposed individuals, healthcare workers and the vulnerable elderly population was implemented, with the vaccination program distributing over 8 million doses of Janssen Ad26.COVS COVID-19 vaccine and over 26 million doses of Pfizer BioNTech (BNT162b2) mRNA COVID-19 vaccine (Department: Health Republic of South Africa, 2024; Gray et al., 2022). As of late 2023, 67% of the world's population has completed COVID-19 vaccine primary series of doses (WHO, 2023c).

In April 2020, a viral strain named SARS-CoV-2 D614G (aspartic acid to glycine in position 614) replaced the original circulating SARS-CoV-2 strain (Korber et al., 2020; Mascola et al., 2021). Subsequently, numerous SARS-CoV-2 variants have emerged from different countries and independently. The variants were classified as variants of concern (VOC) and variants of interest (VOI) based on the increased transmissibility, virulence, diagnostic or vaccine efficacy (WHO, 2022b). These VOCs, namely, the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron parental lineages (B.1.1.529) and sub-lineages (BA.1, BA.2, BA.4, BA.5, XBB, BA.2.86), rapidly dominated regional and/or global waves of infection throughout the COVID-19 pandemic (Carabelli et al., 2023; Fontanet et al., 2021). SA experienced five epidemic infection waves shown

in **Figure 1.1**, dominated by the Ancestral strain (March 2020 - September 2020), Beta (November 2020 - March 2021), Delta (April 2021 - October 2021), Omicron (November 2021 – January 2022) and Omicron sub-lineages (April 2022 - June 2022) variants (Al Hasan et al., 2022; NICD, 2023b; WHO, 2022b; W. Yang & Shaman, 2022).

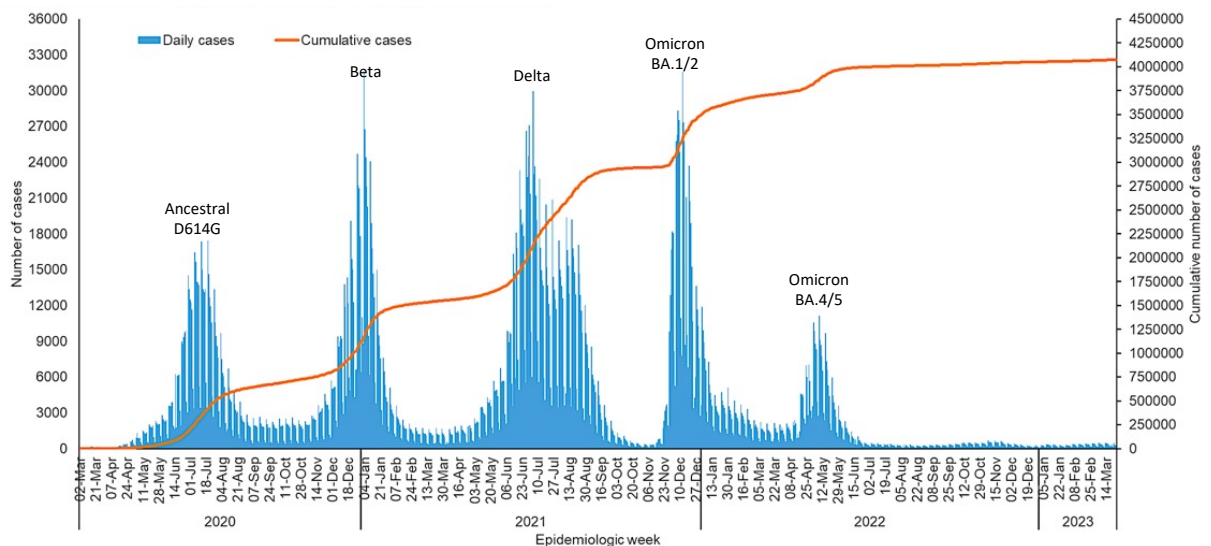


Figure 1.1: The landscape of SARS-CoV-2 epidemic waves in South Africa based on number and cumulative number of laboratory confirmed cases collected from 3 March 2020 until 25 March 2023. Adapted from NICD 2023 (NICD, 2023b).

It is well documented that poor clinical outcome of COVID-19 increases with age along with other risk factors such as pre-existing comorbidities, and children were largely spared severe COVID-19 outcomes (Chou et al., 2022; O’Driscoll et al., 2021). Although children develop severe disease at comparably lower rates than adults, one severe manifestation of SARS-CoV-2 infection in children is a post-infectious syndrome known as multisystem inflammatory syndrome in children (MIS-C) (Chou et al., 2022). The reduced risk of disease in children has resulted in this population remaining the largest unvaccinated population in the world (Naeimi et al., 2023). Other factors that may influence the slow uptake of childhood vaccines include inequities in healthcare quality, vaccine accessibility in low- and middle-income countries, and parental vaccine hesitancy (Naeimi et al., 2023; Nathanielsz et al., 2022; Sam-Agudu et al., 2022; Zar et al., 2020).

Although the immune responses to SARS-CoV-2 in adults have been extensively documented, including the immune landscape of SARS-CoV-2 and responses to emerging VOCs, the immunological mechanisms underlying differences in resistance to SARS-CoV-2 infection and COVID-19 severity in children compared with adults are still not fully understood. This introductory chapter covers the literature on coronaviruses (CoVs), clinical course of COVID-19 in children and adults, COVID-19 complications including MIS-C, immune responses to SARS-CoV-2 infection and MIS-C, and finally cross-reactive immune responses to other human coronaviruses (HCoVs).

1.2 Coronaviruses

CoVs are classified in the order *Nidovirales*, which consists of the *Coronaviridae*, *Arteriviridae*, and *Roniviridae* families (International Committee on Taxonomy of Viruses) (Cui et al., 2019; Fehr & Perlman, 2015). The *Coronaviridae* family is divided into two subfamilies, *Coronavirinae* and *Torovirinae*, and the *Coronavirinae* subfamily is further divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (Fehr & Perlman 2015; Perlman & Netland, 2009). Alpha and beta CoVs generally infect mammals, including humans, whereas gamma CoVs infect birds and delta CoVs infect mammals excluding humans (Asghar et al., 2022; Cui et al., 2019). CoVs are spherical enveloped viruses with a single-stranded positive sense RNA genome of approximately 30 kilobases (kb). The RNA strand contains a 5' cap structure and a 3' poly(A) tail for mRNA translation and replication (Y. Chen et al., 2020; Fehr & Perlman, 2015). The genome organization is similar for all CoVs but differs in virus-specific receptor usage and number of accessory genes (Fehr & Perlman, 2015). The 5' end, two-thirds of the genome, consists of at least 6 open reading frames (ORFs), with the first ORFs (ORF1a/b) encoding non-structural proteins (nsps), which are required for RNA replication and transcription (Fehr & Perlman, 2015; Perlman & Netland, 2009). The remaining one-third of the genome at the 3' end, contains the other ORFs encoding for structural proteins. These include spike (S), envelope (E,) membrane (M) and nucleocapsid (N) proteins as well as accessory proteins that are viral species-specific and are required for viral structure, replication and viral RNA synthesis (Cui et al., 2019; Fehr & Perlman, 2015).

CoVs commonly cause acute and chronic respiratory diseases in many animal species, including livestock and domestic animals. (Cui et al., 2019; Lin et al., 2016; Su et al., 2016; Weiss & Navas-Martin, 2005; P. Zhou et al., 2018). The wide host range of animal CoVs increases the chance of zoonotic spillover into humans. Multiple spill-over events have been documented for CoVs. These include those responsible for the endemic HCoV-NL63, HCoV-229E and HCoV-OC43 and HCoV-HKU1 that cause self-limiting respiratory diseases, as well as highly pathogenic SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) which lead to severe respiratory illness and acute respiratory distress syndrome (ARDS) with high mortality rates (Cui et al., 2019; Gorbalenya et al., 2020; Su et al., 2016; Weiss & Navas-Martin, 2005).

1.2.1 SARS-CoV and MERS-CoV

SARS-CoV caused a large-scale outbreak of SARS between 2002 and 2004 (Fehr R A, 2015; Krammer, 2020). The first cases of severe atypical pneumonia of unknown aetiology were reported in Guangdong Province, China, in late 2002 and spread to several countries (Marra et al., 2003; Rota et al., 2003). The virus was later identified as SARS-CoV, classified as a member of the beta-CoVs genus lineage B, and reported to be phylogenetically distinct from the known CoVs at the time, namely HCoV-OC43 and HCoV 229E, and avian infectious bronchitis virus (IBV) (Marra et al., 2003; Rota et al., 2003). SARS-CoV was proposed to originate from zoonotic sources, supported by the early epidemiological reports of patients who had been exposed to live animals in market transactions prior to developing the disease (Weiss & Navas-Martin, 2005). Civet cats and raccoon dogs and market traders handling live animals had increased seroprevalence of SARS-CoV and SARS-like CoVs despite the absence of SARS disease (Cleri et al., 2010; Guan et al., 2003). The SARS-like CoV isolated from animals had more than 99% homology to SARS-CoV (Song et al., 2004). SARS-like CoV were also isolated from horseshoe bats and market animals, suggesting that bats were natural reservoirs and market animals were intermediate hosts (Balboni et al., 2012; Cleri et al., 2010; Lau et al., 2005; W. Li, Shi, et al., 2005; W. Wang & Han, 2018). The angiotensin-converting enzyme 2 (ACE2) receptor is the key cellular receptor that interacts with SARS-CoV S receptor binding domain (RBD) for efficient viral entry, mainly into epithelial cells (W. Li et al., 2003; Weiss & Navas-Martin, 2005). SARS-CoV infection resulted in a range of clinical outcomes,

including fever, non-productive cough, dyspnoea, pneumonia symptoms and ARDS, ultimately leading to death (Cleri et al., 2010). By the end of the SARS epidemic in 2003, more than 26 countries had reported over 8000 cases and 774 deaths, resulting in a global case fatality rate of approximately 10% (Cleri et al., 2010; Ksiazek et al., 2003; Weiss & Navas-Martin, 2005).

Following the SARS outbreak, a decade later in 2012, the MERS-CoV emerged in Saudi Arabia, Middle East, causing MERS (Cui et al., 2019; Zumla et al., 2015). MERS-CoV was first isolated from a patient's lung, who died of severe respiratory illness in Saudi Arabia in June 2012 (Zaki et al., 2012). The virus was later found to belong to the beta-CoVs genus lineage C and distantly related to SARS-CoV, although phylogenetically related to bat CoVs (Raj et al., 2014; Zaki et al., 2012). MERS-CoV, similar to SARS-CoV was speculated to originate from zoonotic sources (Paden et al., 2018). MERS-CoV-specific antibodies and neutralizing antibodies have been detected in dromedary camels in the Middle East (Meyer et al., 2014; Reusken et al., 2013), indicating that camels were potential intermediate hosts of MERS-CoV (Cui et al., 2019; Raj et al., 2014). Infection is thought to be primarily through contact with infected dromedary camels. Fortunately, person-to-person transmission of MERS is limited to close contact and health care settings thus limiting its spread (Arabi et al., 2017; WHO, 2021, 2022a). The key cell receptor of MERS-CoV differs from SARS-CoV. MERS-CoV utilizes dipeptidyl peptidase 4 (DPP-4, also known as CD26) as a receptor and infects unciliated bronchial epithelial cells and type II pneumocytes (Cui et al., 2019; Raj et al., 2013, 2014). MERS has also been associated with acute renal failure and the most common symptoms are fever, shortness of breath, myalgia, gastrointestinal (GI) symptoms (Raj et al., 2014). Overall, MERS causes a higher mortality rate of 34% compared to SARS-CoV, with 858 deaths out of approximately 2 400 people infected with MERS-CoV since 2012 (R. Lu et al., 2020; Paden et al., 2018; Shereen et al., 2020). The SARS and MERS outbreaks were controlled by public health measures, including quarantine and droplet precautions (Fehr R A, 2015). The outbreaks of SARS-CoV and MERS-CoV ultimately changed the prevailing concept of CoVs causing mild disease posing a low pandemic risk, with SARS considered the most severe human disease caused by HCoVs (Rastogi et al., 2020).

1.2.2 Endemic Human Coronaviruses

Endemic HCoVs are classified as alpha- and beta- CoVs and are speculated to originate from bats or rodents as natural hosts and to have emerged as a result of spill-over events followed by human-to-human transmissions (Van Der Hoek, 2007). HCoVs account for approximately 5-30% of upper and lower respiratory tract (URT and LRT, respectively) infections during winter and early spring (Geller et al., 2012; Su et al., 2016; Wat, 2004). Different geographical regions have different seasonal patterns of circulating HCoVs. In temperate climate regions such as India, some countries in Europe, Middle East, Japan and United States of America (USA), HCoVs infections are primarily detected in winter and spring with low-level of circulation throughout the year (Audi et al., 2020; McIntosh & Perlman, 2015). In tropical/subtropical regions including some countries Africa, Asia, Australia and Central America HCoVs circulate year-round with increased activity during certain months (Audi et al., 2020). In China, HCoVs have been reported to circulate year-round and the highest incidences are reported during spring and autumn (Audi et al., 2020; Z.-Q. Zeng et al., 2018). Meanwhile In SA, it has been reported HCoVs circulates throughout the year, however, the peak of alpha HCoVs infection occurs between November - January during warmer months and peak infections with beta HCoVs occurs in the colder months, May - August (Baillie et al., 2021).

HCoVs have an incubation period of 2-5 days and usually cause common cold illness with symptoms including headache, sneezing, malaise, nasal discharge fever, runny nose, cough and dyspnoea (S. Park et al., 2020; Su et al., 2016; Van Der Hoek, 2007; Woo et al., 2009). In addition to common cold symptoms, neurological complications such as meningoencephalitis and febrile seizures have been associated with all strains of HCoV infections, especially in children (Almqvist et al., 2020; Jevšnik et al., 2016). The first identified HCoVs were HCoV-229E and HCoV-OC43 discovered in the mid-1960s, which belong the alpha and beta- CoVs genus, respectively, and are known to cause mild to severe respiratory disease in young infants, the elderly and immunocompromised individuals (Hamre & Procknow, 1966; McIntosh et al., 1967; Perlman & Netland, 2009). Following the outbreak of the SARS epidemic, HCoV-NL63 and HCoV-HKU1 were identified in 2004 and 2005, respectively (Van Der Hoek et al., 2004; Woo et al., 2005). HCoV-NL63 infection associated LRT disease is common in young children while in adults it mainly

presents with URT disease (Abdul-Rasool & Fielding, 2010; Van Der Hoek, 2007; Van Der Hoek et al., 2005).

1.2.3 SARS-CoV-2

SARS-CoV-2 was first identified from a cluster of pneumonia cases, associated with the local seafood market in Wuhan, China in 2019 and was later confirmed to be a beta-CoV of bat origin related to SARS-CoV (F. Wu et al., 2020; P. Zhou et al., 2020). The SARS-CoV-2 genome was found to be closely related to SARS-like bat CoV with 96% identity, and more distant from SARS-CoV with approximately 80% of sequence homology and 50% to MERS-CoV (R. Lu et al., 2020; P. Zhou et al., 2020). The SARS-CoV-2 genome is approximately 26-32 kb and has 14 ORFs encoding 27 different viral proteins (A. Wu et al., 2020; F. Wu et al., 2020). Generally, RNA viruses, including CoVs have high mutation rates during viral replication (Robson et al., 2020; Saberi et al., 2018; Smith & Denison, 2013).

Viral evolution of SARS-CoV-2 driven by mutations, may have increased viral fitness and spread through the population at higher rates than at the outset of the SARS-CoV pandemic (Markov et al., 2023; A. Wu et al., 2020). The first reported SARS-CoV-2 variant with amino acid changes in the S gene from aspartic acid to glycine at position 614 (D614G), emerged early in the COVID-19 pandemic in March-April 2020 and spread rapidly throughout the world (Korber et al., 2020; Robertson, 2020; Volz et al., 2021). The D614G variant was reported to increase the rate of infectivity and virulence (Gobeil et al., 2021; Korber et al., 2020; Yurkovetskiy et al., 2020; L. Zhang et al., 2020). Subsequently, numerous SARS-CoV-2 variants were reported and spread across the world (WHO, 2022b). The Alpha (B.1.1.7) variant was first reported in the United Kingdom (UK) in October 2020 and contained 8 mutations in the S protein, in addition to D614G (Rambaut et al., 2020). Subsequently the Beta (B.1.351) variant was identified in SA in late August 2020 and contained 9 mutations in the S protein including 2 in the RBD. It was reported to be 1.5 times more transmissible than the Wuhan strain and rapidly spread to many parts of the world (Tegally et al., 2021). In late 2020, the Gamma (P.1) variant was identified in Brazil and reported to share similar mutations in the S-RBD protein with the Alpha and Beta variants (Faria et al., 2021). The

Delta variant was first reported in India in October 2020, containing 13 mutations and was linked to more severe disease (Butt et al., 2022; Cherian et al., 2021; Ong et al., 2022; Twohig et al., 2022). The Omicron variant discovered in SA and Botswana in late November 2021 caused widespread concern due to the greater than 30 mutations in the S protein raising the possibility of vaccine escape (Tegally et al., 2022; Viana et al., 2022). Subsequently, sub-lineages of Omicron emerged rapidly and continue to circulate in countries worldwide (Hadfield et al., 2018; Nextstrain, 2024) (Figure 1.2).

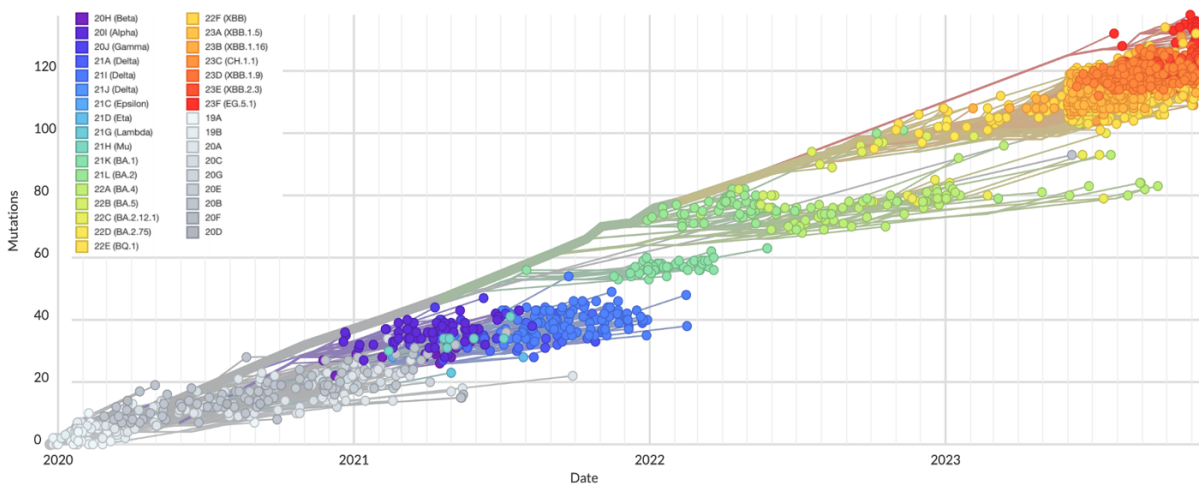


Figure 1.2: Accumulation of SARS-CoV-2 mutations over time (3 years) and color coded is the Pango clade is lineages according to WHO label, Nextstrain clade. The genome samples collected between December 2019 and November 2023 generated by nextstrain.org, (Nextstrain, 2024).

ACE-2 is the host receptor for SARS-CoV-2 resulting in Type II alveolar epithelial cells, alveolar macrophages (AM), airway epithelium and oesophageal epithelial cells being the primary target cells for SARS-CoV-2 entry due to their high expression of ACE-2 receptors (Cui et al., 2019; Harrison et al., 2020; Hoffmann et al., 2020; Rastogi et al., 2020; Yan et al., 2020). Viral entry is a 2-step process comprised of binding to the ACE-2 receptor followed by membrane fusion. This process is mediated by the S protein which consists of S1 and S2 subunits. The S1 subunit contains the RBD which binds to the ACE-2 receptor on host cells. The S2 subunit mediates virus-membrane fusion through fusion peptide (Bergmann & Silverman, 2020; Hoffmann et al., 2020;

Walls et al., 2020; Xia et al., 2020). Viral fusion occurs after two S protein cleavage events, the first at the polybasic furin cleavage site (S1/S2 junction) and the second within the S2 subunit by host cellular serine proteases namely, transmembrane protease serine 2 (TMPRSS2) or cathepsin L (Harrison et al., 2020; Hoffmann et al., 2020). There are two distinct SARS-CoV-2 viral entry mechanisms; endosomal entry and cell surface entry. In cell surface entry, S2 cleavage is mediated by TMPRSS2 at the cell surface. In the endosomal entry, the virus/ACE-2 complex is internalized and S2 cleavage is mediated by cathepsin L thus allowing viral entry in cells without TMPRSS2 (Bestle et al., 2020; Hoffmann et al., 2020; Rastogi et al., 2020). The route of SARS-CoV-2 infection depends on the protease present in the cell types and the protease cleavage sites (Bergmann & Silverman, 2020; W. Li, Zhang, et al., 2005).

SARS-CoV-2 variants arising from multiple mutations have increased transmissibility, suggesting that viral variants infect cells independently of the ACE-2/TMPRSS2 pathway. The Delta variant, similar to the SARS-CoV-2 ancestral strain, utilizes TMPRSS2 mediated viral fusion, whereas Omicron variants uses cathepsin-dependent endocytic pathway as depicted in **Figure 1.3** (Bálint et al., 2022; Meng et al., 2022; Rotulo & Palma, 2023). The change in the preferred viral entry mechanism of the SARS-CoV-2 Omicron variant, increases target cell types in the URT and LRT which could be related to higher transmissibility also seen in children (Bálint et al., 2022; Cloete et al., 2022; Eid et al., 2023).

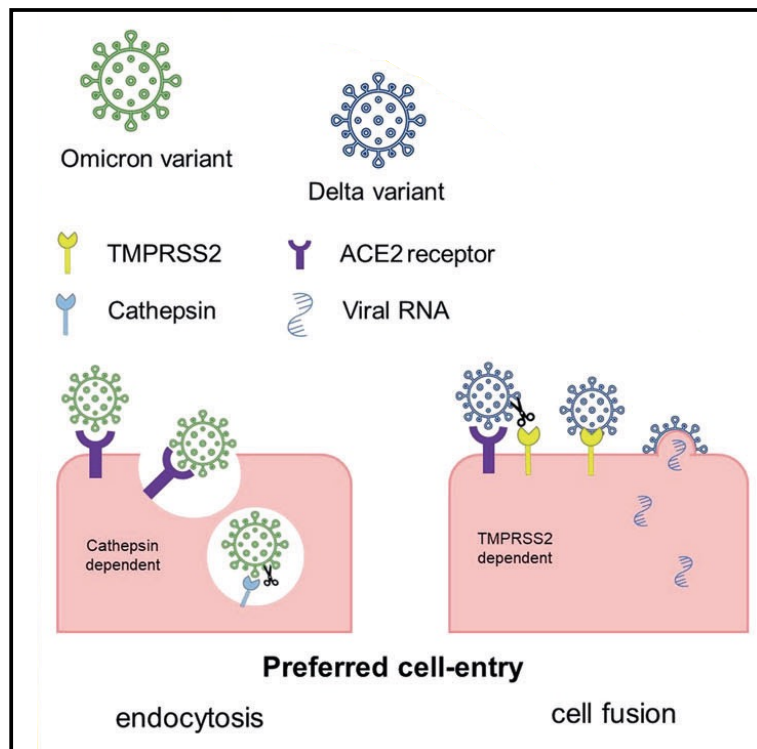


Figure 1.3: SARS-CoV-2 Omicron and Delta variants preferred cell entry. Omicron variant infects the cell through cathepsin-dependent pathway though binding affinity to ACE2 receptor and Delta variants infect cells through TMPRSS2-dependent pathway. Reproduced from Bálint et al., 2022.

1.3 Coronavirus Disease 2019 in adults and children

SARS-CoV-2 infection occurs primarily by person-to-person transmission through close contact with an infected individual via aerosol droplets (Y. R. Guo et al., 2020; C. Huang et al., 2020; Rastogi et al., 2020). The clinical outcomes of COVID-19 are similar to that of SARS-CoV and MERS-CoV infections, ranging from asymptomatic to mild, moderate and severe clinical COVID-19 outcomes (Harrison et al., 2020; D. Wu et al., 2020). The most common symptoms of SARS-CoV-2 infection include fever, dyspnoea, myalgia, dry cough, and gastrointestinal symptoms (Berlin et al., 2020; Chowdhury & Oommen, 2020; Y. R. Guo et al., 2020; C. Huang et al., 2020). Severe manifestations of COVID-19 occur after the onset of dyspnoea which leads to shortness of breath and hypoxemia, progressing to respiratory failure and requiring hospitalization for mechanical ventilation (Lamers & Haagmans, 2022; F. Zhou et al., 2020). Severe manifestations of COVID-19

can also lead to extrapulmonary disease, including neurological complications, cardiac arrhythmias, rhabdomyolysis, kidney and liver injury (Berlin et al., 2020; Harrison et al., 2020). One therapeutic treatment for critical COVID-19 cases other than oxygen therapy is an antiviral drug, remdesivir which was approved by the US Food and drug administration (FDA) for the treatment of hospitalized COVID-19 patients (Aslan et al., 2021; Beigel et al., 2020). Other potential drugs that have been tested and used to treat severe COVID-19 symptoms include convalescent plasma therapy, favipiravir, lopinavir/ritonavir, chloroquine and hydroxychloroquine, immunomodulatory medications such as dexamethasone, tocilizumab (anti-IL-6), anti-TNF- α , baricitinib (Aslan et al., 2021; Beigel et al., 2020; Bergmann & Silverman, 2020; Takahashi et al., 2020; Villar et al., 2020; L. Yang et al., 2020). The incubation period for SARS-CoV-2 infection was estimated to be between 3 and 6 days, which may differ between asymptomatic, symptomatic and severe COVID-19 cases (Chan et al., 2020; Lauer et al., 2020; Linton et al., 2020). Of note, transmission may occur during the asymptomatic phase, which is longer than reported for SARS-CoV and MERS-CoV infection (Chang et al., 2020; Du et al., 2020; Lauer et al., 2020; Q. Li et al., 2020; Su et al., 2016). Several risk factors have been associated with severe clinical COVID-19 outcome, including age and underlying co-morbidities (Chowdhury & Oommen, 2020; Y. R. Guo et al., 2020; J. jin Zhang et al., 2023).

Age

Typically, children are more susceptible to acute respiratory infections such as pneumonia and bronchiolitis caused by respiratory viruses including respiratory syncytial virus (RSV), rhinovirus (RV), influenza virus, human parainfluenza virus (HPIV), HCoVs and human adenovirus (HAdV). These respiratory diseases contribute to morbidity and mortality rates in children depending on age and geographical regions (Kenmoe et al., 2020; Y. Li et al., 2022; Nair et al., 2011; Shi et al., 2017; X. Wang et al., 2020). In contrast, SARS-CoV-2 epidemiological studies reported that children accounted for approximately 2.6% of severe and 0.6% of critical COVID-19 cases, which was less than in adults (Chao et al., 2020; Dong et al., 2020; Q. Li et al., 2020; W. Liu et al., 2020; Rotulo & Palma, 2023).

Comorbidities

Differences in COVID-19 severity could be attributed to the distribution of comorbidities between children and adults, notably hypertension, cardiovascular disease, diabetes, chronic kidney disease, chronic liver disease and chronic pulmonary diseases (Clark et al., 2020; Davies et al., 2020; Gao et al., 2021; Kara et al., 2021; N. Williams et al., 2021; J. jin Zhang et al., 2023). This is supported by the occurrence of symptomatic COVID-19 and hospital admission in some children under 18 years of age with specific comorbidities including type 1 diabetes, neurological disorders, sickle cell disease and congenital heart disease (Antoon et al., 2021; Götzinger et al., 2020; Haji Esmaeil Memar et al., 2021; Kapoor et al., 2021; Kompaniyets et al., 2021; Mann et al., 2022; Mucalo et al., 2021; Shekerdemian et al., 2020; Swann et al., 2020; Tsankov et al., 2021; Zachariah et al., 2020).

1.3.1 Complications of COVID-19

The most common complications of COVID-19 include ARDS and multiple organ failure. Patients with severe COVID-19 have respiratory failure that progresses to ARDS, a severe hypoxemia characterized by inflammation, pulmonary vascular leakage and lung tissue damage, as well as systemic hyperinflammation (Lamers & Haagmans, 2022). In addition, uncontrolled inflammation, including increased levels of inflammatory markers such as C- reactive protein (CRP), ferritin dimer, D-dimer and neutrophil/lymphocyte ratio has been associated with COVID-19 disease severity and death (Lamers & Haagmans, 2022; Mehta et al., 2020; Merad & Martin, 2020). Severe COVID-19 has also been associated with severe lymphopenia, a reduction in CD4+ and CD8+ T cell counts, which appears to correlate with COVID-19 severity and mortality (Vabret et al., 2020; X. Zhang et al., 2020). The cytokine storm seen in critical COVID-19 patients includes elevated inflammatory cytokines, interleukin (IL)-6, IL-8, IL-10, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1 α) and tumor necrosis factor (TNF), which are associated with disease severity and death (Chua et al., 2020; Mathew et al., 2020; Merad & Martin, 2020; Vabret et al., 2020).

Long-term complications of COVID-19 include Long COVID, MIS-C and MIS-Adult (MIS-A). Long COVID is typically diagnosed three or more months after the initial onset of SARS-CoV-2 acute infection and is characterized by persistent symptomatic complications within multiple organs including respiratory, cardiovascular, gastrointestinal, neurological, systemic and psychological (Davis et al., 2023; Desai et al., 2022; Nalbandian et al., 2021; Parums, 2021; Su et al., 2023; Thallapureddy et al., 2022). MIS-A presents four weeks after initial infection with SARS-CoV-2 in young adults over the age of 21 years (Parums, 2021). Individuals initially reported with MIS-A presented with cardiovascular, gastrointestinal, dermatologic, and neurologic symptoms without severe COVID-19 illness and tested positive for SARS-CoV-2, which is similar to the clinical presentation of MIS-C (Davogustto et al., 2021; Morris et al., 2020). MIS-C is a post-COVID-19 complication that occurs in individuals under the age of 21 and is characterized by fever and multi-organ dysfunction (Rowley, 2020b, 2020a).

1.3.2 Multisystemic inflammatory syndrome in children

In April 2020, a cluster cases of children with hyperinflammatory disease associated with SARS-CoV-2 was reported in Europe and later identified as MIS-C (Riphagen et al., 2020). The reported clinical cases of children with MIS-C presented with persistent fever, and clinical features similar to Kawasaki disease (KD) and toxic shock syndrome (TSS) (Riphagen et al., 2020; Viner et al., 2021). The estimated incidence rate of MIS-C cases was less than 1%, which may vary according to ethnic background, age, gender, geographical location and epidemic infection waves driven by SARS-CoV-2 VOC (Butters et al., 2022; Dionne et al., 2022; Payne et al., 2021). Lower incidence and severity of MIS-C following breakthrough infection has been reported in vaccinated children compared to unvaccinated children following Delta and Omicron infection waves (Abraham et al., 2022; Holm et al., 2021; Kenney et al., 2023; Levy et al., 2022). Common symptoms associated with MIS-C include fever, rash, conjunctivitis, gastrointestinal symptoms, shock, peripheral oedema and myocarditis (Esposito & Principi, 2021; Martinez et al., 2020; Rotulo & Palma, 2023; Rowley, 2020a). Children with MIS-C were also reported to have elevated laboratory indicators of inflammation including CRP, D-dimer and serum ferritin and elevated levels of soluble biomarkers associated with macrophage and neutrophil recruitment and activation (Sacco et al., 2022; Vella

et al., 2021). These innate immunity-associated factors have been suggested to contribute to systemic inflammation in the development, severity and complications of MIS-C (de Cevins et al., 2021; Filippatos et al., 2023; Pierce et al., 2022; Ramaswamy et al., 2021; Sacco et al., 2022; Vella et al., 2021). Another proposed factor contributing to the hyperinflammatory state of MIS-C is the presence of elevated autoantibodies against proteins associated with immune cell signaling, endothelial and cardiac development and other targets of autoimmunity in acute MIS-C patients (Consiglio et al., 2020; Gruber et al., 2020; Pfeifer et al., 2022). Finally, along with the GI symptoms, the gut has been proposed to be a viral reservoir, associated with MIS-C pathogenesis (Trottein & Sokol, 2020; Yonker et al., 2020). Yonker et al. showed that children diagnosed with MIS-C had SARS-CoV-2 viral persistence in the GI tract, increased release of zonulin proteins associated with intestinal permeability, and elevated SARS-CoV-2 antigens in the periphery, concluding that hyperinflammatory responses in MIS-C is associated with viral persistence in the GI tract (Yonker et al., 2021). Therapeutic management of critical COVID-19 in children is similar to that in adults, with the addition of glucocorticoids (methylprednisolone) in combination with intravenous immunoglobulin (IVIg) for MIS-C treatment (McArdle et al., 2021; Rotulo & Palma, 2023).

1.4 Immune responses to SARS-CoV-2 infection

The immune system consists of two complementary arms: the innate and adaptive immune systems, which interact and work in a coordinated manner to respond to pathogen invasion, aided by circulating factors such as cytokines and chemokines. Several theories have been proposed as potential contributors to the age-related differences in clinical outcomes of COVID-19 between children and adults, including underlying comorbidities, expression of viral entry receptors, robust innate immunity, differences in adaptive immunity and cross-reactive HCoVs immunity (Chou et al., 2022; Fischer, 2020; Pierce et al., 2022; J. jin Zhang et al., 2023; Zimmermann & Curtis, 2021). This section discusses the immune responses to SARS-CoV-2 with a focus on children compared to adults, and the final **section 1.4.3** discusses the immune responses in MIS-C.

1.4.1 Innate immunity

Innate immunity initiates local inflammatory responses to viral infection, directly eliminates virus-infected cells and primes adaptive immune responses. The initial cellular antiviral defense upon viral infection is mediated by the induction of interferon (IFN) responses and upregulation of IFN-stimulated genes (ISG) to regulate the immune response, after which the antiviral response involves the recruitment of immune cells coordinated by the secretions of cytokines and chemokines (Blanco-Melo et al., 2020; Jensen & Thomsen, 2012; Karki & Kanneganti, 2022; A. J. Lee & Ashkar, 2018). Innate immune sensing of SARS-CoV-2 is mediated by the recognition of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs), which induce antiviral immune responses (Karki & Kanneganti, 2022; Y. Lim et al., 2016; Minkoff & tenOever, 2023; Vabret et al., 2020). Among the PRRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), including RIG-1 and melanoma differentiation-associated antigen 5 (MDA5) are of particular importance for controlling viral infection and induction of IFNs (Kindler et al., 2016; Madden & Diamond, 2022; Newton et al., 2016; D.-M. Yang et al., 2021). SARS-CoV-2 viral intermediates are also likely recognized by a family of toll-like receptors (TLR), which sense single-stranded RNA (TLR7 and TLR8) and double-stranded RNA intermediates (TLR3) (Birra et al., 2020; Karki & Kanneganti, 2022; Khanmohammadi & Rezaei, 2021; Madden & Diamond, 2022; D.-M. Yang et al., 2021). Transcription of type I and III IFNs and several proinflammatory cytokines occurs after PRR sensing, thereafter cytokines and chemokines are released that play a critical role in viral clearance and recruitment of other immune cells (Birra et al., 2020; Khanmohammadi & Rezaei, 2021; Sahu et al., 2021). Type I and III IFNs are master antiviral signaling factors, the main drivers of innate immune responses to viral infection and induce antiviral effectors in an autocrine and paracrine manner (A. Park & Iwasaki, 2020). The role of IFN-I during SARS-CoV-2 infection is complex, although delayed and impaired IFN-I and IFN-III are hallmarks of COVID-19 disease severity and likely contribute to viral persistence, inflammation and lung injury in critically ill patients (Blanco-Melo et al., 2020; Galani & Andreakos, 2021; Hadjadj et al., 2020; Karki & Kanneganti, 2022; D.-M. Yang et al., 2021).

Children have been shown to possess higher expression of MDA5 and RIG-I receptor genes in upper airway epithelial cells compared to adults (Loske et al., 2022). The pre-activated cell-intrinsic immune responses due to increased receptors in children suggested robust induction of antiviral IFN production which may contribute to early clearance of SARS-CoV-2 (Loske et al., 2022; Speranza, 2021; D.-M. Yang et al., 2021; Yoshida et al., 2021). It is widely reported that children infected with SARS-CoV-2 have more robust innate immunity characterized by activated IFN gene signatures, inflammasome complex activation, activated innate immune cells such as natural killer (NK) cells, neutrophils and monocytes involved in viral clearance and cell recruitment (Loske et al., 2022; Neeland et al., 2021a; Pierce et al., 2020; Yoshida et al., 2021). Lastly, a study by Yoshida et al. showed that healthy SARS-CoV-2 uninfected children (aged 3 days - 16 years) had stronger local IFN immune responses along with systemic responses enriched with naïve lymphocyte populations compared to uninfected adults, altogether these responses may contribute to early viral clearance in children (**Figure 1.4**) (Yoshida et al., 2021).

Overall, these studies of the innate immune response in children have shown that early clearance of SARS-CoV-2 may contribute to the different clinical outcomes of COVID-19 compared to adults. The importance of innate immune responses against SARS-CoV-2 in children cannot be overlooked, however, the adaptive immune system is also important as it provides a coordinated cellular immune response critical for viral control and establishes immunological memory.

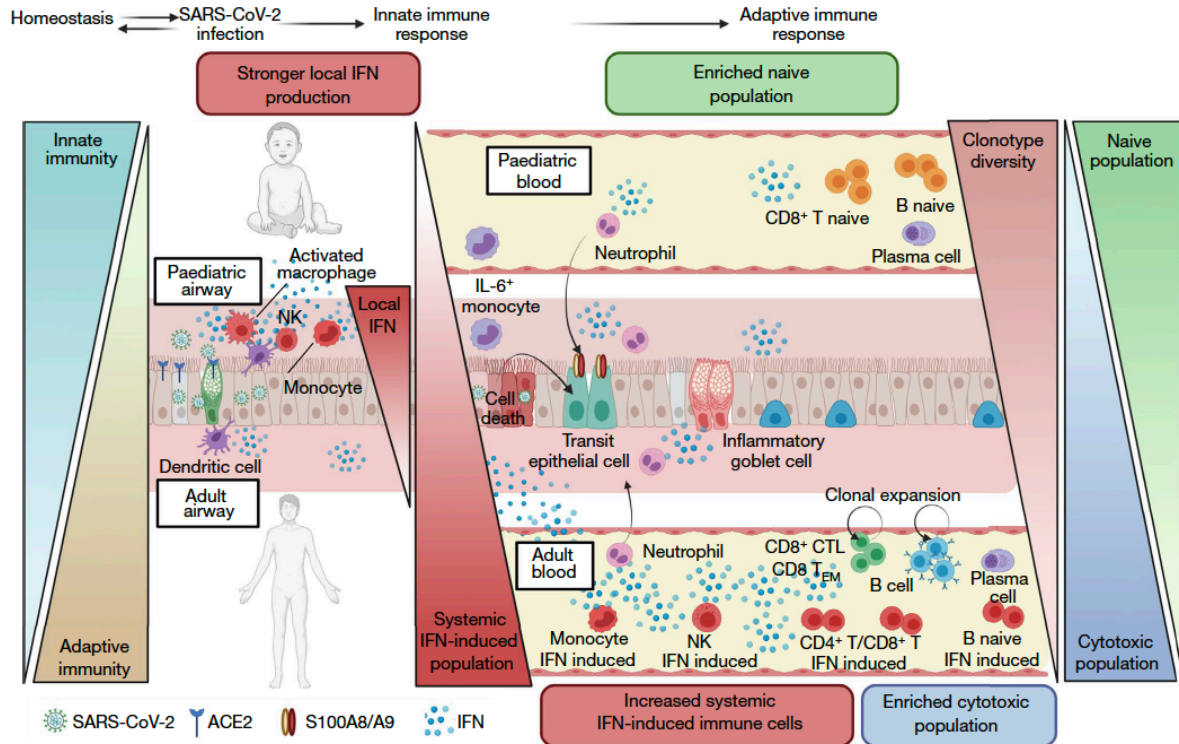


Figure 1.4: SARS-CoV-2 local and systemic immune responses in children and adults. Local immunity from airways on right, shows children have stronger local IFN immunity, overall higher innate immunity, and less adaptive immunity than adults. Systemic responses in peripheral blood showed adults have increased systemic IFN-induced immune cells along with cytotoxic enriched population, less naïve population and clonotype diversity compared to children. Reproduced from Yoshida et al., 2021.

1.4.2 Adaptive immunity

The role of adaptive immunity in controlling primary SARS-CoV-2 infection is critical to disease course as well as the generation of a robust immunological memory for protective immunity against subsequent re-infection (Bartleson et al., 2021). Three lymphocyte populations make up the adaptive immune system, B lymphocytes (B cells), CD4+ and CD8+ T lymphocytes (T cells). Several studies have reported the importance of the adaptive immune response as a major determinant of the clinical outcome following SARS-CoV-2 infection (Bertoletti et al., 2021b; C. Chen et al., 2023; Grifoni et al., 2020; Kedzierska & Thomas, 2022; Melenotte et al., 2020; Moss, 2022; Sette et al., 2023; Sette & Crotty, 2021). The role of each adaptive component during SARS-CoV-2 infection in children and adults is discussed below under humoral and cellular immune responses.

1.4.2.1 Humoral immune responses

Humoral or antibody-mediated immunity to SARS-CoV-2 proteins is rapid and elicits robust B cell responses with the detection of virus-specific IgM, IgG, IgA and neutralizing IgG antibodies, approximately 7-14 days after infection (Rotulo & Palma, 2023; Vabret et al., 2020). Antibody responses to SARS-CoV-2 S protein and highly immunogenic RBD can potentially block viral interaction with the host ACE-2 receptor, thereby limiting viral entry into cells (Amanat et al., 2020; Bartsch et al., 2021; Ju et al., 2020; Secchi et al., 2020; Wölfel et al., 2020; Zost et al., 2020). Humoral immune responses decline with age with B cells displaying a diminished potential to undergo somatic hypermutation resulting in aged individuals generating less robust antibody responses compared to younger individuals (Bartleson et al., 2021; Frasca, 2018; Gibson et al., 2009; Weksler & Szabo, 2000). Children have been reported to possess higher proportions of naïve lymphocytes including B cells (Simon et al., 2015; Yoshida et al., 2021), the diversity of naïve B cell receptors enables recognition of wide varieties of novel pathogens including SARS-CoV-2 and antibody repertoires evolve with exposure to infectious pathogens (Bajaj et al., 2021; Ciocca et al., 2021; Nielsen et al., 2019; Rodda & Pepper, 2020; Semmes et al., 2021; Tabibian-Keissar et al., 2016).

Children mount a significantly higher IgG antibody responses to SARS-CoV-2-S, RBD, S1 domain and N protein compared to adults, whereas no differences in antibody IgG titres were found when comparing asymptomatic and symptomatic children (ranging from 3 – 11 years, 6 - 13 years and 0.2 - 17.9 years) (Dowell et al., 2022a; Renk et al., 2022; Ruedas-López et al., 2022). Similarly, antibody neutralizing activity was found increased in children compared to adults (Bonfante et al., 2021; Garrido et al., 2021; P. Liu et al., 2020; Ruedas-López et al., 2022; H. S. Yang et al., 2021). Several studies have also shown that children and adolescents develop robust and durable antibody responses to SARS-CoV-2 which are maintained up to 6 months to 1 year after mild or asymptomatic infection (Garrido et al., 2021; Jacobsen et al., 2022; P. Liu et al., 2020; Méndez-Echevarría et al., 2021; Ruedas-López et al., 2022). Higher initial SARS-CoV-2 antibody titers, hospitalization and LRTI were associated with improved antibody durability (Méndez-Echevarría et al., 2021; Renk et al., 2022).

SARS-CoV-2 escape from infection and vaccination induced antibody-mediated immunity has been extensively reported for VOCs, in particular Omicron sub-lineages in adults (Bekliz et al., 2022; Cao et al., 2022; Carreño et al., 2022; Cele et al., 2022; Dejnirattisai et al., 2022; Khan et al., 2022; Richardson et al., 2022; Rössler et al., 2022; Wilhelm et al., 2022). Partial viral escape of antibody immunity from prior SARS-CoV-2 infection or vaccination has also been reported for Beta, Gamma and Delta variants (Carabelli et al., 2023; Markov et al., 2023). Although, it has been reported that vaccine-induced immunity against Beta and Delta variants was largely preserved (Abu-Raddad et al., 2021; Andrews et al., 2022; Bekker et al., 2022; Lopez Bernal et al., 2021; P. Tang et al., 2021; Zeng et al., 2022), the emergence of Omicron variants with approximately 30 mutations in S-RBD region compared to other VOCs has resulted in greater antibody evasion (Tegally et al., 2022; Viana et al., 2022). Prior SARS-CoV-2 infection elicited lower neutralization capacity against Omicron BA.1, suggesting limited protection against Omicron BA.1 re-infection (Carreño et al., 2022; Dejnirattisai et al., 2022; Planas et al., 2022; Richardson et al., 2022). Several studies investigating VOC antibody immune escape in convalescent/uninfected children also demonstrating varying cross-reactive neutralizing capacity against VOC Alpha, Beta, Delta and Omicron variants (Bellusci et al., 2023; L.-L. Chen et al., 2022; Dowell et al., 2022a; Paniskaki et al., 2023; W. Peng et al., 2023; Renk et al., 2022; Seidel et al., 2023; Sieber et al., 2022; J. Tang et al., 2022; Toh et al., 2022). A study by Sieber et al. reported that convalescent children lacked neutralization antibodies against Omicron (B.1.1.529) variant 12 months post-infection (Sieber et al., 2022). Similarly, a study by Paniskaki et al. which found that $\approx 66\%$ and 93% loss of cross-reactive neutralizing potential against Delta (B.1.617.2) and Omicron (B.1.1.529) variants, respectively, in children 6 months post infection (Paniskaki et al., 2023). Altogether, these studies show that neutralization potential to Omicron VOC is poor or reduced when compared to earlier VOCs including ancestral (D614G) variant.

The partial immune escape of SARS-CoV-2 VOC from neutralizing antibodies raised major concerns on the effectiveness of first-generation SARS-CoV-2-S vaccines in eliciting robust immune responses to provide herd and long-term immunity. Protective immunity elicited by prior

SARS-CoV-2 vaccination against VOCs in children remains to be studied extensively since this population is the largest unvaccinated population globally (Naeimi et al., 2023).

1.4.2.2 T cell mediated immune responses

Several studies from the USA, Netherlands, Singapore, UK, Sweden and Germany early in the pandemic reported robust T cell responses following SARS-CoV-2 infection, with CD4⁺ T cell responses being more dominant than CD8⁺ T cell responses (Altmann & Boyton, 2020; Bertoletti et al., 2022; Grifoni et al., 2020; Y. Peng et al., 2020; Rydzynski Moderbacher et al., 2020; Sekine et al., 2020; Sette & Crotty, 2021; Thieme et al., 2020; Vabret et al., 2020). Extensive subsequent research was directed at determining the role of T cell immune responses in controlling SARS-CoV-2 infection and disease severity. The most common effector responses elicited by SARS-CoV-2 infections are T helper cell 1 (Th1) secreting IFN- γ , IL-2, TNF- α cytokines, which enhance cell-intrinsic antiviral activity, follicular helper (Tfh) CD4⁺ T cells that induce B cells to produce high-affinity antibodies and cytotoxic CD8⁺ T cells with effector molecules, perforin, CD107a degranulation and Granzyme B/A which kill pathogen-infected cells (Altmann & Boyton, 2020; Goldblatt et al., 2022; Jarjour et al., 2021; Sette & Crotty, 2021; Vabret et al., 2020). The timing, magnitude and quality of SARS-CoV-2-specific T cell responses have been shown to be important determinants of COVID-19 outcomes during primary acute infection. Early CD4 T cell responses exhibiting high polyfunctionality in combination with prompt type 1 IFN responses and strong CD8 T cell responses are all associated with asymptomatic to mild disease, while low frequencies of naïve T cells is associated with severe disease (Chandran et al., 2022; Rydzynski Moderbacher et al., 2020; Sette et al., 2023; A. T. Tan et al., 2021; Tarke et al., 2022b). Naïve T cells have been shown at high frequencies in SARS-CoV-2 infected children compared to infected adults. This was also accompanied by diverse TCR repertoires capable to recognizing multiple epitopes of the viral proteins leading to early adaptive immunity in children (Khoo et al., 2023; Yoshida et al., 2021).

Several studies have shown that children with asymptomatic or symptomatic SARS-CoV-2 infection have detectable CD4⁺ and CD8⁺ T cell responses to SARS-CoV-2 viral structural proteins (C. A. Cohen et al., 2021; Cooper et al., 2021; Cotugno et al., 2021; Dowell et al., 2022a; Goenka et al., 2021; Jacobsen et al., 2022; Kaaijk et al., 2022; Paul et al., 2022; Rowntree et al., 2022;

Tsang et al., 2021), although some discordance with respect to the magnitude of these responses compared to adults remains. SARS-CoV-2-specific T cell responses against S, were more robust in infected adults with higher frequencies of IFN- γ + CD4+ T cells compared to pediatric groups (Pierce et al., 2020). Similarly, a study by Cohen C.A et al. showed SARS-CoV-2 infected children (aged 2 - 13 years) had lower IFN- γ + CD4+ and CD8+ T cell responses to SARS-CoV-2 structural proteins compared to infected adults, which were proposed to be as a result of lower baseline immune activation and possibly due to disparate pre-existing immunity to endemic human CoVs (C. A. Cohen et al., 2021). A study by Rowntree et al. utilised peptide-HLA tetramers to measure SARS-CoV-2-epitope-specific CD4+ directed against 1 epitope DPB4/S₁₆₇ and CD8+ T cells directed against 6 epitopes (A1/ORF1a₁₆₃₇, A2/S₂₆₉, A3/N₃₆₁, A24/S₁₂₀₈, B7/N₁₀₅, and B40/N₃₂₂) to provide further immunological features of virus-specific T cells (Rowntree et al., 2022). The study also found SARS-CoV-2 RBD IgG+ children (aged 0.4 - 7 years) had lower circulating SARS-CoV-2-tetramer specific CD4+ and CD8+ T cells compared to adults and concluded T cells are not fully activated to similar extent as adults (Rowntree et al., 2022). In contrast, Dowell et al. reported children (ages 3 - 11 years) with prior SARS-CoV-2 infection have more robust cellular responses against SARS-CoV-2 structural proteins compared to adults. The magnitude of responses against SARS-CoV-2-S was higher in the SARS-CoV-2 seropositive children compared to seropositive adults (Dowell et al., 2022a). T cell responses in children showed differential cytokine responses, with reduced IL-2 productions. The study concluded that children have a highly differentiated profile which contribute to enhance cellular immunity compared to adults (Dowell et al., 2022a). The differences in the magnitude of SARS-CoV-2-specific cellular responses in children compared to adults in these studies could be accounted by the disease severity between children and adults, differences in assay functional readouts and peptide pools used for stimulation.

In addition to the role of T cells in ameliorating disease severity, they play an important role in immunological memory for protection from disease associated with reinfection. Memory T cell development depends on the effectiveness of the primary response to allow differentiation of memory T cell precursors into fully formed memory populations (Altmann & Boyton, 2020). SARS-CoV-specific memory T cells have been shown to persist for 17 years following primary infection,

highlighting the potential for long lasting T cell immunity for protection from reinfection and disease (Le Bert et al., 2020). To date, SARS-CoV-2-specific memory T cells have been reported to persist for 6-15 months after primary infection (K. W. Cohen et al., 2021; Dan et al., 2021; L. Guo et al., 2022; Marcotte et al., 2022; Zuo et al., 2021). Several studies have demonstrated that the predominant memory subset of SARS-CoV-2-specific CD4⁺ T cells induced by infection or vaccination are of the early differentiated phenotype (ED: CD45RA-CD27⁺), which comprises both central memory (TCM: CD45RA-CD27⁺CCR7⁺) and effector memory 1 (EM1: CD45RA-CD27⁺CCR7⁻) (Dan et al., 2021; Goel et al., 2021b; Moyo-Gwete et al., 2023; Nesamari et al., 2024; Painter et al., 2021; Rowntree et al., 2022). Dan et al. specifically showed the trajectory of antigen-specific TCM increased, while TEM reduced over time such that by 6 months post-infection the TCM subset was dominant (Dan et al., 2021). Of note, the study by Goel et al. demonstrated that an increased proportion of EM1 cells, a subset of ED memory T cells, at the peak of the CD4 T cell response was associated with improved durability (Goel et al., 2021b). Comparisons of SARS-CoV-2-specific T cell memory phenotypes between children and adults has revealed discordant data. A study by Cohen C.A. et al. reported that infected children exhibited reduced proportions of TEM (CD45RA-CCR7⁻) but similar TCM (CD45RA-CCR7⁺) phenotype within SARS-CoV-2-specific CD4⁺ T cells compared to adults (C. A. Cohen et al., 2021), which was in contrast to a studies by Jacobsen et al. and Rowntree et al (Jacobsen et al., 2022; Rowntree et al., 2022). The study by Jacobsen et al. reported a reduced proportion of TCM (CD45RO⁺CCR7⁺) within SARS-CoV-2-specific CD4⁺ T cells (Jacobsen et al., 2022), while the study by Rowntree et al. showed that early differentiated T cells (CD45RA-CD27⁺ which they termed TCM-like) increased with age (Rowntree et al., 2022). The discordant data reported by these studies require more extensive study on SARS-CoV-2-specific memory profile in children.

Studies have also investigated the persistence of SARS-CoV-2-specific cellular immunity following asymptomatic infection in children also lack concordance in the data (Dowell et al., 2022a; Jacobsen et al., 2022; Kaaijk et al., 2022; Sieber et al., 2022). A study by Kaaijk et al. showed detectable SARS-CoV-2-S specific T cells 10 months post-infection in 44% of infected children compared to 81% in adults (Kaaijk et al., 2022). This represented a 4-fold reduction in SARS-CoV-

2-specific T cells at 10 months vs 3 weeks post infection in children at which point the responses were no longer significantly higher than those in uninfected children (Kaaijk et al., 2022). Similarly, a study by Jacobsen et al. showed a significant reduction in SARS-CoV-2-specific CD4+ T cells in children over 8-month period although, in disparity to the Kaaijk study, these responses remained detectable in most children albeit at a lower frequency than in adults (Jacobsen et al., 2022). In contrast, a study by Dowell et al. showed higher SARS-CoV-2-S specific T cell responses in children compared to adults at 6 months post infection, although SARS-CoV-2-S specific T cell responses were lower at 12 months compared to 6 months in children (Dowell et al., 2022a), while a study by Sieber et al. showed no differences in the CD4+ T cell levels at 6 months compared to 12 months in children (Sieber et al., 2022).

The literature on cross-reactive SARS-CoV-2-specific T cell responses to VOC elicited from prior SARS-CoV-2 infection and/or vaccination is continuously growing, and numerous studies have reported on adult cohorts (Alter et al., 2021; Gao et al., 2022; Geers et al., 2021; GeurtsvanKessel et al., 2022; L. Guo et al., 2022; Keeton et al., 2021, 2022, 2023; Nesamari et al., 2024; Reynolds et al., 2021; Riou et al., 2021; Skelly et al., 2021; Tarke et al., 2022a; Tarke et al., 2021b; Woldemeskel et al., 2021). Overall, these studies found that SARS-CoV-2-specific CD4+ and CD8+ T cell responses were largely preserved against VOCs in adults. However, fewer studies have investigated cross-reactive T cell responses against SARS-CoV-2 VOC in children and the emerging data is conflicting. A study by Seidel et al. demonstrated that one year after infection with Ancestral Wuhan strain SARS-CoV-2 the magnitude of the SARS-CoV-2 BA.1 S CD4+ T cell responses was significantly lower compared to CD4+ T cell responses directed against ancestral strain S (Seidel et al., 2023). In contrast, studies by Sieber et al. and Paniskaki et al. demonstrated no differences in the magnitude of the SARS-CoV-2 BA.1 S compared to ancestral S CD4+ T cell responses 6 months (Paniskaki et al., 2023) or 1 year (Sieber et al., 2022) after infection.

1.4.3 Immune responses in MIS-C

Several studies have evaluated SARS-CoV-2-specific T cell responses in children with MIS-C compared to severe COVID-19 or convalescent children (Hsieh et al., 2022a; Hsieh et al., 2022b;

Lam et al., 2022; Rybkina et al., 2023; Singh et al., 2022; Vella et al., 2021). However, there is conflicting findings on the magnitude of SARS-CoV-2-specific T cells in MIS-C patients. A study Singh et al. found children with MIS-C had inadequate antiviral T cell responses compared to COVID-19 convalescent children, suggesting that constrained SARS-CoV-2-specific T cell responses play a role in MIS-C clinical outcome and pathogenesis (Singh et al., 2022). In contrast another study by Conway et al., found SARS-CoV-2-specific T cell responses in MIS-C were higher compared to COVID-19 convalescent children, which suggests the syndrome is not associated with ineffective antiviral responses following SARS-CoV-2 infection (Conway et al., 2022). Rybkina et al. showed SARS-CoV-2-specific T cell responses following recovery from MIS-C were distinct, with increased frequency and Th1 polarization profiles compared to those children that recovered from COVID-19 (Rybkina et al., 2023). The study also found that circulating T cells in acute MIS-C exhibited multiple features of tissue-resident T cells (Trm) that correlated with markers indicative of cardiac dysfunction, indicating a possible role of T cells in immunopathology (Rybkina et al., 2023). Although there is contradicting literature on the contribution of SARS-CoV-2-specific T cells in MIS-C pathogenesis, studies have established T cell responses have a potential role in MIS-C pathogenesis, more importantly in immunopathology and overall effector function for viral control.

SARS-CoV-2 S protein was reported to have superantigen-like properties, that induce non-specific activation of T cells. This was reported in some severe COVID-19 patients with hyperinflammation (Cheng et al., 2020; Hamdy & Leonardi, 2022; Notarbartolo et al., 2021) and patients diagnosed with MIS-C (Lam et al., 2022; D. Lee et al., 2023; Moreews et al., 2021; Porritt et al., 2021b; Ramaswamy et al., 2021; Yonker et al., 2021). Superantigens bypass the normal antigen specificity of TCR through direct binding to the invariant region of major histocompatibility class II (MHC-II) molecules and specific TCR variable domain β chains to induce polyclonal expansion and activation of T cells (Amormino et al., 2022; Brodin, 2023; Cheng et al., 2020; Filippatos et al., 2023). The superantigen hypothesis is backed by reports that a proportion of children with MIS-C displayed expansion of T cells with TCR β variable region, *TRBV11.2* gene, encoding TCR V β 21.3. This is further supported by the similarity of MIS-C to TSS. TSS has been shown to be associated

with bacterial superantigen-mediated responses leading to non-specific T cell activation (Brodin, 2022, 2023; Moreews et al., 2021; Rivas et al., 2021, 2022). In addition, two studies have reported that expression of TCR V β 21.3 or the *TRBV11.2* gene, can differentiate between children with MIS-C and those with non-MIS-C inflammatory conditions (Lam et al., 2022; Moreews et al., 2021).

In a recent study, Lee et al. reported that a subset of children with MIS-C (ages 0.3-14 years) lack autosomal recessive genes encoding 2'-5'-oligoadenylate synthetase (OAS)-RNase L, which primarily function in the viral RNA sensing pathway leading to enhances excessive inflammatory responses in myeloid cells (D. Lee et al., 2023). **Figure 1.5** Brodin et al. (Brodin, 2023) shows a proposed model of hyperinflammation and delayed presentation of MIS-C as a continuous loop of hyperactivation that involves SARS-CoV-2 viral persistence in the GI tract (Yonker et al., 2021), tissue resident myeloid and T cell activation (Rybkina et al., 2023), inhibition of RNase-OAS pathway (D. Lee et al., 2023) and lastly T cell activation by SARS-CoV-2 superantigen-structure (D. Lee et al., 2023; Moreews et al., 2021).

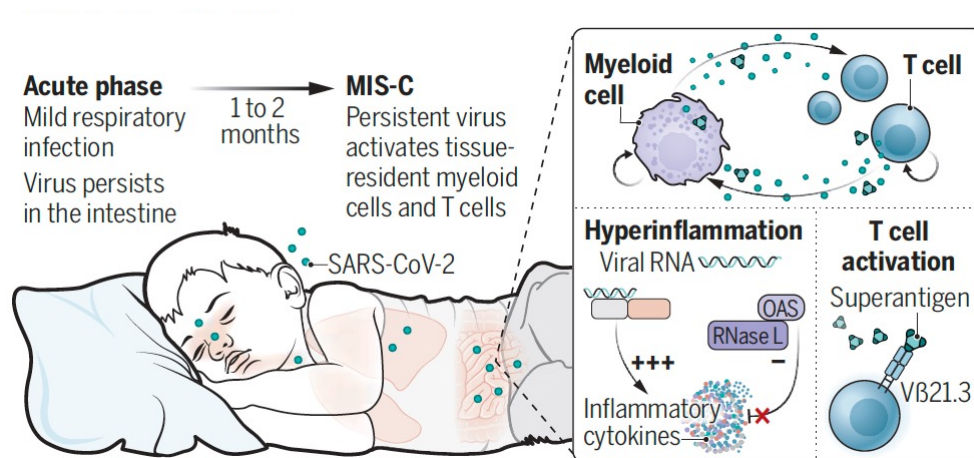


Figure 1.5: MIS-C pathogenesis involving GI viral persistence, hyperinflammation and T cell activation in children with MIS-C. Following SARS-CoV-2 infection the virus persists in the GI tract causing tissue-resident myeloid and T cells hyperactivation. The cytokines storm contributed by mutations in OAS-RNase viral RNA pathways and superantigen-like responses though SARS-CoV-2 spike-superantigen-like protein binding on T cells with TCR-V β 21.3 resulting in non-specific T cell activation seen in MIS-C cases. Reproduced from Brodin, 2023.

1.5 Cross-reactivity to other coronaviruses

Numerous studies have reported SARS-CoV-2 uninfected or unexposed individuals have detectable SARS-CoV-2-specific T cells, which could be attributed to memory T cells generated to some extent by circulating endemic HCoVs in children and adults (Braun et al., 2020; Grifoni et al., 2020; Humbert et al., 2023; Kundu et al., 2022; Le Bert et al., 2020; Meckiff et al., 2020; Sagar et al., 2021; Saletti et al., 2020; Sette et al., 2023; Woldemeskel et al., 2020; Yu et al., 2022). This theory has been proposed as a mechanism for age-related differences in COVID-19 clinical outcomes. Pre-existing T cell immunity against SARS-CoV-2 from endemic HCoVs in children is discussed below.

1.5.1 Pre-existing T cell immunity from endemic coronaviruses

Early in the pandemic, prior infection with endemic HCoVs was been hypothesized to provide cross-protection against SARS-CoV-2 (Aldridge et al., 2020; Aydillo et al., 2021; Sette & Crotty, 2020; Tso et al., 2021), with numerous groups reporting the presence of SARS-CoV-2 responding T cells in unexposed individuals (Braun et al., 2020; Grifoni et al., 2020; Le Bert et al., 2020).

Subsequent studies have shown T cells are capable of recognising homologous epitopes shared between endemic HCoVs, therefore memory T cells due to prior or recent infection with endemic HCoVs contribute to humoral cross-reactivity (Da Silva Antunes et al., 2021; de Vries, 2020; Humbert et al., 2023; Kundu et al., 2022; Mateus et al., 2020; Nelde et al., 2021; H. X. Tan et al., 2021). Mateus et al. showed that SARS-CoV-2 unexposed adults exhibited pre-existing memory T cells, cross-reactive to SARS-CoV-2, as a result of recognition of peptides with a high degree of homology between SARS-CoV-2 and seasonal endemic HCoVs (Mateus et al., 2020). Dissimilar levels of pre-existing immunity from prior exposure to endemic HCoVs may therefore contribute to differing humoral and cellular immune profiles against SARS-CoV-2 in children compared to adults (Dowell et al., 2022a; Humbert et al., 2023; Lavinder & Ippolito, 2022). Dowell et al. demonstrated that 60% of SARS-CoV-2 seronegative children with cellular immune responses have antibody responses against 1 or 2 endemic HCoVs, providing further evidence that the SARS-CoV-2 responding T cells in unexposed individuals is due to pre-existing HCoV-specific T cell responses that cross-react with SARS-CoV-2 (Dowell et al., 2022a). SARS-CoV-2 protective

immunity was shown to be elicited by the presence of cross-reactivity to endemic HCoV in children during the Omicron (BA.1) infection wave, as seronegative T cell non-responders showed a higher test positivity compared to seronegative T cell responders (Dowell et al., 2022b). This further supported the hypothesis that there is relative protection from SARS-CoV-2 associated with pre-existing immunity against endemic HCoVs in children.

1.6 Aims and objectives of the thesis.

The overall aim of this study was to investigate SARS-CoV-2-specific antibody and T cell responses in a diverse cohort of children compared to adults, by characterizing SARS-CoV-2-specific CD4+ and CD8+ T cell responses and phenotypic profiles and lastly to investigate durability and cross-reactive immunity to SARS-CoV-2 VOC in children.

Aim 1: To investigate the magnitude and polyfunctional profile of SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive and seronegative unvaccinated children compared to adults and the association with pre-existing immunity to endemic human coronaviruses.

Rationale: Early in the COVID-19 pandemic, it was shown that children predominantly exhibited less severe COVID-19 compared to adults. Proposed theories for lower COVID-19 severity in children include early viral clearance by robust innate immunity, less robust adaptive immune responses resulting in less inflammatory damage or pre-existing immunity due to endemic HCoVs. Numerous studies have investigated and demonstrated that adults exhibit robust and high magnitude of SARS-CoV-2-specific cellular immune response despite disease status (Moss, 2022; Sette & Crotty, 2021, 2022). The literature on differential SARS-CoV-2 immune profiles between convalescent children and adults has yielded some contradictory findings. To gain more insight into cross-reactive immunity in children, we compared the magnitude of SARS-CoV-2-specific T cell responses between SARS-CoV-2 seropositive and seronegative children and determined the polyfunctional SARS-CoV-2-specific T cell profile in the context of pre-existing immunity to endemic HCoVs. We also compared the magnitude of SARS-CoV-2-specific T cell responses between seropositive children and healthcare workers (HCW), to assess the relationship between

SARS-CoV-2-specific T cells and age to understand the role of virus-specific T cell immunity in children compared to adults. We hypothesise that SARS-CoV-2 seropositive children will have a higher magnitude of SARS-CoV-2-specific T cell responses compared to SARS-CoV-2 seronegative children and adults will have a more robust SARS-CoV-2-specific T cell responses compared to children.

This aspect of the study is presented in Chapter 3 and has been published as:

Ntombi Benede, Marius B. Tincho, Avril Walters, Vennesa Subbiah, et al ..., Wendy A. Burgers & Roanne S. Keeton. **Distinct T cell polyfunctional profile in SARS-CoV-2 seronegative children associated with endemic human coronavirus cross-reactivity.** *iScience* 27, 108728, January 2024. DOI: [10.1016/j.isci.2023.108728](https://doi.org/10.1016/j.isci.2023.108728).

Ntombi Benede contributions: *Received and processed samples using whole blood assays and stored the samples. Performed the antibody panel building and optimization for flow cytometry, generated the data through sample staining followed by acquisition on the flow cytometer and performed the SARS-CoV-2 serology assays. Analyzed the serology and flow cytometry data, contributed to the data interpretation, wrote and drafted the manuscript.*

Aim 2: **To investigate the durability of SARS-CoV-2-specific T cell responses after 16 months and determine T cell cross-reactivity to SARS-CoV-2 Delta and Omicron BA.1 variants in children.**

Rationale: SARS-CoV-specific T cells have been detected after 17 years since SARS-CoV outbreak and several studies have since shown that SARS-CoV-2-specific T cells are durable 15 months post-symptoms onset in adults (Bertoletti et al., 2022; Le Bert et al., 2020; Sette et al., 2023; Sette & Crotty, 2022). It has also been widely reported that T cell responses are preserved across SARS-CoV-2 VOCs in adult cohorts whether vaccinated and/or previously infected with SARS-CoV-2 (Kedzierska & Thomas, 2022; Keeton et al., 2021, 2023; Moss, 2022; Nesamari et al., 2024; Petrone et al., 2023; Sette et al., 2023). Although the data are discordant, a number of studies have shown reduced SARS-CoV-2-specific T cell responses in children compared to adults, raising the question of whether T cell responses in children would be as durable or cross-reactive as observed in adults. Given the discordant data on SARS-CoV-2-specific age-related differences in

adaptive immunity and the important role of memory T cells on durability, we assessed the memory profiles of SARS-CoV-2-specific T cells in children in comparison to adults with a similar time of SARS-CoV-2 exposure and subsequently investigated the maintenance of SARS-CoV-2-specific T cell responses over a 16-month period and cross-reactivity to SARS-CoV-2 VOC in children using a large SA based birth-cohort, Drakenstein Child Health Study (DCHS) study (Zar et al., 2015). We investigated SARS-CoV-2 T cell responses against Wuhan strain (Ancestral/ANC), Delta and Omicron (BA.1) S peptide pools in children that were infected with ancestral or Beta (n=31) and those possibly infected with Delta or Omicron (n=19) to assess whether the T cell responses in children cross-recognize Delta and BA.1 variants as reported in adult cohorts. We hypothesise that children will retain SARS-CoV-2-specific T cell responses 16 months after primary exposure to SARS-CoV-2 and SARS-CoV-2 Ancestral-specific T cells will cross-recognize SARS-CoV-2 Delta and Omicron variants in children.

This aspect of the study is presented in Chapter 4.

Aim 3: To investigate the immune pathology of MIS-C by determining SARS-CoV-2-specific adaptive immune responses in children with MIS-C compared to those with febrile inflammatory diseases and SARS-CoV-2 seropositive healthy children.

Rationale: MIS-C is a severe post-COVID-19 sequelae that develops in minority of previously healthy children (Feldstein et al., 2020). Adaptive T cell immunity is crucial for viral control, but can also cause immunopathology. The role of SARS-CoV-2-specific T cells in MIS-C outcome remains to be understood. Expansion CD4+ and CD8+ T cells expressing TCR V β 21.3 have been reported in patients with MIS-C and proposed as a hallmark for MIS-C (Moreews et al., 2021). The immunological differences in SARS-CoV-2-specific T cell responses in children with MIS-C compared to children with non-MIS-C inflammatory conditions and healthy children have not been thoroughly investigated. In this study, to determine whether antigen-specific T cells contribute to the pathogenesis of MIS-C and to investigate if polyclonal V β 21.3+ T cells are associated with MIS-C, we compared the functional profiles of SARS-CoV-2-responsive T cell responses and expression of TCR V β 21.3 on SARS-CoV-2-responding T cells in MIS-C cases, non-MIS-C and SARS-CoV-2 seropositive controls. We hypothesise that children with MIS-C will mount

robust SARS-CoV-2-specific T cell responses compared to children with non-MISC inflammatory conditions and healthy children.

This aspect of the study is presented in Chapter 5 and has been published as:

Claire Butters*, **Ntombi Benede***, et al ... Roanne S. Keeton* & Kate Webb*. **Comparing The Immune Abnormalities In MIS-C To Healthy Children And Those With Inflammatory Disease Reveals Distinct Inflammatory Cytokine Production And A Monofunctional T Cell Response.** *Clinical Immunology* 259, 109877, February 2024. DOI: [10.1016/j.clim.2023.109877](https://doi.org/10.1016/j.clim.2023.109877). *These authors contributed equally.

Ntombi Benede contributions: *Received and processed samples using whole blood assays and stored the samples. Performed the antibody panel building and optimization for flow cytometry, generated the data through sample staining followed by acquisition on the flow cytometer and analyzed flow cytometry data. Contributed to the flow cytometry data interpretation, wrote and drafted the manuscript.*

CHAPTER 2: MATERIALS AND METHODS

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2.1 Study participants

2.1.1 Recruitment and enrollment

Participants included in this study were recruited from three clinical study sites during the COVID-19 pandemic from 7 January 2021 – 7 December 2022 in the Western Cape Province, South Africa (SA). In total the study included 166 children aged less than 18 years and 103 adults over the age of 18 years.

The first cohort consisted of children recruited from Red Cross War Memorial Children's Hospital (RC), Cape Town from 7 January 2021 – 6 September 2021, this included 50 healthy children hospitalized for elective surgeries unrelated to infectious diseases and were classified as healthy controls, 8 children diagnosed with (MIS-C) based on the WHO criteria, and 6 children with febrile inflammatory disease (**Table 2.1**). Children with febrile inflammatory diseases were diagnosed with abnormal sepsis (n=1), dysentery (n=1), systemic juvenile idiopathic arthritis (SJIA) with macrophage activation syndrome (MAS) (n=1), gastroenteritis (n=2) and salmonella (n=1). These children were classified as inflammatory control (IC) children. The study was approved by the University of Cape Town (UCT), Faculty of Health Sciences Human Research Ethics Committee (HREC 599/2020, HREC 640/2023). Parental or legal guardians provided written informed consent for the children participants.

Table 2.1: Demographics of participants included from the first study cohort.

	Healthy controls	MIS-C	Inflammatory controls
N	50	8	6
Age, median (range)	5.4 (0.3 – 15)	6.1 (1.4 – 13.9)	8.6 (0.3 – 12)
Gender (F: M ratio)	12:38	5:3	4:2
SARS-CoV-2 seropositivity (n, %)	27 (54%)	8 (100%)	6 (100%)
SARS-CoV-2 PCR positivity (n,%)	ND	ND	ND
SARS-CoV-2 vaccination (n,%)	0 (0%)	0 (0%)	0 (0%)
Days Post-treatment, (range)	N/A	5 (1– 54)	N/A
Collection date range	1 Feb -20 May 2021	7 Jan - 6 Sep 2021	7 Jan - 5 Jul 2021

The second cohort consisted of adult participants that were COVID-19 convalescent unvaccinated healthcare workers (HCW) based at the Groote Schuur hospital (GSH) in Cape Town, recruited between July 2020 and January 2021, sample collection occurred from 22 January 2021 - 23 February 2021. HCW (n=30) included in the study were enrolled for this sub-study of parental protocol HREC 291/2020. The full cohort description and demographic information of this cohort is detailed in **Chapter 3, Figure 4.A**.

The last cohort of this study consisted of 102 children and 73 matched-maternal adults that were enrolled and recruited from the Drakenstein Child Health Study (DCHS) (Paarl, Western Cape, SA), a SA birth cohort study (Zar et al., 2015). Blood samples were collected from 15 March to 19 May in 2021. A second longitudinal sample was collected in a subset of 98 children between 13 June and 7 December in 2022. The DCHS birth cohort was established to investigate the epidemiology, risk factors, aetiology and long-term impact on childhood lower respiratory diseases in low and middle-income countries. The birth cohort study follows 1000 mother-child pairs over multiple years. The mothers were enrolled during 20-28 weeks of pregnancy and mothers and children were followed up annually for longitudinal measures. The study participant demographics are listed in detail in **Chapter 4, Table 4.1**. The study was approved by UCT Human Research Ethics Committee (HREC 401/2009, HREC 640/2023). Parental or legal guardians provided written informed consent for all the children participants which was renewed annually along with the ethical approval.

Each cohort description used in the overall study is provided in **Chapter 3, 4 and 5**. All participants included in this study were asymptomatic or had mild COVID-19 illness during the recruitment and did not require hospitalization.

2.2 Assays

2.2.1 Antigens and peptides

Antigens used in this study for serological analysis by Enzyme-Linked Immunosorbent Assay (ELISA) include commercially available recombinant SARS-CoV-2 spike (S)-S1 (Cape Bio Pharms, SA) and nucleocapsid (N) proteins (BioTech Africa, SA). Proteins were reconstituted in phosphate-buffered saline (1xPBS, Sigma-Aldrich, USA) to a stock concentration of 500 µg/mL and frozen at -80°C until use. Endemic human coronaviruses (HCoVs) HKU1 and OC43 S proteins were expressed in Human Embryonic Kidney (HEK) 293F suspension cells (Dr Nicole Doria-Rose, VRC, USA) by transfecting the cells with the S plasmids (Dr Gaurav Kwatra, Prof Shabir A. Madhi, Wits VIDA, SA and Prof Barney Graham, VRC, USA) and incubated for 6 days at 37°C, 10% CO₂ and 70% humidity. Following incubation, proteins were purified using a nickel resin followed by size-exclusion chromatography and relevant fractions were collected and frozen at -80°C until use.

SARS-CoV-2 S peptides used for the stimulation of whole bloods were 15-mer sequences with 11 amino acid overlap, covering then immunodominant parts of the SARS-CoV-2 S proteins (PepTivator, Miltenyi Biotech, USA), and SARS-CoV-2 N and membrane (M) peptides consisted of 17-mers with 10 amino acid overlap (BEI resources, USA). SARS-CoV-2 S, N and M peptides were based on Wuhan-1 strain sequence proteins. The commercial peptides from PepTivator, Miltenyi Biotech were resuspended in distilled water to a concentration of 50 µg/mL, while N and M peptides from BEI resources were resuspended in 1xPBS with dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a final concentration of 100 µg/mL. Combined peptide pools covering S, N and M were used at 1 µg/mL peptide in a final concentration of 0.05% DMSO for the stimulation of whole bloods.

Additional peptides that were used for the stimulation of whole bloods from children during the second-year visit, included SARS-CoV-2 variants of concern (VOC) S peptide pools consisting of 15-mers overlapping by 10 amino acids. The pools were covering the SARS-CoV-2 ancestral (Wuhan, GenBank:MN908947), Delta (B.1.617.2; GISAID: EPI_ISL_2020950), and Omicron (B.1.1.529, carrying in the S sequence of 38 described mutations A67V, H69del, V70del, T95I,

G142D, V143del, Y144del, Y145del, S152W, N211del, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F) S proteins (TC Peptide Lab, San Diego, CA). Commercially available mitogens, either Phytohemagglutinin (PHA) or a combination of phorbol 12-myristate 13-acetate (PMA) and Ionomycin were used for whole blood stimulation, while performing antibody titration and had been previously titrated in the laboratory group. PHA was used at the final concentration of 10 µg/mL, PMA at 0.02 µg/mL and Ionomycin at 1 µg/mL. All the mitogens were manufactured from Sigma-Aldrich. All reconstituted peptide pools and mitogens were stored at -20°C.

2.2.2 Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a common serology assay used for the detection and quantification of pathogen specific antibodies (Gan & Patel, 2013). During the course of the COVID-19 pandemic in 2020, ELISA was commonly used for screening and identification of SARS-CoV-2 seroconverters and understanding the humoral immunity to SARS-CoV-2 (Amanat et al., 2020; Stadlbauer et al., 2020). In the current study, the measurement of SARS-CoV-2-S and N-specific antibodies was performed using indirect ELISA previously established in our group (Makatsa et al., 2021). This assay was used to characterize the serostatus of the cohorts.

For this study, the immunoglobulin G (IgG) responses against SARS-CoV-2-S-S1 domain and N and HCoV-HKU1 and OC43-S IgG were measured using indirect ELISA. For the SARS-CoV-2 specific ELISAs, the viral proteins were reconstituted in 1xPBS and stored frozen in the -80°C Ultra freezer. High binding plates (MaxiSorp Nunc-Immuno 96-well plate, Sigma-Aldrich) were coated with SARS-CoV-2 proteins (2 µg/mL) and incubated overnight at 4°C. Plates were then treated with a blocking buffer containing 1% casein (Sigma-Aldrich) in PBS 0.1% Tween 20 (PBS-T, Sigma-Aldrich) for 1 hour. Thereafter, plasma samples (1:50 dilution in 0.5% casein) were added and incubated at room temperature (RT) for 2 hours. Plates were washed and incubated with secondary antibody anti-human IgG (Fc-specific) horseradish peroxidase (Sigma-Aldrich) diluted 1:5000 in dilution buffer for 1 hour followed by O-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich) for 12 minutes. Hydrochloric acid (50 µL, 3M HCl, Sigma Aldrich) was then added

to stop the reaction, the plates were read at 490nm using Versamax microplate reader using SoftMax Pro software (version 5.3). Pre-pandemic samples (n=40) were used to set a cut-off for positivity at two standard deviations (SD) above the mean optical density (OD).

For the HCoV ELISAs, 2 µg/mL of HKU1 and OC43 S proteins were coated in 96-well high binding plates (Corning® 96-well EIA/RIA assay ELISA high-binding 96-well plates, Sigma-Aldrich) and incubated at 4°C overnight. Following incubation, plates were blocked with blocking buffer containing 1xPBS, 5% skimmed milk powder and 0.05% Tween 20 (all manufactured from Sigma-Aldrich) for 1 hour. Plasma samples were diluted to 1:100 dilution in blocking buffer and added to the plates for 2 hours at RT. The secondary antibody, Anti-human IgG (Fc-specific) peroxidase secondary antibody, (Sigma-Aldrich), was diluted to 1:3000 dilution in blocking buffer and added to the plates for 1 hour, followed by 3,3',5,5'-tetramethylbenzidine (TMB) substrate (ThermoFisher Scientific, USA) for 5 minutes. The reaction was then halted with 50 µL of sulfuric acid (1M H₂SO₄, Sigma-Aldrich) and the absorbance measured at 450nm using the SpectraMax® ABS Plus Microplate Reader, SoftMax Pro software (version 5.3).

2.2.3 Whole blood assay (WBA)

Antigen-specific T cells can be quantified using various methods including Enzyme-Linked Immunosorbent Spot (ELISPOT) assay, activation-induced marker (AIM) assay, intracellular cytokine staining (ICS) and proliferation assays. The assays mentioned, have different functional readouts including cytokine secretion, cytotoxicity and expression of activation markers (Inthawong et al., 2023; Maecker et al., 2000). Whole blood assay (WBA) was developed to evaluate antigen-specific responses for clinical applications, in which the presence of autologous cellular components may be physiologically relevant for specific responses in comparison to peripheral blood mononuclear cell (PBMC) (Hanekom et al., 2004; Kagina et al., 2015; Suni et al., 1998). PBMC based assays require large amount of blood and good cell viability which can be impacted by freezing and thawing (Appay et al., 2006; Braudeau et al., 2021). WBAs used in combination with ICS have been used for the measurement and detection of antigen-specific T cell responses to infectious diseases and vaccines (Braudeau et al., 2021; Hanekom et al., 2004;

Inthawong et al., 2023; Meddows-Taylor et al., 2007). Due to the limited blood which can be safely drawn from children we elected to use WBAs in the current study. The WBA used in this study was adapted from Mutavhatsindi & Riou and Riou et al. (Mutavhatsindi & Riou, 2022; Riou et al., 2022).

Blood was collected in sodium heparin tubes and WBA were set up within 4-8 hours of collection. Heparinized whole blood (500 µl) was aliquoted into O-ring tubes and stimulated with combined peptide pools covering the ancestral strain SARS-CoV-2 S (Miltenyi Biotech), N and M proteins (BEI resources) for 24 hours at 37°C. For cross-reactive T cell analysis, blood was stimulated with peptide pools consisting of 15-mers overlapping by 10 amino acids, covering the SARS-CoV-2 ancestral strain (Wuhan, GenBank:MN908947), Delta (B.1.617.2), and Omicron (B.1.1.529) S proteins (TC Peptide Lab, San Diego, CA, USA). All the peptides were used at a final concentration of 1 µg/mL. Stimulation was done in the presence of co-stimulatory antibodies against anti-CD28 (clone 28.2, BD Biosciences, USA) and anti-CD49d (clone L25, BD Biosciences) at final concentration of 1 µg/mL and Brefeldin-A (BFA, Sigma Aldrich) at final concentration of 10 µg/mL. The unstimulated blood served as a positive control and was treated with co-stimulatory antibodies, BFA, and an equimolar amount of DMSO (Sigma-Aldrich). Following 24-hour stimulation, the blood was treated with 2mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) for 15 minutes followed by red blood cell lysis and cell fixation by treating the blood suspension with BD FACS lysing solution (BD Biosciences) for 10 minutes. Samples were then centrifuged at 1500rpm for 10 minutes and the cell pellet was resuspended in freezing media (90% foetal bovine serum [FBS] and 10% DMSO; all manufactured from Sigma-Aldrich) for cryopreservation and stored in the -80°C Ultra freezer until batched analysis.

2.3 Flow cytometry

Flow cytometry is an instrumental tool, commonly used for immunological studies (Adan et al., 2017). It allows for the measurement of multiple features of immune cells using fluorochromes or fluorophores conjugated to antibodies that bind to and detect cellular molecules or cellular

components. For immunoassays these markers are used for identifying and quantifying cell populations, cell surface receptors, intracellular targets, cell sorting, nucleic acid content, measuring enzymic activity and apoptotic markers (Adan et al., 2017; Macey, 2007; Mair & Tyznik, 2019).

2.3.1 Cell staining and acquisition.

ICS followed by flow cytometry is used to measure qualitative and quantitative profiles of antigen-specific cellular responses and can be performed using PBMC or whole blood samples (Horton et al., 2007; Maecker et al., 2000).

2.3.1.1 Surface and intracellular cytokine staining

Cryopreserved cells were thawed at 37°C and washed with FACS buffer (1% FBS in 1xPBS) followed by centrifuging at 1500rpm for 10 minutes and transferred to a 96-well V-bottom. The plate was centrifuged at 2100rpm for 5 minutes and the supernatant was discarded. Thereafter cell pellets were resuspended in 50 µL per well of cell surface antibody master mix and incubated in the dark for 20 minutes at RT. The surface markers are shown in **Table 2.2** and **Table 2.3**. Following incubation, cells were washed twice with FACS buffer and then permeabilized with 100 µL BD Perm buffer (BD Biosciences) for 30 minutes in the dark, at RT. The plate was centrifuged at 2100rpm for 5 minutes, the supernatant discarded and 50 µL of intracellular antibody master mix was added to each well and incubated for 30 minutes at RT, in the dark. Intracellular antibody markers are shown in Table 2.2 and Table 2.3. Finally, cells were washed and fixed in 150 µL BD CellFIX buffer (BD Biosciences) and transferred to polypropylene cluster tubes and placed in FACS tubes (BD Biosciences). **Table 2.2** shows the first panel (Panel 1) of antibody-conjugates used in **Chapter 3 and 5** and **Table 2.3** is for second panel (Panel 2) of the 8-color antibody-conjugates used in **Chapter 4**. Respective gating strategy of Panel 1 and 2 are shown in **Figure 2.2** and **Figure 2.3**, respectively.

Table 2.2: 7 colour-antibody Panel 1 used for cell staining in Chapter 3 and 5

Marker	Fluorochrome	Clone	Function	Manufacture	Staining
CD3	BV650	OKT3	T cell lineage	Biocom	IC
CD4	ECD	SFC12T4D11	T cell lineage	Beckman Coulter	Surface
CD8	BV510	RPA-T8	T cell lineage	Biocom	Surface
TCRV β 21.3	FITC	REA894	T cell receptor	Biocom	Surface
IFN- γ	AF700	B27	Cytokine	BD Biosciences	ICS
IL-2	APC	MQ1-17H12	Cytokine	Biocom	ICS
TNF- α	BV785	Mab11	Cytokine	Biocom	ICS

Table 2.3: 8 colour-antibody panel 2 used for cell staining and flow cytometry for Chapter 4

Marker	Fluorochrome	Clone	Function	Manufacture	Staining
CD3	BV786	OKT3	T cell lineage	Biocom	IC
CD4	PE-Cy7	L200	T cell lineage	BD Biosciences	Surface
CD8	BV510	RPA-T8	T cell lineage	Biocom	Surface
CD45RA	BV605	H100	Memory	Biocom	Surface
CD27	PE-Cy5	1A4CD27	Memory	Beckman Coulter	Surface
IFN- γ	AF700	B27	Cytokine	BD Biosciences	ICS
IL-2	PE-dazzle	MQ1-17H12	Cytokine	Biocom	ICS
TNF- α	FITC	Mab11	Cytokine	Biocom	ICS

2.3.1.2 Compensation controls

Compensation is performed during acquisition to correct the spectral overlap that may occur between fluorophores (Roederer, 2001). An algorithm is performed by the cytometer software using the positive and negative populations of compensation controls to reveal the level of spectral overlap between different fluorophores (Mahnke & Roederer, 2007). Compensation can also be done at the analysis stage using FlowJo. Compensation controls are tubes of beads which have been individually stained with each of the fluorophore conjugated antibodies as the stained samples and need to have a higher median fluorescent intensity than the stained cells to work optimally. Beads are advantageous as compensation controls because they have no biological materials which may interfere with calculations or corrected spectral overlap (Cossarizza et al., 2019).

Compensation controls of single-stained tubes of beads were acquired for each experiment before sample acquisition. Anti-mouse and anti-rat compensation beads (BD Biosciences) were used according to manufactures instructions. Briefly a series of BD FACS tubes were prepared by adding one drop each of positive and negative compensation beads to 100 μ L FACS wash buffer followed by adding the respective volume of each individual fluorochrome-labelled antibody to the bead mix to a tube. These were incubated for 10 minutes at RT in the dark. The stained beads were washed in 2 mL FACS washing buffer by centrifugation at 1800rpm for 8 minutes and thereafter resuspended in 200 μ L of BD CellFIX (BD Biosciences).

2.3.1.3 Sample acquisition and gating strategy

Prepared compensation beads and stained samples were acquired on BD LSRFortessa flow cytometer (**Figure 2.1**) using FACSDiva software. Following acquisition, flow cytometry data were analyzed using FlowJo v10 (FlowJo LLC, USA). **Figure 2.2** shows the gating strategy for panel 1 used in **Chapter 3 and 5**, and **Figure 2.3** shows the gating strategy for panel 2 used in **Chapter 4**. During flow analysis, each sample is checked for stability on each laser and to exclude any discrepancies in the florescent intensity during the acquisition (**Figure 2.2. A-C** & **Figure 2.3. A-B**). This is plotted as time gate against a fluorochrome for each of the lasers used. Next single cells are gating using the linear portion on the forward scatter- (FSC) height vs area plot (**Figure 2.2.D** & **Figure 2.3.C**). This was followed by gating FSC and side scatter (SSC) to distinguish lymphocytes based on size and granularity (**Figure 2.2.E** & **Figure 2.3.D**). T cells were further identified based on expression of CD3 (**Figure 2.2.F** & **Figure 2.3.E**), CD4 and CD8 (**Figure 2.2.G** & **Figure 2.3.F**). Thereafter, the cytokines IFN- γ , TNF- α and IL-2 were gated on CD4+ and CD8+ T cells for both panels as shown in **Figure 2.2.H-I** and **Figure 2.3.G-H**. A positive cytokine response was determined using the criteria of a frequency in the stimulated sample twice the frequency of the unstimulated sample and a minimum of 5 cytokine events. All graphs are plotted using the net cytokine responses. These were calculated as the frequency of the stimulated sample minus the frequency of the unstimulated sample per participant. **Figure 2.2 J-K** shows the gating of TCR V β 21.3-FITC against CD4 and CD8 markers for panel 1. This analysis is shown further in Chapter 5. Phenotypic memory profiles were analysed for panel 2 (**Figure 2.3 I-J**). These were classified

based on the expression of CD45RA and CD27. Four subsets were delineated as naïve (CD45RA+CD27+), early differentiated (ED:CD45RA-CD27+), late differentiated (LD:CD45RA-CD27-) and effector (Eff:CD45RA+CD27-) cells. A cut-off of 20 cytokine events was applied for memory phenotyping of the cytokine-producing cells.

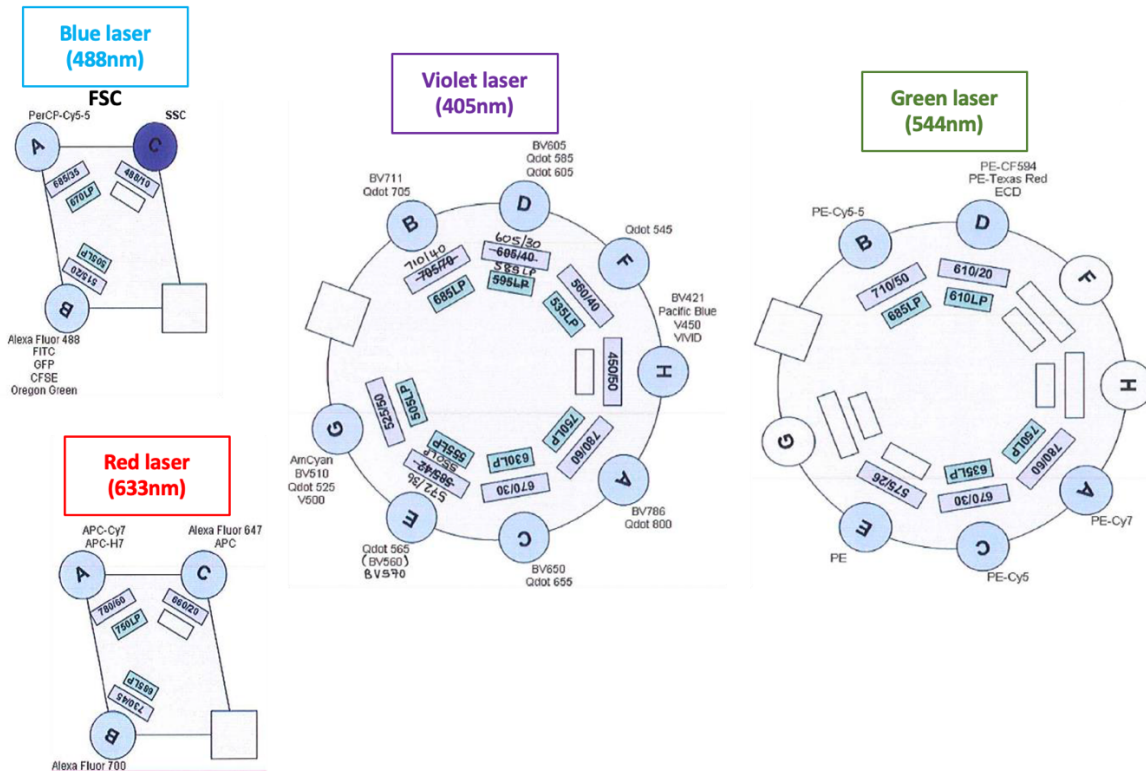


Figure 2.1: The filter configuration of the BDFortessa used in the study.

The filter arrangements are shown for each of the 4 lasers. These include two trigons (blue and red lasers) and two octagons (violet and green) and the respective detectors.

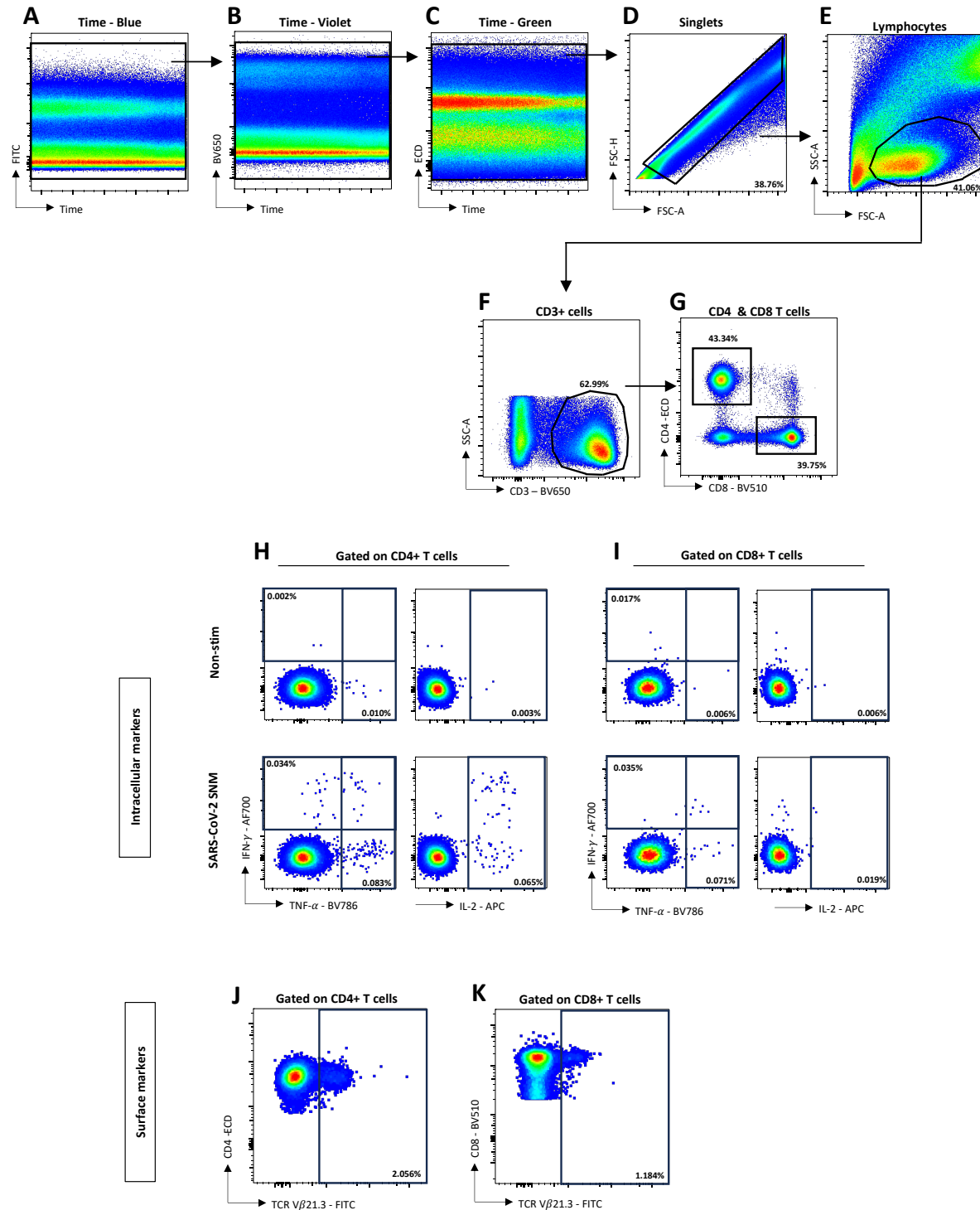


Figure 2.2: Gating strategy (Panel 1) used to identify cytokine expression in CD4+ and CD8+ T cells in response to SARS-CoV-2 SNM peptide pools and phenotype profile.

Representative flow plots showing **(A-C)** time gates, **(D)** singlets, **(E)** lymphocytes, **(F)** CD3+ cells, **(G)** CD4+, CD8+ cells, **(H-I)** intracellular (IFN- γ , IL-2, TNF- α) and **(J-K)** surface (TCRV β 21.3) markers. FSC; Forward scatter, SSC; side scatter, H; Height, A; area, SNM; SARS-CoV-2-spike, nucleocapsid and membrane proteins.

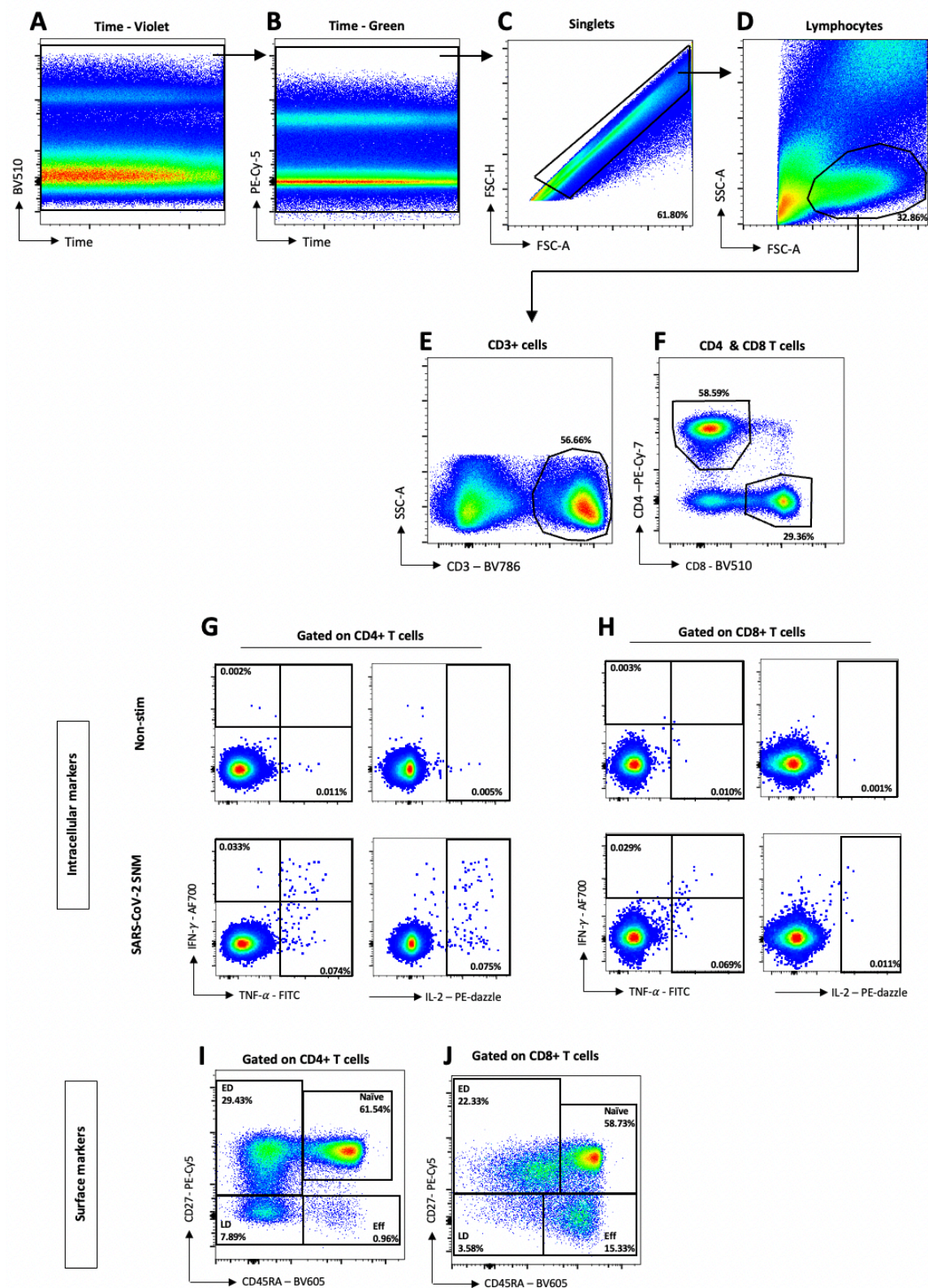


Figure 2.3: Gating strategy (Panel 2) used to identify cytokine expression in CD4+ and CD8+ T cells in response to SARS-CoV-2 SNM peptide pools and memory profiles.

Representative flow plots that show **(A-B)** time gates, **(C)** singlets, **(D)** lymphocytes, **(E)** CD3+ cells, **(F)** CD4+ CD8+ cells, **(G-H)** intracellular (IFN- γ , IL-2, TNF- α) and **(I-J)** surface (CD27 and CD45RA) markers. FSC; Forward scatter, SSC; side scatter, H; Height, A; area, SNM; SARS-CoV-2-spike, nucleocapsid and membrane proteins, ED; early differentiated, LD; late differentiated and Eff; effector.

2.4 Statistical analysis

GraphPad Prism (Version 9, GraphPad Software Inc, San Diego, CA, USA) was used for statistical tests. Non-parametric tests were used for all comparisons. The Mann-Whitney U test, Wilcoxon Signed-Rank test and Friedmann test were used to compare unmatched and paired samples, respectively. Multiple comparisons were performed using a one-way ANOVA and Dunn's multiple comparison test. Chi-square or Fisher's exact test were used for comparisons between proportion of responders and permutation test were used to compare the median cytokine contribution of each combination to the T cell response. A Spearman Rank correlation test was used to determine correlations between antibody responses, T cell responses and age. For all statistical analyses, *p values* of less than 0.05 were considered statistically significant. Analysis of cytokine co-expressing populations were performed using Pestle and SPICE version 6.1 (<https://niaid.github.io/spice>) (Roederer et al., 2011). Details of statistical analyses performed are described in the figure legends.

CHAPTER 3: DISTINCT T CELL POLYFUNCTIONAL PROFILE IN SARS-CoV-2 SERONEGATIVE CHILDREN ASSOCIATED WITH ENDEMIC HUMAN CORONAVIRUS CROSS-REACTIVITY.

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Contributions: Ntombi Benede performed the experimental work, analyzed the data and co-wrote the manuscript with her supervisors. All contributions are stipulated in Appendix.

3.1 Abstract

SARS-CoV-2 infection in children typically results in asymptomatic or mild disease. There is a paucity of studies on SARS-CoV-2 antiviral immunity in African children. We investigated SARS-CoV-2-specific T cell responses in 71 unvaccinated asymptomatic South African children who were seropositive or seronegative for SARS-CoV-2. SARS-CoV-2-specific CD4⁺ T cell responses were detectable in 83% of seropositive and 60% of seronegative children. Although the magnitude of the CD4⁺ T cell response did not differ significantly between the two groups, their functional profiles were distinct, with SARS-CoV-2 seropositive children exhibiting a higher proportion of polyfunctional T cells compared to their seronegative counterparts. The frequency of SARS-CoV-2-specific CD4⁺ T cells in seronegative children was associated with the endemic human coronavirus (HCoV) HKU1 IgG response. Overall, the presence of SARS-CoV-2-responding T cells in seronegative children may result from cross-reactivity to endemic coronaviruses and could contribute to the relative protection from disease observed in SARS-CoV-2-infected children.

3.2 Introduction

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection in children usually causes asymptomatic or mild illness, contrasting with the high rate of severe disease reported in older adults (Aleebrahim-Dehkordi et al., 2021; Chou et al., 2022). As a result, global reports of coronavirus disease 2019 (COVID-19) cases among children and adolescents are underreported. The United Nations International Children's Emergency Fund (UNICEF) estimates that 21% of all reported confirmed cases occur in individuals younger than 20 years (UNICEF, 2023). In the USA, it has been documented that COVID-19-associated hospitalization rates among children less than 18 years were lower compared to those in older individuals (Kim et al., 2020). Indeed, the COVID-19-Associated Hospitalization Surveillance Network (COVID-NET) reported that children younger than 18 years accounted for only 4.2% of COVID-19-associated hospitalization and 0.2% of COVID-19-associated in-hospital deaths (COVID-NET, 2023). In South Africa, during the first 2 years of the

pandemic, 12.5% of confirmed cases and 0.7% of COVID-19 associated in-hospital deaths were in individuals younger than 19 years (NICD, 2022).

In contrast to the low COVID-19 severity in the majority of younger individuals, it is known that children are more susceptible than adults to other acute viral respiratory tract infections including respiratory syncytial virus (RSV), rhinovirus (RV), influenza virus and common circulating endemic human coronaviruses (HCoV) (Nair et al., 2011). Several age-associated factors have been proposed to play a role in the reduction of severity to SARS-CoV-2 infection in children (Bogunovic & Merad, 2021; Chou et al., 2022), including limited comorbidities (Sandoval et al., 2021; Tsankov et al., 2021; N. Williams et al., 2021), differences in the expression of SARS-CoV-2 viral entry factors (Koch et al., 2022; Muus et al., 2021; Schuler et al., 2021), robust innate immune responses (Loske et al., 2022; Neeland et al., 2021a; Pierce et al., 2021; Speranza, 2021), humoral and cellular immunity (Cotugno et al., 2021; Méndez-Echevarría et al., 2021; Yoshida et al., 2021) and pre-existing immunity against common cold circulating endemic HCoVs (C. A. Cohen et al., 2021; Dowell et al., 2022a; Humbert et al., 2023; Nickbakhsh et al., 2020; Woudenberg et al., 2021). Endemic HCoVs account for 15 to 30% of respiratory infections reported annually in children (Fehr R A, 2015; Zimmermann & Curtis, 2020). These HCoVs belong to the alpha-coronavirus subfamily (HCoV-229E and HCoV-NL63) and the beta-coronavirus subfamily (HCoV-OC43 and HCoV-HKU1), are generally considered to have seasonal infection peaks during the winter season and are responsible for high rates of infection among children (Audi et al., 2020). In contrast, the influenza surveillance data from India showed that HCoV-HKU1 peaked during spring to summer over the 2015-2016 period (Friedman et al., 2018).

It is well established that protective immune responses to SARS-CoV-2 encompass both an antibody and T cell component (Sette & Crotty, 2021). Several studies have reported robust and durable antibody and T cell responses against SARS-CoV-2, which are maintained up to 6-12 months following infection (Dan et al., 2021; Garrido et al., 2021; L. Guo et al., 2022; Méndez-Echevarría et al., 2021). However, data on T cell responses in children compared to adults are conflicting, with studies reporting lower SARS-CoV-2-specific T cell responses in children (C. A.

Cohen et al., 2021; Kaaijk et al., 2022; Pierce et al., 2020), no differences (Tosif et al., 2020) or greater T cell responses in children (Dowell et al., 2022a). Many of these studies make use of ELISPOT assays which preclude analysis of T cell phenotypes or functional cytokine profiles.

In this study, we prospectively characterized specific T cell responses in SARS-CoV-2 seropositive and seronegative children during the COVID-19 pandemic and determined the functional profiles of SARS-CoV-2-specific T cells, in the context of their pre-existing immunity against endemic beta-HCoVs. This study provides insights into cross-reactive immunity to SARS-CoV-2 in children.

3.3 Materials and methods

3.3.1 Study participants.

Pediatric participants (n=71) were recruited from two cohorts in the Western Cape Province, South Africa. The first cohort enrolled 50 children from the Red Cross War Memorial Children's Hospital (Cape Town, Western Cape, South Africa). In this cohort 50/71 children were hospitalized for non-COVID-19-related elective procedures and were confirmed to be clinically well, with no chronic or intercurrent infectious or inflammatory disease. This study is approved by University of Cape Town Human Research Ethics Committee as a Paediatric Immunology Biorepository (UCT HREC 112/2012) and MIS-C substudy (UCT HREC 599/2020). A further 21 children were enrolled from the Drakenstein Child Health Study (DCHS) (Cape Winelands Western Cape, South Africa), a birth cohort study (Zar et al., 2015). The participants from this study were recruited between 1 February 2021 and 20 May 2021, after the first and the second infection waves with Ancestral strain and Beta variant in South Africa (Zar et al., 2022). The study was approved by the University of Cape Town Human Research Ethics Committee and renewed annually (HREC 401/2009). Parents or legal guardians provided written informed consent for all pediatrics participants.

COVID-19 convalescent unvaccinated adults from a longitudinal study of healthcare workers (HCW) enrolled from Groote Schuur Hospital (Cape Town, Western Cape, South Africa) were included in the study (n=30). HCW in this cohort were recruited between July 2020 and January

2021 and were selected for inclusion based on a prior PCR-confirmed SARS-CoV-2 infection at least 3 months earlier. All participants were asymptomatic or had mild symptoms and did not require hospitalization for COVID-19 and were symptom-free at the time of sampling. Written informed consent was obtained from all participants and the study was approved by the University of Cape Town Human Research Ethics Committee (HREC 190/2020 and 209/2020).

3.3.2 SARS-CoV-2 and HCoV antigens

For serology assays, recombinant SARS-CoV-2 spike (S) S1 (Cape Bio Pharms), and SARS-CoV-2 nucleocapsid (N) (BioTech Africa) proteins were used for this study. HKU1 and OC43 S proteins were expressed in Human Embryonic Kidney (HEK) 293F suspension cells by transfecting the cells with the S plasmid. After 6 days, proteins were purified using a nickel resin followed by size-exclusion chromatography. Relevant fractions were collected and frozen at -80°C until use. SARS-CoV-2 peptides used for T cell assays included a commercially available peptide pool (15-mers peptides with 11 amino acid overlap) covering the immunodominant regions of SARS-CoV-2 S protein (PepTivator SARS-CoV-2 Prot_S, Miltenyi Biotech) based on the Wuhan-1 strain. The S peptide pool was prepared by resuspending in distilled water and used at a final concentration of 1 µg/mL. SARS-CoV-2 (Wuhan-1) N and membrane (M) peptides (17-mers with 10 amino acid overlap spanning the full proteins) were obtained from BEI Resources and were prepared by resuspending in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and used at a concentration of 1 µg/mL.

3.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

SARS-CoV-2-specific Enzyme-Linked Immunosorbent Assay (ELISA) was performed to characterize the serostatus of participants. Two µg/mL of S or N protein was used to coat 96-well high-binding plates and incubated overnight at 4°C. The plates were incubated in a blocking buffer consisting of 1% casein, 0.05% Tween 20, 1xPBS for SARS-CoV-2 or 1xPBS, 5% skimmed milk powder, 0.05% Tween 20 for HCoVs for 1 hour. Plasma samples were diluted to 1:50 for SARS-CoV-2 or 1:100 for HCoVs in the respective blocking buffer and added to the plates for 2 hours. For the SARS-CoV-2

ELISA, secondary antibody was diluted to 1:5000 in dilution buffer and added to the plates for 1 hour followed by SigmaFast O-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich) for 12 minutes. For the HCoV ELISAs, secondary antibody was diluted to 1:3000 in blocking buffer and added to the plates followed by TMB substrate (ThermoFisher Scientific). Upon stopping the reaction with 1-3 M sulfuric acid, absorbance was measured at a wavelength of 490 nm for SARS-CoV-2 or 450 nm for HCoVs. A cut-off for positivity was set at two standard deviations (SD) above the mean optical density (OD) of prepandemic samples (n=40) for SARS-CoV-2 ELISAs

3.3.4 Whole blood-based T cell assay

Blood was collected in sodium heparin tubes and processed within 4-6 hours of collection. The whole blood assay sample processing used for this study was adapted from a whole blood intracellular cytokine detection assay designed to detect SARS-CoV-2 specific T cells in adults (Mutavhatsindi & Riou, 2022; Riou et al., 2022). For this study, 500 μ L of blood was stimulated for 24 hours at 37°C with a combined pool of SARS-CoV-2 peptides including S, N and M, all at 1 μ g/mL in the presence of costimulatory antibodies against CD28 (clone 28.2) and CD49d (clone L25) (1 μ g/mL each; BD Biosciences) and Brefeldin A (10 μ g/mL, Sigma-Aldrich). Unstimulated blood was incubated with costimulatory antibodies, Brefeldin A and an equimolar amount of DMSO as a background control. After 24 hours, blood was treated with EDTA (2 mM) for 15 minutes followed by red blood cell lysis and white cell fixation using FACS lysing solution (BD Biosciences) for 10 minutes. Cells were then cryopreserved in freezing media (90% fetal bovine serum (FBS) and 10% DMSO) and stored at -80°C until batched analysis.

3.3.5 Cell staining and flow cytometry

Cell staining was performed on cryopreserved fixed cells that were thawed and washed with 1% FACS washing buffer (1% FBS in PBS). A viability dye was not included during the staining process, as it has been reported that the omission of a viability dye during the staining process does not impact the detection and quantification of cytokines when using the whole blood-based T cell

assay (Mutavhatsindi & Riou, 2022). Cells were stained with the following surface antibody markers: CD4 ECD (SFC112T4D11, Beckman Coulter), CD8 BV510 (RPA-8, Biolegend) and incubated at room temperature for 20 minutes. Cells were permeabilized and stained with intracellular antibody markers CD3 BV650 (OKT3), interferon- γ (IFN- γ) AlexaFluor® 700 (B27), tumor necrosis factor- α (TNF- α) BV786 (Mab11) and interleukin-2 (IL-2) APC (MQ1-17H12) (all from Biolegend). Finally, cells were washed and fixed with Cellfix (BD Biosciences). Samples were acquired on a multiparameter BD Fortessa flow cytometer using Diva software version 9 and analyzed using FlowJo v10. Results are expressed as the frequency of CD4+ or CD8+ T cells expressing IFN- γ , TNF- α or IL-2. Cytokine responses presented are background subtracted values (from the frequency of cytokine produced by unstimulated cells). The gating strategy used for this study is depicted in **Chapter 2** under **section 2.3.1.3, Figure 2.2.A-I**.

3.3.6 Quantification and statistical analysis

Statistical analyses were performed in Prism (v9.4.1; GraphPad Software Inc, San Diego, CA, USA). Non-parametric tests were used for all comparisons. The Mann-Whitney and Wilcoxon signed rank tests were used for unmatched and paired samples, respectively. Chi-square tests were used for comparisons between the proportion of responders represented as pie charts. All correlations reported are non-parametric Spearman's correlations. Analysis of cytokine co-expressing populations was performed using SPICE version 5.1. *P* values less than 0.05 were considered statistically significant. Details of analyses performed for each experiment are described in the figure legends.

3.4 Results

3.4.1 Study cohort.

To investigate SARS-CoV-2-specific immune responses, we measured immunoglobulin (Ig) G and T cell responses in 71 healthy children recruited in Cape Town, Western Cape, South Africa. The participants are described in (**Figure 3.1.A**). The median age of the children was 7 years (interquartile range (IQR) 2.8-9 years) and 34% (24/71) were female. The children included in this

study had not received any SARS-CoV-2 vaccine prior to recruitment and no PCR-confirmed infection data were available, although 58% showed SARS-CoV-2 seropositivity (defined as being positive for either anti-S or anti-N IgG). Samples were collected between 1 February 2021 – 20 May 2021, after two infection waves in South Africa, that were dominated by the ancestral D614G strain followed by the Beta variant of concern (Tegally et al., 2021).

3.4.2 SARS-CoV-2-specific antibody responses in children

To characterize the children serologically, we measured IgG responses against SARS-CoV-2 S and N proteins using an indirect Enzyme-Linked Immunosorbent Assay (ELISA) (**Figure 3.1.B**). A large proportion (58%, 41/71) of unvaccinated children had detectable SARS-CoV-2-specific IgG against S and/or N proteins and were classified as seropositive. Of the seropositive children, 51% (21/41) had S- and N-specific IgG, 39% (16/41) had only S-specific IgG and 10% (4/41) had only N-specific IgG (**Figure 3.1.B**). The remaining 42% (30/71) had undetectable SARS-CoV-2-S and N-specific IgG and were classified as seronegative (**Figure 3.1.B**). IgG responses to S and N are shown for the 41 seropositive children in **Figure 3.1.C**. Moreover, we compared SARS-CoV-2-S- and N-specific IgG levels among SARS-CoV-2 seropositive (n=41) children stratified by age. Children under the age of 5 years (n=13) had significantly higher SARS-CoV-2-S-specific IgG responses ($p=0.0007$) compared to children older than 5 years old (n=28) (**Figure 3.2.A**). However, no significant differences ($p=0.972$) were observed for SARS-CoV-2-N-specific IgG responses (**Figure 3.2.B**).

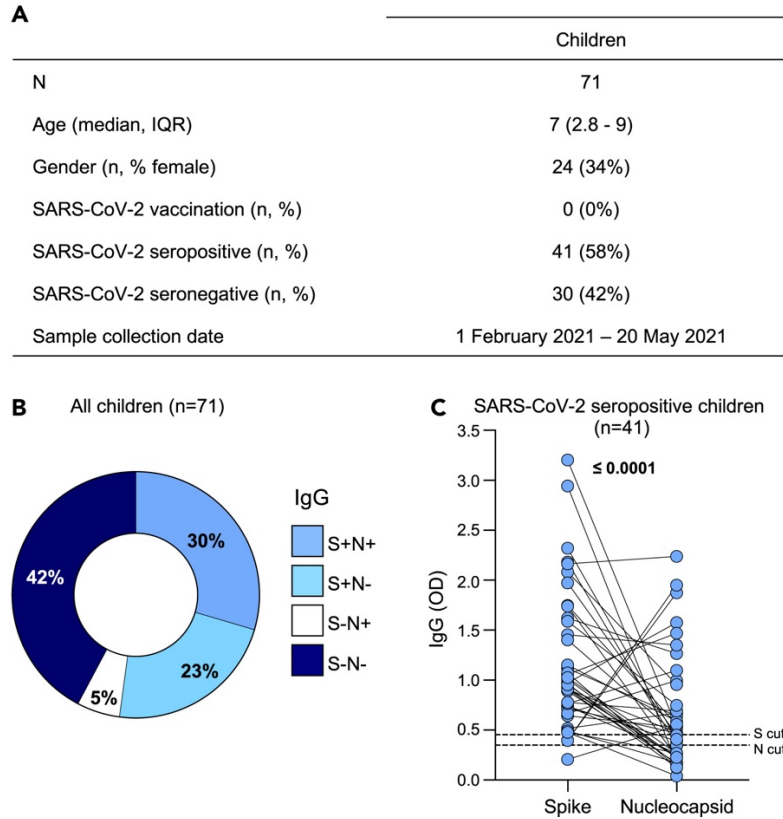


Figure 3.1: SARS-CoV-2-specific antibody responses in children.

(A) The demographic characteristics of 71 unvaccinated children included in this study. Age, gender, SARS-CoV-2 vaccination status, SARS-CoV-2 serology and collection date are shown. **(B)** Proportion of children exhibiting antibody responses to SARS-CoV-2 spike (S) and nucleocapsid (N) proteins. **(C)** The magnitude of SARS-CoV-2 S and N IgG antibodies (OD_{490nm}) measured by ELISA in seropositive children (n=41). The dotted lines indicate the cut-off for positivity which was calculated as the mean optical density of COVID-19 prepandemic control samples. Statistical analysis was performed using the Wilcoxon signed rank test; *p* values <0.05 were considered statistically significant and are bolded.

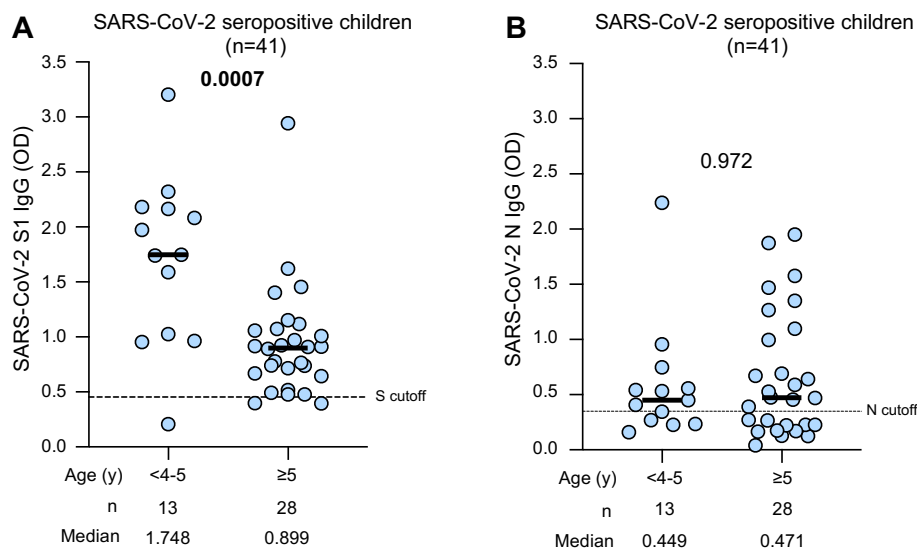


Figure 3.2: SARS-CoV-2-specific antibody responses in children stratified by age.

(A) The magnitude of SARS-CoV-2 spike-specific IgG measured by ELISA in SARS-CoV-2 seropositive children younger than 5 years (n=13) and 5 years or older (n=28). **(B)** The magnitude of SARS-CoV-2-nucleocapsid-specific IgG in SARS-CoV-2 seropositive children younger than 5 years (n=13) and 5 years or older (n=28). Statistical comparisons in **(A)** and **(B)** were performed using the Mann-Whitney test between groups; *p* values <0.05 were considered statistically significant and are bolded.

3.4.3 SARS-CoV-2-specific T cell responses in children

The magnitude of SARS-CoV-2-specific T cell responses in children was quantified using a whole blood assay and intracellular cytokine staining followed by flow cytometry. SARS-CoV-2-specific CD4+ and CD8+ T cell responses were measured as total cytokine production of IFN- γ , TNF- α or IL-2 in response to a combined peptide pool covering SARS-CoV-2 S, N and M proteins (**Figure 3.3.A**).

We assessed the proportion of responders and magnitude of SARS-CoV-2-specific T cell responses according to the serostatus of the children (**Figure 3.3.B-C**). As expected, 83% (34/41) of seropositive children had detectable SARS-CoV-2-specific CD4+ T cells. Interestingly, 60% (18/30) of seronegative children also had detectable SARS-CoV-2-specific CD4+ T cells, although this was

a significantly lower proportion than observed in the seropositive group ($p=0.031$). The magnitude of the SARS-CoV-2-specific CD4⁺ T cell response in seronegative responders was comparable to that observed in seropositive children (median of responders: 0.04% and 0.057% respectively; $p=0.437$, **Figure 3.3.B**). Conversely, we did not observe significant differences in the proportion of SARS-CoV-2 CD8⁺ T cell responders (~50%) or the frequency of SARS-CoV-2-specific CD8⁺ T cells between seropositive and seronegative children (median of responders: 0.019% and 0.025% respectively; $p=0.214$, **Figure 3.3.C**).

Next, we evaluated the polyfunctional profile of SARS-CoV-2-responding CD4⁺ and CD8⁺ T cells based on the co-production of IFN- γ , IL-2 or TNF- α (**Figure 3.3.D-E**). The overall functional profile of SARS-CoV-2-specific CD4⁺ T cells in seropositive children was distinct from that of seronegative children ($p=0.007$, **Figure 3.3.D**). SARS-CoV-2-specific CD4⁺ T cells in seropositive children were more polyfunctional, exhibiting a higher proportion of triple functional IFN- γ +IL-2+TNF- α +/- producing CD4⁺ T cells ($p=0.0044$), and dual expressing cells (IL-2+TNF- α +, $p=0.0048$; IFN- γ +IL-2+, $p\leq 0.0001$) compared to the seronegative children. In contrast, seronegative children were characterized by an increased proportion of IFN- γ monofunctional CD4⁺ T cells ($p=0.0048$). Considering the CD8 compartment, we detected no significant differences in the overall functional profile of SARS-CoV-2-responding CD8⁺ T cells between the two groups ($p=0.098$), although seropositive children did have a larger proportion of CD8⁺ T cells producing both IFN- γ and IL-2 ($p=0.027$) and single IFN- γ ($p=0.011$) (**Figure 3.3.E**).

Taken together, these data show that a significant proportion of seronegative children have detectable SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells. Notably, these CD4⁺ T cells exhibit a distinct polyfunctional cytokine profile compared to those found in SARS-CoV-2-exposed children.

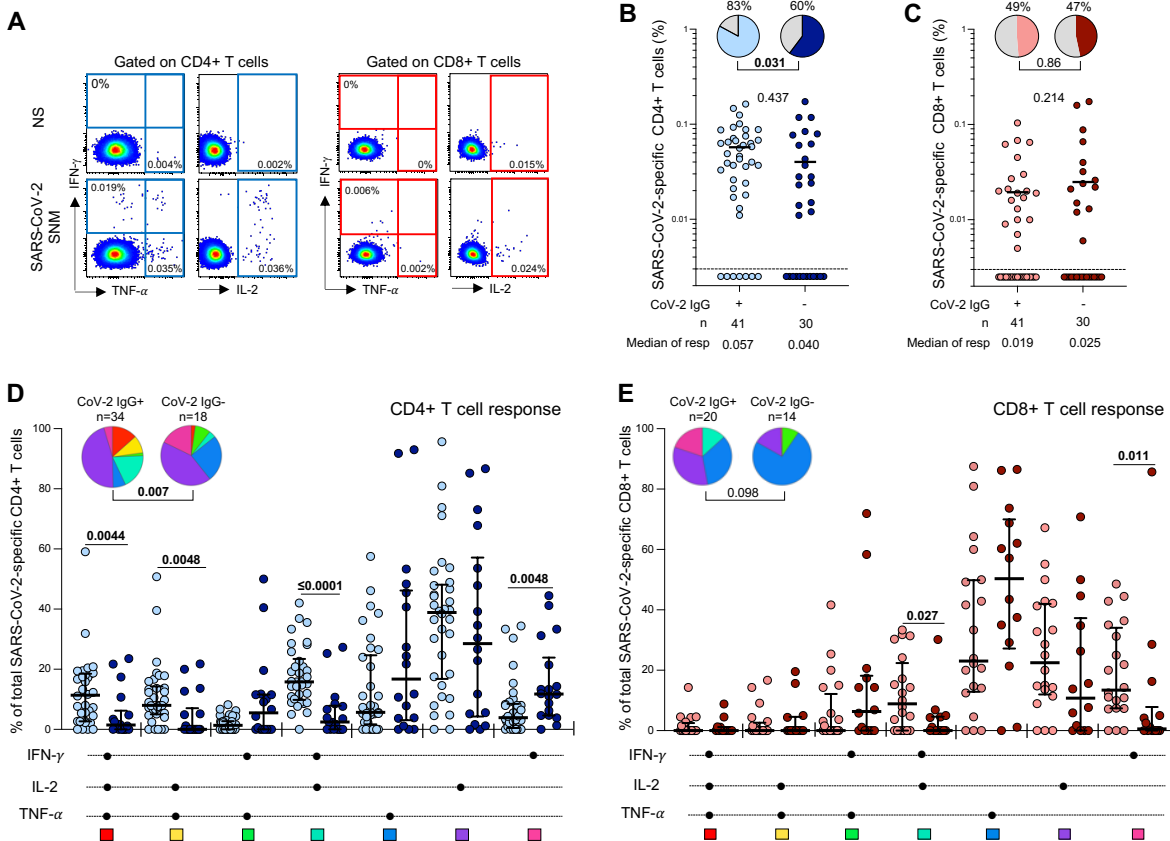


Figure 3.3: SARS-CoV-2-specific T cell responses in children.

(A) Representative flow cytometry plots of SARS-CoV-2-specific interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and interleukin-2 (IL-2) cytokine production from CD4+ (left) and CD8+ (right) T cells in response to SARS-CoV-2 peptide stimulation. NS: no stimulation, SNM: combined peptide pool of SARS-CoV-2-spike, nucleocapsid and membrane proteins. **(B)** Frequency of SARS-CoV-2-specific CD4+ T cells producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). Children were grouped according to SARS-CoV-2 serostatus (light blue: n=41 SARS-CoV-2 seropositive; dark blue: n=30 SARS-CoV-2 seronegative). Bars represent the median of the responders, and median values are indicated. The pie charts represent the proportion of responders with detectable T cell response to SARS-CoV-2 SNM peptides. **(C)** Frequency of SARS-CoV-2-specific CD8+ T cells producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). Children were grouped according to SARS-CoV-2 serostatus (light red: n=41 SARS-CoV-2 seropositive children; dark red: n=30 SARS-CoV-2 seronegative children). Statistical comparisons in **(B)** and **(C)** were performed using the Mann-Whitney test between seropositive and seronegative children and the Chi-square test to compare the percentage of responders; *p* values <0.05 were considered statistically significant and are bolded. **(D)**

Polyfunctional profile of SARS-CoV-2-specific CD4+ and **(E)** CD8+ T cells in seropositive and seronegative unvaccinated children. The x-axis illustrates each combination which is indicated with a black circle for the presence of IFN- γ , IL-2 and TNF- α . The medians and interquartile range are shown. Each response pattern (any possible combination of IFN- γ , IL-2 and TNF- α production) is color coded and summarized in the pie charts, with each pie slice representing the median contribution of each combination to the total SARS-CoV-2 responses. The permutation test was used to compare the statistical differences between the pie charts and the Mann Whitney Sum Test to compare response patterns between seropositive and seronegative children; *p* values <0.05 were considered statistically significant and are bolded.

3.4.4 Pre-existing immunity to endemic common circulating human coronaviruses

The presence of SARS-CoV-2-responding CD4+ T cells primarily exhibiting a monofunctional profile observed in seronegative children led us to hypothesize that the T cell response might be due to cross-reactivity resulting from prior infection with common circulating endemic HCoVs. Therefore, we measured endemic beta HCoV-HKU1 and HCoV-OC43 S IgG in SARS-CoV-2 seropositive and seronegative children. The magnitude of both HCoV-HKU1 (**Figure 3.4.A**) and HCoV-OC43 (**Figure 3.5.A**) S IgG was comparable in the seropositive and seronegative groups (median OD: 0.746 vs 0.420; *p*=0.153 for HCoV-HKU1 and 1.769 vs 1.281; *p*=0.07 for HCoV-OC43, respectively).

We found a moderate correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-HKU1-S IgG in seronegative children (*p*=0.036, *r*=0.392, **Figure 3.4.B**), while no correlation was observed in seropositive children (*p*=0.936, *r*=-0.013, **Figure 3.4.C**). For HCoV-OC43, no correlation was detected in either the seronegative (*p*=0.324, *r*=-0.187, **Figure 3.5.B**) or the seropositive group (*p*=0.171, *r*=-0.218, **Figure 3.5.C**). Overall, these results may suggest the SARS-CoV-2-reactive CD4+ T cell responses in a proportion seronegative children could be due in part to cross-reactive T cell immunity resulting from prior infection with common cold HCoV-HKU1.

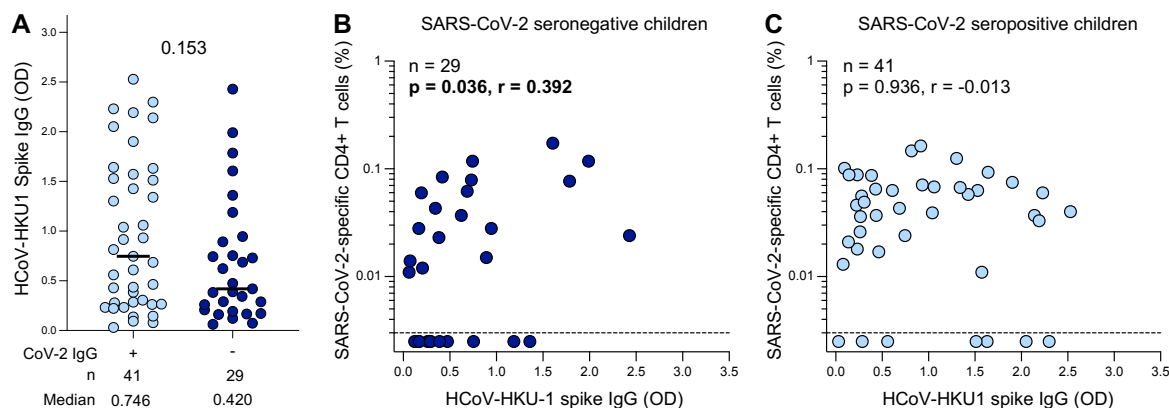


Figure 3.4: SARS-CoV-2 cross-reactivity to endemic beta-HCoV in children.

(A) The magnitude of HCoV-HKU-1 spike IgG levels were measured by ELISA in SARS-CoV-2 seropositive (light blue; n=41) and seronegative (dark blue; n=29) children. Plasma sample was insufficient for one seronegative child therefore OD for HCoV-HKU1 was not measured for this participant. The bars represent the median values. A statistical comparison was performed using the Mann-Whitney test between seropositive and seronegative children; a p value <0.05 was considered statistically significant. **(B)** Correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-HKU-1-spike IgG levels in SARS-CoV-2 seronegative children (n=29). One participant had insufficient sample available to be included in this assay. **(C)** Correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-HKU-1-spike IgG levels in SARS-CoV-2 seropositive children (n=41). Statistical comparisons for **(B)** and **(C)** were performed using a two-tailed non-parametric Spearman rank tests; p values <0.05 were considered statistically significant and are bolded and correlation coefficients values are shown.

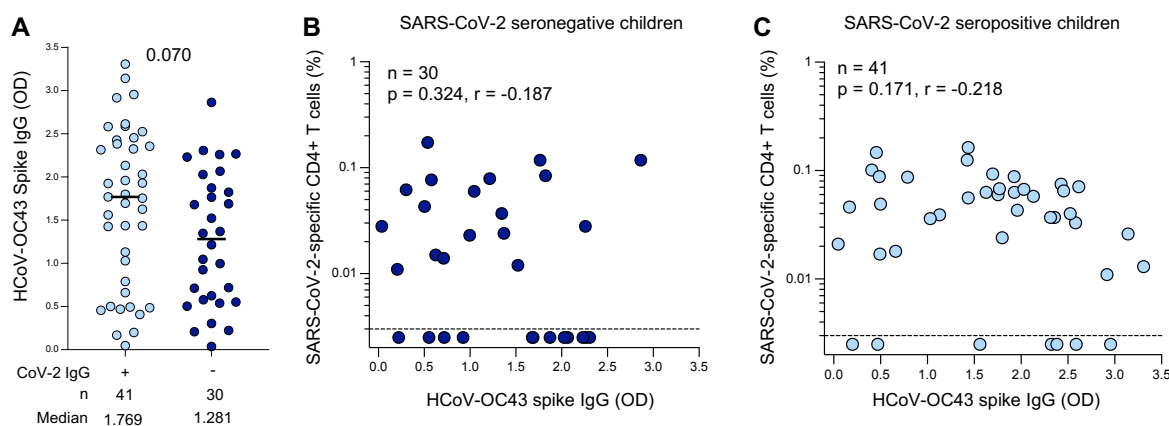


Figure 3.5: SARS-CoV-2 cross-reactivity to endemic HCoV-OC43 in unvaccinated children.

(A) The magnitude of HCoV-OC43 spike IgG levels were measured by ELISA in SARS-CoV-2 seropositive (light blue; n=41) and seronegative (dark blue; n=30) children. The bars represent the median values. Statistical comparisons were performed using the Mann-Whitney test between seropositive and seronegative children; *p values* <0.05 were considered statistically significant. **(B)** Correlation between the frequency of SARS-CoV-2-specific CD4⁺ T cells and HCoV-OC43-spike IgG levels in SARS-CoV-2 seronegative. **(C)** Correlation between the frequency of SARS-CoV-2-specific CD4⁺ T cells and HCoV-OC43-spike IgG levels in SARS-CoV-2 seropositive children (n=41). Statistical comparisons in **(B)** and **(C)** were performed using a two-tailed non-parametric Spearman rank tests; *p values* <0.05 were considered statistically significant and are bolded and correlation coefficients values are shown.

3.4.5 SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive children compared to COVID-19 convalescent adults

Blood and plasma samples were collected from 30 COVID-19 convalescent HCWs participating in a longitudinal study at Groote Schuur Hospital (Cape Town, Western Cape, South Africa) (**Figure 3.6.A**). All convalescent adults had a prior mild/asymptomatic PCR-confirmed SARS-CoV-2 infection (median 224 days prior to sampling) and had not received a COVID-19 vaccine at the time of sampling. Samples were collected between 22 January 2021 – 23 February 2021.

A trend towards a higher proportion of SARS-CoV-2 CD4 responders was observed in convalescent adults (97%) compared to seropositive children (83%) (*p*=0.07, **Figure 3.6.B**). Moreover, in SARS-CoV-2 responders, convalescent adults did have a significantly higher frequency of SARS-CoV-2-specific CD4⁺ T cells compared to seropositive children (median of responders: 0.092% vs 0.057%, respectively, *p*=0.0004). Unlike CD4⁺ T cell responses, there were no significant differences in either the proportion of CD8 responders (53% vs 49%, *p*=0.705) or the frequency of SARS-CoV-2-specific CD8⁺ T cells (median of responders: 0.038% vs 0.019%; *p*=0.096) between convalescent HCWs and seropositive children (**Figure 3.6.C**). Furthermore, we observed a moderate correlation between age and the frequency of SARS-CoV-2-specific CD4⁺ T cells (*p*=0.0006, *r*=0.396, **Figure 3.6.D**). No correlation was observed with SARS-CoV-2-specific CD8⁺ T cells and age (*p*=0.116, *r*=0.188, **Figure 3.6.D**). Overall, these results show that convalescent adults mount higher SARS-

CoV-2-specific CD4+ T cell responses compared to children. This disparity may, in part be attributed to the incomplete maturation of the adaptive immune system in children (Kloc et al., 2020; Simon et al., 2015).

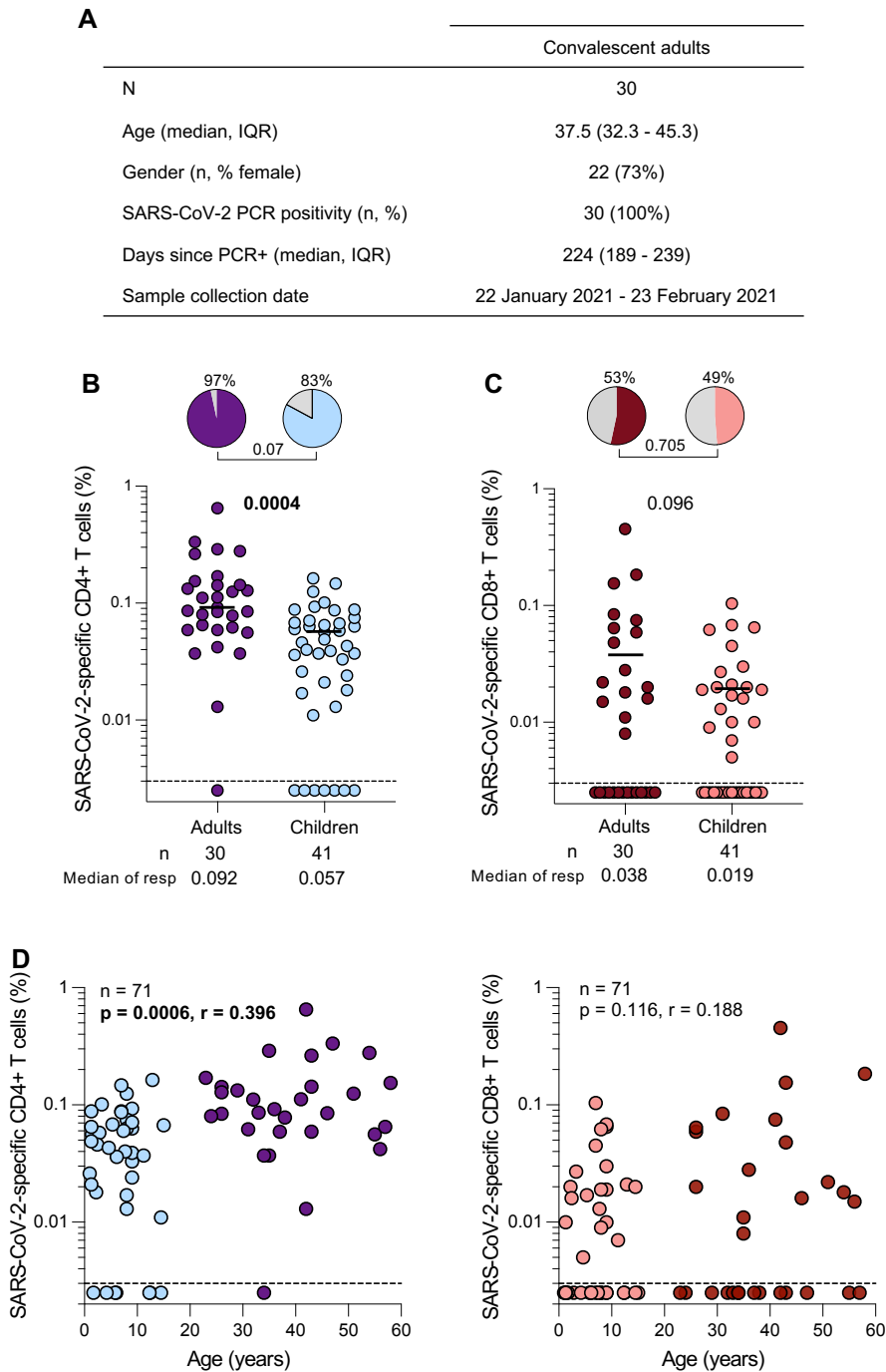


Figure 3.6: SARS-CoV-2-specific T cell responses in children compared to convalescent adults.

(A) The demographic characteristics of 30 unvaccinated convalescent adults included in this study. Age, sex, SARS-CoV-2 PCR-positivity, days since PCR test and collection date are shown. **(B)** Frequency of SARS-CoV-2-specific CD4⁺ T cells producing any of the measured cytokines (IFN- γ , TNF- α , or IL-2) in SARS-CoV-2 convalescent HCW (purple; n=30) and seropositive children (light blue; n=41). The pie charts represent the proportion of responders with a detectable T cell response to SARS-CoV-2 SNM combined peptide pools. Bars represent median of the responders. **(C)** Frequency of SARS-CoV-2-specific CD8⁺ T cells producing any of the measured cytokines (IFN- γ , TNF- α , or IL-2) in SARS-CoV-2 convalescent HCW (dark red; n=30) and seropositive children (light red; n=41). The bars represent the median of the responders and median values are indicated. Statistical comparisons were performed using the Mann-Whitney test between seropositive children and adults and the Chi-square test was used to compare the percentage of responders for **(B)** and **(C)**; *p values* <0.05 were considered statistically significant and are bolded. **(D)** Correlations between SARS-CoV-2-specific CD4⁺ or CD8⁺ T cells and age in convalescent HCW (purple and dark red; n=30) and seropositive (light blue and light red; n=41) children. Statistical comparisons were performed using a two-tailed non-parametric Spearman rank tests; *p values* <0.05 were considered statistically significant and are bolded and correlation coefficients are shown.

3.5 Discussion

T cells have been associated with protection from severe COVID-19 (Moss, 2022). It is therefore important to understand the nature of T cell responses targeting SARS-CoV-2 in children, who have been largely spared from severe COVID-19. In this study, comparing SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive and seronegative children, a sizable proportion of seronegative children had SARS-CoV-2-reactive CD4⁺ and CD8⁺ T cells, but their CD4⁺ T cells exhibited a distinct functional cytokine profile compared to seropositive children. Importantly, in seronegative children, the frequency of SARS-CoV-2-reactive CD4⁺ T cells was positively associated with HCoV-HKU1 S-specific IgG antibodies. Additionally, we showed that convalescent adults had a higher magnitude of CD4⁺ T cell responses against SARS-CoV-2 compared to seropositive children, which associated positively with age.

Our data are in accordance with several studies showing that children develop robust humoral and cellular immunity to SARS-CoV-2 infection (Cotugno et al., 2021; Dowell et al., 2022a; Paul et al., 2022; Tosif et al., 2020; Tsang et al., 2021) and BNT162b2 vaccine (Cinicola et al., 2022). In addition, SARS-CoV-2-specific T cell responses have been detected in 40-60% of SARS-CoV-2 unexposed individuals including children and adults (C. A. Cohen et al., 2021; Dowell et al., 2022a; Grifoni et al., 2020; Le Bert et al., 2020; Mateus et al., 2020; Singh et al., 2022; Swadling et al., 2021; H. X. Tan et al., 2021) suggesting possible cross reactivity to HCoVs. One study demonstrated that the proportion of T cell responders was higher in children than adults (Dowell et al., 2022a). In contrast, a study by Tsang et al. found that SARS-CoV-2 uninfected children failed to mount detectable T cell responses (Tsang et al., 2021). A further study showed that only a small proportion of seronegative children mounted a SARS-CoV-2-specific CD4+ T cell response (13%) compared to 60% in seropositive siblings, despite similar exposure in shared households (Paul et al., 2022). These two studies differ from our findings, where we showed that 60% of seronegative children had detectable SARS-CoV-2-specific T cell responses, consistent with Dowell et al (Dowell et al., 2022a). The discrepancies between the studies could be due to a difference in the seasonal prevalence of circulating HCoVs in each study setting (Audi et al., 2020; S. Park et al., 2020; Shah et al., 2022), different T cell assays used to analyze T cell responses, and/or the cohort demographics.

Although the source of SARS-CoV-2 reactive T cells in seronegative children remains unclear, mounting evidence argues for cross-reactivity to endemic HCoVs. HCoVs (including beta HCoVs-HKU1 and OC43, and alpha HCoVs-NL63 and 229E) have partial sequence homology with SARS-CoV-2 (de Vries, 2020; Mateus et al., 2020). While cross reactive humoral responses have been shown not to underly this protection (Zar et al., 2021), studies have reported pre-existing T cell responses to endemic HCoVs with cross-reactivity to SARS-CoV-2 in unexposed adults (Da Silva Antunes et al., 2021; Kundu et al., 2022; Loyal et al., 2021; Nelde et al., 2021; H. X. Tan et al., 2021; Woldemeskel et al., 2020; Yu et al., 2022) and children (Dowell et al., 2022a; Humbert et al., 2023; Niessl et al., 2021b; Saletti et al., 2020). These cellular responses were targeted mainly towards the S2 subunit which is highly conserved among coronaviruses (Dowell et al., 2022a;

Woudenberg et al., 2021). In our study, we showed a positive correlation between the frequency of SARS-CoV-2 CD4⁺ T cells and the magnitude of HKU1 S-specific IgG in SARS-CoV-2 seronegative children. Our findings are supported by similar findings showing that 58% (7/12) seronegative children had binding antibodies against alpha and/or beta HCoV S proteins, which they concluded were likely due to recent HCoV infection (Dowell et al., 2022a).

Pre-existing immunity from endemic HCoV infection or exposure has been shown to be associated with a protective effect against COVID-19 in adults (Da Silva Antunes et al., 2021; Kundu et al., 2022; Sagar et al., 2021; Woldemeskel et al., 2020; Yu et al., 2022). Sagar et al. showed that individuals with a documented recent or ongoing HCoV infection and SARS-CoV-2 infection had fewer COVID-19-associated complications when hospitalized, compared to individuals without HCoV infection (Sagar et al., 2021). Previously, the clinical relevance of pre-existing immunity and cross-reactive cellular responses from endemic HCoVs in providing protection from SARS-CoV-2 infection in children was unclear. Recently however, Dowell et al. demonstrated that SARS-CoV-2-reactive T cell responses in SARS-CoV-2 seronegative children were associated with relative protection against Omicron infection, suggesting a protective role in SARS-CoV-2 unexposed children (Dowell et al., 2022b).

Polyfunctional T cells have been reported to be associated with protection against several viral diseases (Betts et al., 2006; Ciuffreda et al., 2008; Seder et al., 2008; Snyder et al., 2016). These include protection against cytomegalovirus (CMV) infection after lung transplantation (Snyder et al., 2016), improved viral control of hepatitis C virus (HCV) (Ciuffreda et al., 2008) and detection during human immunodeficiency virus (HIV) infection in long term non-progressors (Betts et al., 2006). We found that seropositive children had a more polyfunctional profile of SARS-CoV-2-specific CD4⁺ T cells while CD4 responses in seronegative children exhibited a predominantly monofunctional profile. The SARS-CoV-2-specific polyfunctional Th1 CD4 T cell response (characterized by co-expression of IFN- γ , TNF- α and/or IL-2), as seen in seropositive children, may be necessary for effective viral control and has been documented in COVID-19 convalescent adults (Altmann & Boyton, 2020; Bertoletti et al., 2022).

The distinct CD4 and CD8 polyfunctional profiles reported in our findings could be a result of differing degrees of memory T cell differentiation and/or lower SARS-CoV-2 peptide binding affinity with HCoV-specific T cells resulting in low T cell activation. The affinity between MHC molecules presenting peptides (pMHC) and the T cell receptor (TCR) plays a role in antigen recognition. It has been reported high TCR-pMHC complex affinity is associated with increased overall T cell functional efficacy in vitro and in vivo and therefore an increased polyfunctional profile compared to low affinity TCRs (Allard et al., 2017; Spear et al., 2019; Stone et al., 2009). TCR cross-recognition of pMHCs that are not structurally identical results in lower binding affinity which decreases T cell polyfunctionality (Stone et al., 2009; M. P. Tan et al., 2015). It is therefore plausible that the distinct polyfunctional profile of SARS-CoV-2 CD4+ T cell responses in seronegative children observed in our study may be mediated by cross-recognition of pre-existing T cell immunity to HCoV-HKU1, where partial sequence homology between HCoV-HKU1 and SARS-CoV-2 results in low peptide binding affinity and a different polyfunctional T cell profile (Jarjour et al., 2021; Mateus et al., 2020; Nelde et al., 2021; Spear et al., 2019; M. P. Tan et al., 2015). It is noteworthy to report that we did not find any association with the other beta HCoV-OC43 S-specific IgG and SARS-CoV-2-specific T cells in seronegative children, which could be related to differences in seasonal prevalence of types of HCoV infections. Indeed, multiple studies have shown differences in the prevalence of HCoV infections which change yearly and depend on regional environment (Dare et al., 2007; Friedman et al., 2018; Lau et al., 2006; Mackay et al., 2012; Theamboonlers et al., 2007). In particular, a study by Friedman et al showed HCoV-OC43 was the most prevalent HCoV in winter, while HCoV-HKU1 was the most prevalent in spring to summer during the 2015-2016 time period (Friedman et al., 2018).

Numerous studies have now compared the magnitude of the SARS-CoV-2 specific T cell response in children to those detectable in adults (C. A. Cohen et al., 2021; Dowell et al., 2022a; Kaaijk et al., 2022; Pierce et al., 2020; Tosif et al., 2020; Tsang et al., 2021). Initially it was thought that children may have a higher magnitude of T cell responses given their relative resistance to severe disease and the link between T cell responses and protection from severe disease (Bogunovic &

Merad, 2021; Rydyznski Moderbacher et al., 2020). However, children were shown to have lower T cell responses than adults in most studies, including the current study (C. A. Cohen et al., 2021; Kaaijk et al., 2022; Pierce et al., 2020). Several hypotheses could possibly explain the differences in the magnitude of SARS-CoV-2-specific CD4⁺ T cell responses between children and adults in our study. These include 1) differences in disease severity between the groups, 2) the immaturity of children's immune system and 3) the timing of infection in relation to sampling. Adults have a mature immune system with more differentiated memory T cell subsets endowed with increased cytokine capacity, whereas children have an immature immune system, with many more naïve T cells which have reduced cytokine producing capacity, are enriched for monofunctional responses, and have increased antigen dependence (Mahnke et al., 2013). However, as the exact time of SARS-CoV-2 infection in the children group could not be defined, we cannot fully exclude that the higher magnitude of SARS-CoV-2-specific CD4⁺ T cells observed in the adult group could be related to a more recent infection compared to the children group. Despite lower circulating SARS-CoV-2-specific T cells, children and adults may also have different responses in the respiratory tract. There is evidence in adults demonstrating the presence of SARS-CoV-2-specific T cell responses in the nasal mucosa after infection and vaccination (J. M. E. Lim et al., 2022; Ssemaganda et al., 2022). However, comparative studies have yet to be performed in children. Additionally, several studies have proposed that one potential contributing factor to age-related COVID-19 clinical outcome is the robust innate immune responses observed in children, which may contribute to early control of viral replication (Neeland et al., 2021a; Speranza, 2021; Weatherhead et al., 2020). Recent studies have shown that, compared to adults, SARS-CoV-2 seronegative children exhibited an increased number of innate immune cells with pre-activated signatures leading to early production of interferon-mediated antiviral effects in the upper respiratory tract (Loske et al., 2022; Yoshida et al., 2021).

In conclusion, our study shows that a robust SARS-CoV-2-specific T cell response is observed in children, including a proportion of those with no evidence of prior SARS-CoV-2 infection. We demonstrate that the magnitude of SARS-CoV-2-reactive CD4⁺ T cells in seronegative children correlates with HCoV-HKU1 exposure. This, together with a distinct functional profile of SARS-

CoV-2-specific responding CD4+ T cells observed between seropositive and seronegative children may provide further evidence for pre-existing T cell responses cross-reactive to SARS-CoV-2.

Limitations of the study

This study relied on serology to determine SARS-CoV-2 exposure in the pediatric cohort. With no PCR confirmation of SARS-CoV-2 infection, the exact time of infection of the children could not be determined. This caveat in our study may have an impact in the differences of SARS-CoV-2-specific T cell responses seen in adults compared to seropositive children. Additionally, our study investigated T cell responses against SARS-CoV-2 S, N and M proteins, and not the non-structural viral proteins that can also serve as targets for the T cell response, thus not capturing the full extent of T cell reactivity in infected participants. While our study focused solely on Th1-associated cytokines, it remains to define whether other Th CD4 subtypes such Th2 or Th17 could also play a role in COVID-19 protection. Furthermore, we did not address durability of the T cell responses in children due to the cross-sectional design of the study. Moreover, it would be of interest to assess the HCoV-HKU1-specific memory T cell responses and define the relationship with SARS-CoV-2 specific immune responses and their potential protective effect against SARS-CoV-2. Further experiments assessing HCoV-IgG responses as well as the neutralizing antibody activity against HCoVs, SARS-CoV-2 and its variants of concern are warranted to gain a better understanding of the relative protection from disease observed in SARS-CoV-2-infected children compared to adults.

CHAPTER 4: DURABILITY OF SARS-CoV-2-SPECIFIC T CELL RESPONSES AND CROSS-REACTIVITY TO SARS-CoV-2 DELTA AND OMICRON BA.1 VARIANTS IN CHILDREN

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4.1 Introduction

Early studies reported that severe acute respiratory syndrome coronavirus (SARS-CoV)-2-specific antibody responses decline over time (Anand et al., 2021; Cromer et al., 2021; Gallais et al., 2021; He et al., 2021; Ibarrondo et al., 2020). This raised concerns in the global health sector since the waning of humoral immunity potentially affects long-term immunity induced by both SARS-CoV-2 infection and COVID-19 vaccination. T cells have been reported to mediate SARS-CoV-2 control and have been associated with limiting the severity of COVID-19 (Bertoletti et al., 2022; Moss, 2022; Rydyznski Moderbacher et al., 2020; Sette & Crotty, 2021). Memory T cells have been shown to persist for up to 17 years, far longer than antibodies, in patients previously infected with SARS-CoV during the 2003 epidemic outbreak (Künzli & Masopust, 2023; Le Bert et al., 2020). To date, it has been reported that SARS-CoV-2-specific memory T cells are maintained from 6 - 15 months post infection (K. W. Cohen et al., 2021; Dan et al., 2021; Marcotte et al., 2022; Rodda et al., 2021; Zuo et al., 2021). This provides evidence that SARS-CoV-2-specific memory T cells may also persist for years after primary SARS-CoV-2 infection (Bertoletti et al., 2021a; Kedzierska & Thomas, 2022; Sette & Crotty, 2021).

The emergence of the highly transmissible SARS-CoV-2 Omicron variant, which has been shown to be extremely evasive of neutralizing antibodies, initially caused great concern about the efficacy of immunity acquired from infection with earlier variants or ancestral, first-generation vaccination and the increased risk of re-infections (Carabelli et al., 2023; Cele et al., 2022; Garcia-Beltran et al., 2021; Khan et al., 2022; L. Liu et al., 2022; Planas et al., 2022). However, it has since been widely documented that SARS-CoV-2 variants do not cause viral escape from T cell immunity, induced by either natural infection or vaccination (Geers et al., 2021; Goel et al., 2021b; Keeton et al., 2021, 2022; Riou et al., 2021; Sette et al., 2023; Sette & Crotty, 2022; Tarke et al., 2021a; Tarke et al., 2021b). This persistence of cellular immunity in the face of ongoing viral evolution, together with the increased durability of T cells compared to antibody responses, has led to a shift in focus to cellular memory to provide long-term immunity against severe disease during re-infections. Most of these studies report on adult populations, while the literature on the

durability and cross-reactivity of SARS-CoV-2-specific immunity in children is more limited and remains under investigated (C. A. Cohen et al., 2021; Dowell et al., 2022a; Jacobsen et al., 2022; Kedzierska & Thomas, 2022; Paul et al., 2022; Sette et al., 2023; Sette & Crotty, 2022).

We, Benede et al., and others have previously shown that children exhibit lower SARS-CoV-2-specific T cell responses compared to adults (Benede et al., 2024; C. A. Cohen et al., 2021; Goenka et al., 2021; Kaaijk et al., 2022; Moratto et al., 2020; Pierce et al., 2020; Rowntree et al., 2022). This may have implications for both durability and cross-reactivity to variants of concern (VOC). Unfortunately, the emergence of SARS-CoV-2 variants including Delta and Omicron subvariants, resulted in increased incidence rates and a surge in COVID-19 hospitalizations and risk of infections in unvaccinated children and adolescents (Cloete et al., 2022; Delahoy et al., 2021; Elliott et al., 2022; Marks et al., 2022; L. Wang et al., 2022). Several studies have shown that children have poor cross-neutralizing potential against SARS-CoV-2 VOC, including Alpha, Beta Delta (Dowell et al., 2022a; Jacobsen et al., 2022; Renk et al., 2022) and Omicron subvariants (Bellusci et al., 2023; W. Peng et al., 2023; Seidel et al., 2023; Sieber et al., 2022; J. Tang et al., 2022; Toh et al., 2022) compared to adults or adolescents. However, in contrast to studies focusing extensively on adult cohorts, there are limited studies focusing on the cross-reactivity of T cell responses to SARS-CoV-2 VOC, including Delta and Omicron, in unvaccinated children (Paniskaki et al., 2023; Paul et al., 2022; Seidel et al., 2023; Sieber et al., 2022).

Factors possibly contributing to differences in T cell responses between children and adults include cross-reactivity to endemic HCoV, timing of SARS-CoV-2 infection, differences in disease severity, and age-related T cell development (Chou et al., 2022). T cell biology differs over the course of human life such that early-life T cell function differs from that of adult T cells (B. V. Kumar et al., 2018; Simon et al., 2015). In early life and during childhood, there is an increased output of naïve T cells by the thymus, this compartment has a high receptor diversity that provides the adaptive immunity with the potential to respond to novel and diverse antigens (B. V. Kumar et al., 2018; Naylor et al., 2005; Qi et al., 2014).

Understanding the immunological memory and dynamics of adaptive T cell immunity against SARS-CoV-2 after infection is important for understanding the future trajectory of long-term immunity in children. This is particularly relevant in South Africa (SA) given the limited SARS-CoV-2 vaccination coverage of children under 12 years of age (Department: Health Republic of South Africa, 2023). In this study, we address and expand on a caveat of the previous study in Chapter 3, that differences in the timing of SARS-CoV-2 infection may influence differences in responses between children and adults. Here, we included participants from a birth cohort study consisting of mother-child pairs living within the same household (Zar et al., 2015). In addition, we investigated the T cell maintenance of SARS-CoV-2 responses over 16 months at a follow-up visit (V2) in children who were SARS-CoV-2 seropositive or seronegative at the baseline visit (BL). Finally, we analyzed T cell cross-reactivity to SARS-CoV-2 Delta and Omicron variants in children following two Omicron BA.1/2 and BA.4/5 waves in SA.

4.2 Materials and methods

4.2.1 Study participants.

Participants included in this study consisted of 102 children and 73 matched maternal adults that were enrolled and recruited from the Drakenstein Child Health Study (DCHS) (Paarl, Western Cape, SA) a SA birth cohort study (Zar et al., 2015). The 73 maternal adults had 1 child each included in the study therefore 73 children were paired with the matched maternal adults. Of the 102 children, the 29 children were not paired with the maternal adults due to no available blood samples from the maternal adults. The study participant demographics are listed in detail in **Figure 4.1**. All participants were asymptomatic or had mild COVID-19 illness and did not require hospitalization. Previous infection was determined by a positive SARS-CoV-2 Spike (S) Enzyme-Linked Immunosorbent Assay (ELISA) IgG response and was not confirmed by PCR testing. The study was approved by the Human Research Ethics Committee, University of Cape Town (HREC 401/2009, HREC 640/2023). Parental or legal guardians provided written informed consent for all paediatric participants which was renewed annually.

4.2.2 Enzyme-linked immunosorbent assay (ELISA).

SARS-CoV-2-S IgG responses were measured in all participants, using indirect ELISA as described in **Chapter 2, section 2.2.2**.

4.2.3 Whole blood stimulation and processing.

Blood was collected and stimulated as described in **Chapter 2 section 2.2.3**. Additionally, 98 children came back for the follow up visit between 13 June 2022 until 7 December 2022. Of these, blood from the first 50 children in which we had sufficient sample was also stimulated with peptide pools targeting SARS-CoV-2 ancestral strain (Wuhan, GenBank:MN908947), Delta (B.1.617.2), and Omicron BA.1 (B.1.1.529) S proteins (TC Peptide Lab, San Diego, CA), as described in **Chapter 2, section 2.2.1**.

4.2.4 Cell staining and flow cytometry.

Cell staining and acquisition was performed as described in **Chapter 2, section 2.3.1** and the antibody panel used for this study is listed under **Chapter 2, Table 2.2**. The gating strategy used to analyze the flow cytometry data for this chapter is depicted in **Chapter 2, Figure 2.3.A-J**.

4.2.5 Statistical analysis.

Statistical tests were performed as described in **Chapter 2, section 2.4**. Further details of analysis performed for each experiment is described in the figure legends.

4.3 Results

4.3.1 Study cohort.

A total of 102 children and 73 matched maternal participants were included in this study (**Table 4.1**). Samples were collected between 15 March 2021 and 19 May 2021, following two epidemic waves dominated by the ancestral D614G strain (March 2020 - September 2020) and the Beta

variant (October 2020 - May 2021) in SA (**Figure 4.1**) (Tegally et al., 2021; W. Yang & Shaman, 2022). The median age of the children was 7 years (range 5 - 8 years) and for adults was 33 years (range 25 - 47 years) (**Table 4.1**). More than 50% of the children (60/102) and 100% (73/73) of the adults were female, in accordance with the cohort study design. Of the children, 22% (23/102) were HIV exposed but uninfected and 19% (14/73) of the adults were living with HIV. Overall, 61% (62/102) of the children and 88% (64/73) of the maternal adults were seropositive for SARS-CoV-2 at BL, indicating previous SARS-CoV-2 infection. None of the participants had been vaccinated against SARS-CoV-2 at BL (**Table 4.1**).

Follow-up samples (V2) were collected between 13 June 2022 to 7 December 2022, a median of 16 months (range 13 - 21 months) after the initial BL visit (**Figure 4.1**), to assess the maintenance or *de novo* SARS-CoV-2-specific T cell responses and cross-reactivity to SARS-CoV-2 Ancestral, Delta and Omicron BA.1 variants. A total of 98 unvaccinated children returned for the follow-up visit and were included in the longitudinal analysis. Due the peptide availability, only blood from the first 50 children in which we received sufficient sample for additional analysis were stimulated with VOC peptides for the cross-reactivity study. Adults were excluded at this timepoint because a large proportion had received the COVID-19 vaccine prior to the V2 sample collection timepoint.

Table 4.1: The demographics characteristics of unvaccinated children and mothers included in this study. Collection dates, age, gender, HIV, SARS-CoV-2 serology and vaccinations are shown.

	Baseline (BL)		V2 - follow-up
Collection time range	15 Mar 2021 – 19 May 2021		13 Jun 2022 – 07 Dec 2022
Median date of collection	16 April 2021		24 September 2022
	Children	Mother	Children
n	102	73	98
Mother & child pairs	73		NA
Age (median, range)	7 (5-8)	33 (25-47)	8 (6-10)
Gender (n, %, female)	60 (59%)	73 (100%)	58 (59%)
HIV exposed uninfected (n, %)	23 (22%)	N/A	23 (23%)
HIV infected (n, %)	N/A	14 (19%)	N/A
SARS-CoV-2 Seropositive (n, %)	62 (61%)	64 (88%)	ND
SARS-CoV-2 Vaccination	0 (0%)	0 (0%)	0 (0%)

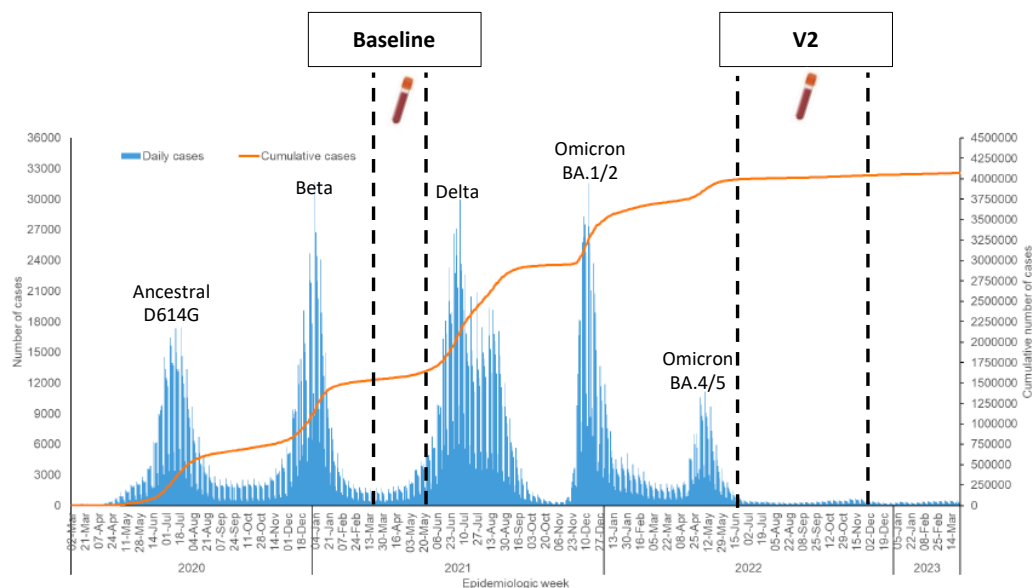


Figure 4.1: Study cohort demographics and COVID-19 epidemiological infection waves in South Africa.

Number of cases and cumulative COVID-19 cases from 3 March 2020 – 25 March 2023, along with sample collection duration. Baseline samples were collected approximately 12 months after the onset of the COVID-19 pandemic and V2 samples were collected approximately 20 months after the BL visit (source NICD 2023; <https://www.nicd.ac.za/diseases-a-z-index/disease-index-covid-19/sars-cov-2-genomic-surveillance-update/>).

4.3.2 Magnitude, polyfunctional, and memory profile of SARS-CoV-2-specific T cells in SARS-CoV-2 seropositive and seronegative children at BL visit.

In Chapter 3, we showed that 83% of SARS-CoV-2 seropositive and 60% of seronegative children had detectable SARS-CoV-2-specific T cells. Here, we sought to confirm the data in another cohort with a larger sample size and to further characterize the phenotypic memory profiles that were not analyzed in the previous study.

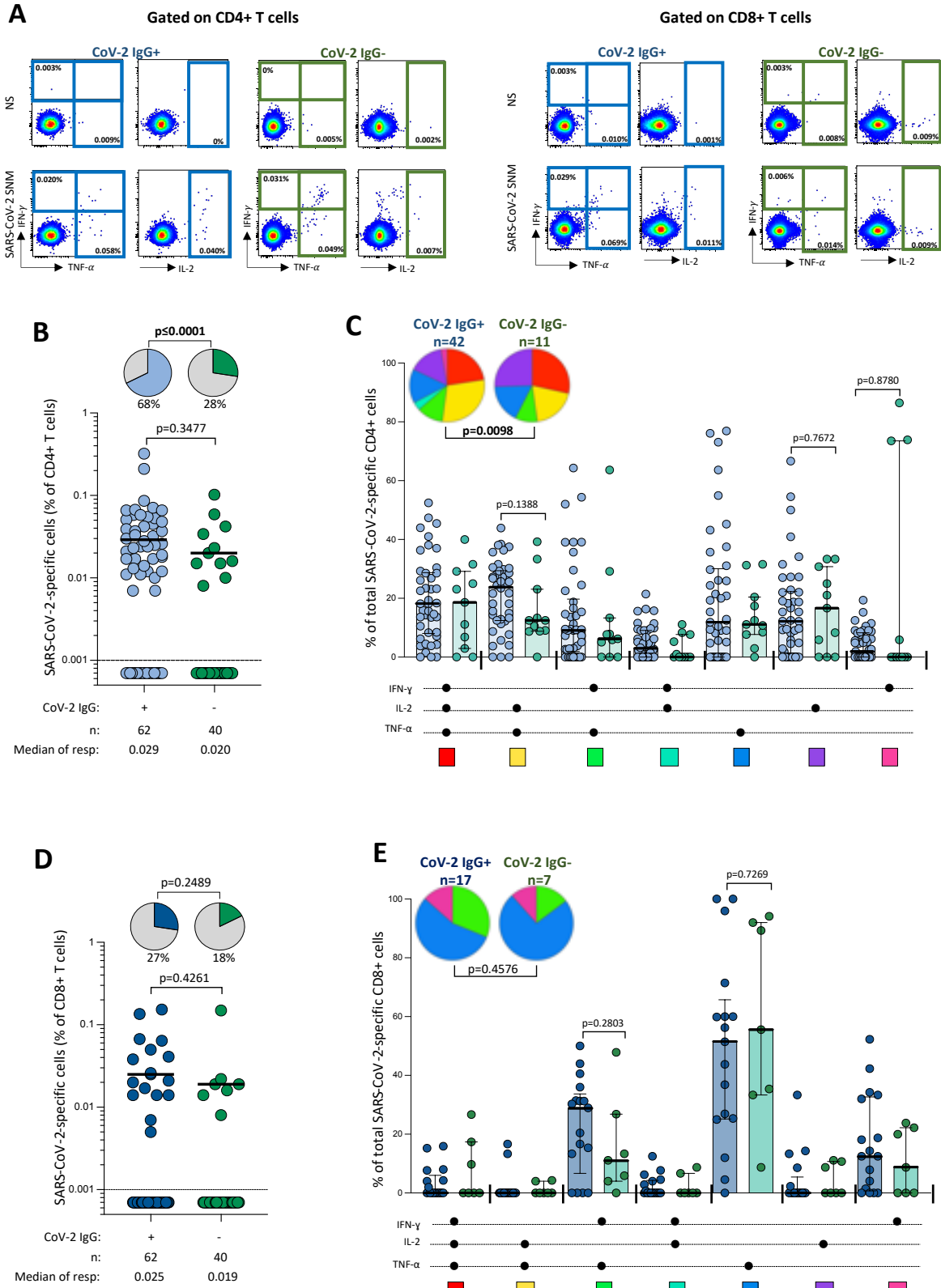
First, we compared the magnitude of SARS-CoV-2-specific CD4⁺ T cell responses between SARS-CoV-2 seropositive and seronegative children (**Figure 4.2A**). The frequencies of SARS-CoV-2-specific CD4⁺ T cells were comparable between SARS-CoV-2 seropositive and seronegative children (median of responders: 0.029% vs 0.020%, respectively, **Figure 4.2B**). However, as expected, the proportion of CD4 responders was significantly higher at 68% (42/62) in seropositive children and 28% (11/40) in seronegative children ($p \leq 0.0001$, **Figure 4.2B**). The proportion of SARS-CoV-2-specific CD8 responders was lower than CD4 responders in both groups, with 27% (17/65) of seropositive and 18% (7/40) of seronegative children having a detectable CD8 response ($p = 0.2489$, **Figure 4.2D**). No significant differences were found in the frequency of SARS-CoV-2-specific CD8⁺ T cells between seropositive and seronegative children (median of responders: 0.025% vs 0.019%, respectively, **Figure 4.2D**).

Thereafter, we investigated whether SARS-CoV-2 serostatus in children influences the polyfunctional profile of SARS-CoV-2-responding T cells, as reported in Chapter 3. The polyfunctional profile of SARS-CoV-2-specific CD4⁺ T cells was defined by the co-expression of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) or interleukin-2 (IL-2) cytokines in responders using the Boolean gating strategy (**Figure 4.2C & E**). The overall polyfunctional profile of SARS-CoV-2-specific CD4⁺ T cells in seropositive (n=42) and seronegative (n=11) children was distinct ($p = 0.0098$, **Figure 4.2C**). No differences were detected in the proportions of SARS-CoV-2-specific CD4⁺ T cells producing different combinations of cytokines between seropositive and seronegative children and the response was distributed between triple (median: 18% vs 19% for seropositive and seronegative children), dual (24% vs 18% for seropositive and seronegative

children) and monofunctional cells (27% vs 28% for seropositive and seronegative children). It should, however, be noted that seropositive children had a 1.85-fold higher proportion of CD4⁺ T cells co-producing IL-2 and TNF- α compared to seronegative children (median: 24% for seropositive vs 13% for seronegative, respectively, $p=0.1388$, **Figure 4.2C**). The polyfunctional profile of SARS-CoV-2-specific CD8⁺ T cells showed no significant differences between seropositive and seronegative children (**Figure 4.2E**). In contrast to CD4 responses, the dominant CD8⁺ T cells were monofunctional TNF- α producing cells in both seropositive and seronegative children (median: 51% and 56%, respectively). However, SARS-CoV-2-responding CD8⁺ T cells with a dual functional profile co-expressing IFN- γ and TNF- α were 2.6-fold higher in seropositive children compared to seronegative children (median: 29% vs 11%, respectively, $p=0.2803$, **Figure 4.2E**). These data confirm our previous findings in Chapter 3, even though no statistically significant differences were reported in the proportion of triple, dual, or monofunctional expressing cells in this chapter. In both cohorts we showed that the magnitude of SARS-CoV-2-specific T cells were comparable between SARS-CoV-2 seropositive and seronegative children, but CD4⁺ T cell response displayed a distinctive functional profile of SARS-CoV-2-responding cells. The polyfunctional CD8 profile in both seropositive and seronegative children, were predominantly monofunctional TNF- α producing cells, which is somewhat different from our findings reported in Chapter 3.

Next, we evaluated the memory phenotypic profile of SARS-CoV-2-specific T cells in SARS-CoV-2 seropositive ($n=40$) and seronegative ($n=8$) children with detectable T cell responses (with a minimum of 20 cytokine events), to define the memory phenotype of cytokine-producing cells. Based on the expression of CD27 and CD45RA markers, four memory subsets were identified, namely naïve-like (CD45RA⁺CD27⁺), early differentiated (ED; CD45RA⁺CD27⁺), late differentiated (LD; CD45RA⁺CD27⁺) and effector (Eff; CD45RA⁺CD27⁺) T cells (**Figure 4.2F&H**). Although no significant differences were observed between SARS-CoV-2 seropositive and seronegative children for any of the memory subsets, seronegative children had a 2.7-fold higher proportion of SARS-CoV-2-responding naïve-like CD4⁺ T cells in comparison to seropositive children (median: 35% vs 13.20%, respectively, $p=0.1553$, **Figure 4.2G**). Over 60% of SARS-CoV-2-responding CD4⁺

T cells displayed an ED memory phenotype in SARS-CoV-2 seropositive children and 49.7% in seronegative counterparts, representing the dominant phenotype of both groups (**Figure 4.2G**). LD memory profiles of SARS-CoV-2-specific CD4⁺ T cells were comparable in both groups (median: 15.77% for seropositive and 10.56% for seronegative children respectively, **Figure 4.2G**). Memory phenotype of SARS-CoV-2-specific CD8⁺ T cells was also assessed (**Figure 4.2H**). Similar to CD4 T cell responses and possibly due to the study being underpowered (n=12 and n=6 for seropositive and seronegative children respectively), no significant differences were detected between the different memory subsets in seropositive compared to seronegative children (**Figure 4.2I**). However, we found an 8.4-fold higher proportion of ED CD8⁺ T cells in seropositive children (46.01%) compared to seronegative children (5.45%) and a 2.3-fold lower proportion of effector T cells in seropositive children (23.78%) compared to seronegative children (54.55%) (**Figure 4.2I**). These data show that in seropositive children, SARS-CoV-2-responding CD4 and CD8 T cells predominantly display an ED memory profile while seronegative children have ED dominance in CD4 T cells but a predominant Eff memory CD8 profile.



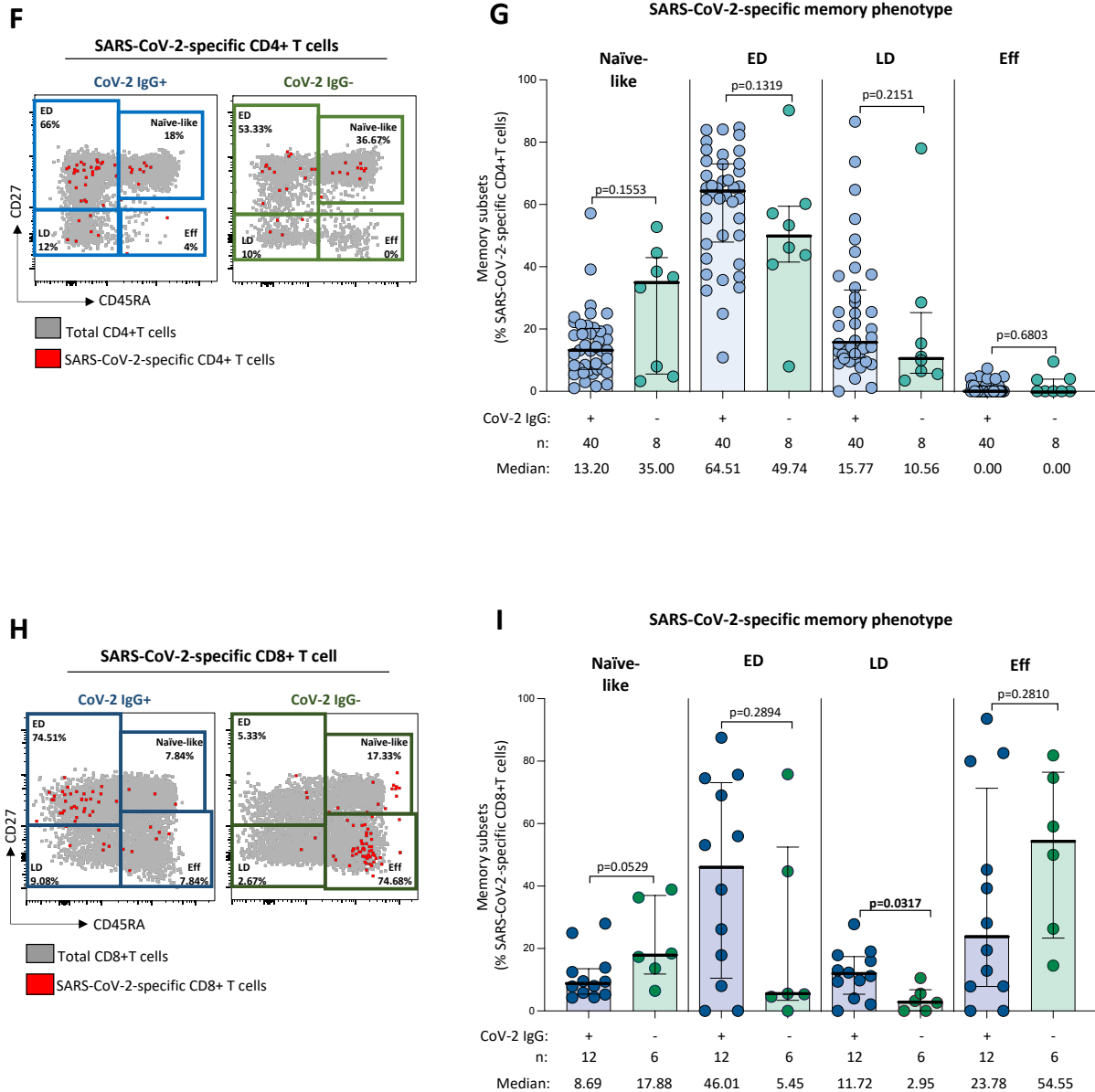


Figure 4.2: Magnitude, polyfunctional, and memory profiles of SARS-CoV-2-specific T cells in SARS-CoV-2 seropositive and seronegative children at BL.

(A) Representative examples of flow cytometry plots of IFN- γ , TNF- α and IL-2 production in CD4+ and CD8+ T cells in response to SARS-CoV-2 peptide stimulation in seropositive (CoV-2 IgG+) and seronegative (CoV-2 IgG-) children. NS: no stimulation; SNM: combined peptide pools of SARS-CoV-2-spike, nucleocapsid and membrane proteins. (B) Frequency of SARS-CoV-2-specific CD4+ T cells producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. SARS-CoV-2-spike serology was used to classify children into seropositive (blue: n=62) and seronegative (green: n=40) groups. Bars represent median and median values are indicated below the graph. The proportion of participants with detectable T cell responses to SARS-CoV-2 are presented as pie charts and percentage of responders are indicated. (C) The polyfunctional profile of SARS-CoV-2-specific CD4+ T cells in SARS-CoV-2

seropositive (n=42) and seronegative (n=11) children. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 response. **(D)** Frequency of SARS-CoV-2-specific CD8+ T cells producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. SARS-CoV-2 seropositive (blue: n=62) and seronegative (green: n=40) groups. Bars represent median and median values are indicated. The proportion of participants with detectable T cell responses to SARS-CoV-2 are presented as pie charts and percentage of responders are indicated. **(E)** The polyfunctional profile of SARS-CoV-2-specific CD8+ T cells in SARS-CoV-2 seropositive (n=17) and seronegative (n=7) children. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. **(F)** Representative flow plots of the memory differentiation profile in SARS-CoV-2-specific CD4+ T cells based on the expression of CD27 and CD45RA in seropositive and seronegative children. The overlay flow plot depicts SARS-CoV-2-specific CD4+ T cells presented as red dots and grey dots represent total CD4+ T cells. The memory subsets are presented as naïve-like (CD45RA+CD27+), early differentiated (ED:CD45RA-CD27+), late differentiated (LD:CD45RA-CD27-) and effector (Eff:CD45RA+CD27-) cells. **(G)** The proportion of each memory subsets in SARS-CoV-2-specific CD4+ T cells in SARS-CoV-2 seropositive (n=40) and seronegative (n=8) children with detectable responses. The median, IQR are shown, and median values are indicated. **(H)** Representative flow plot examples of the memory differentiation profile in SARS-CoV-2-specific CD8+ T cells in seropositive and seronegative children. **(I)** The proportion of each memory subsets in SARS-CoV-2-specific CD8+ T cells in SARS-CoV-2 seropositive (n=12) and seronegative (n=6) children with detectable responses. The median, IQR are shown, and median values are indicated. Statistical comparisons in **(B)** and **(D)** was performed using Mann-Whitney test between seropositive and seronegative children and the Chi-square test compare the percentage of responders. In figures **(C)** and **(E)** statistical analysis were performed using the SPICE software with the permutation test for the pie charts and the Wilcoxon unpaired t test to compare response patterns between seropositive and seronegative children. Statistical comparisons in **(G)** and **(I)** were performed using Mann-Whitney test between SARS-CoV-2 seropositive and seronegative children; *p values* <0.05 were considered statistically significant and are bolded.

4.3.3 Children exhibit lower T cell responses, but similar polyfunctional profiles of SARS-CoV-2-specific T cells compared to mothers at BL visit.

A limiting factor highlighted by the reviewers for the publication under Chapter 3 of this thesis was that the reported differences in SARS-CoV-2-specific responses between children and adults could be due to different timing of infection in each group. The date of infection was confirmed by PCR in adults (HCW) but no PCR data was available for the RC child cohort. To determine whether time of infection influences T cell responses and profiles, we compared T cell responses in shared household contacts, assuming a similar time of SARS-CoV-2 exposure in matched children and mother pairs (n=73). The children and mother pairs were grouped according to SARS-

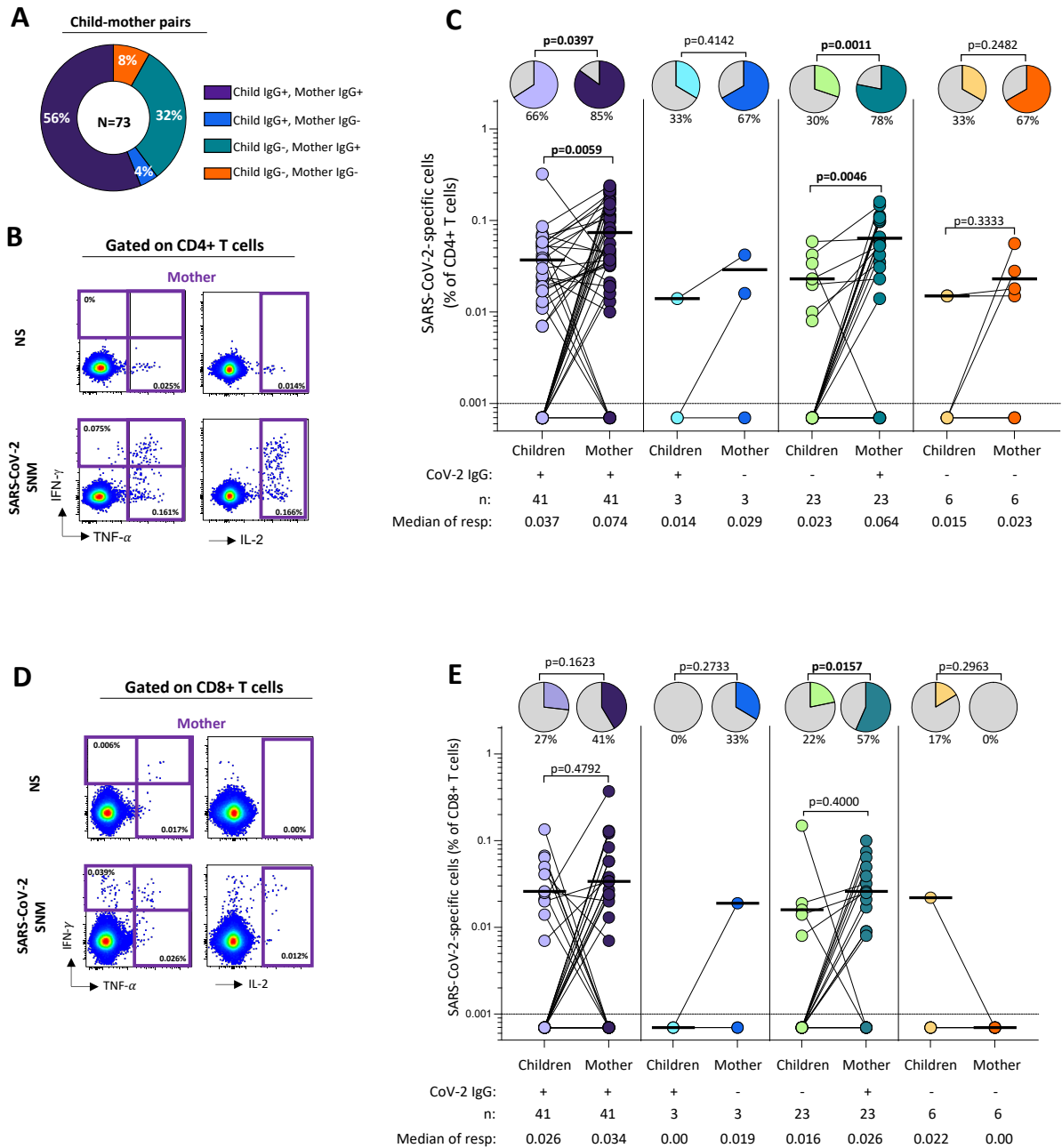
CoV-2-S serostatus. A proportion of 56% (41/73) were concordant with both children and mothers SARS-CoV-2 seropositive, while 8% (6/73) of matched pairs were SARS-CoV-2 seronegative which were likely unexposed to SARS-CoV-2 (**Figure 4.3A**). The remaining 26 matched pairs were discordant and further subdivided into 32% (23/73) being seronegative children with seropositive mothers and 4% (3/73) of seropositive children with seronegative mothers (**Figure 4.3A**). It is interesting to note that 23/64 (36%) of children were seronegative despite living in a household with a seropositive mother.

Firstly, we compared the magnitude of SARS-CoV-2-specific CD4⁺ T cells in matched child and mother pairs (**Figure 4.3B**). In the seropositive child-mother matched pairs (n=41 pairs), we found a significantly lower proportion of SARS-CoV-2 CD4 responders in children compared to mothers (66% vs 85% respectively) (p=0.0397; pie charts, **Figure 4.3C**). Likewise, the frequencies of SARS-CoV-2-specific CD4⁺ T cells were significantly lower in seropositive children in comparison to the seropositive mothers (median of responders: 0.037% and 0.074%, respectively, p=0.0059, **Figure 4.3C**). A similar trend was observed between seronegative children (n=23) and seropositive mothers (n=23), such that a lower proportion of seronegative children had SARS-CoV-2-specific CD4⁺ T cells compared to seropositive mothers (30% vs 78%, p=0.0011) which were of lower magnitude (median of responders: 0.023% vs 0.064%, respectively, p=0.0046, **Figure 4.3C**). Within groups of seropositive (n=3, light blue) and seronegative children (n=6, light orange) with seronegative (n=3; dark blue and n=6; dark orange) mothers, no significant differences were found in either the proportion of CD4 responders or the frequency of SARS-CoV-2-specific CD4⁺ T cells (**Figure 4.3C**) likely due to these groups being underpowered. In the CD8⁺ compartment (**Figure 4.3D**), we found similar proportions of 27% (11/41) of seropositive children and 41% (17/41) of seropositive mothers had comparable frequencies of SARS-CoV-2-specific CD8⁺ T cells (median of responders: 0.026% vs 0.034% for children and mothers, respectively, **Figure 4.3E**). We found significantly lower proportions of CD8 responders in seronegative children (22%) compared to seropositive mothers (57%) (p=0.0157, **Figure 4.3E**). The frequency of SARS-CoV-2-specific CD8⁺ T cell responses were also lower in seronegative children compared to seropositive mothers (median of responders: 0.016% vs 0.026%, respectively, p=0.4000, **Figure 4.3E**).

We next compared the polyfunctional profiles of SARS-CoV-2-responding CD4 cells (**Figure 4.3.F-G**) in the seropositive child/seropositive mother group (n=27 vs n=35 respectively) (**Figure 4.3F**) and seronegative child/seropositive mother group (n=7 vs n=18 respectively) (**Figure 4.3G**) with detectable CD4+ T cell responses. This analysis was done unpaired on all participants with a T cell response to increase the statistical power. The child-mother pairs which consisted of seronegative mother with seropositive children (n=3) and both seronegative mothers and children (n=6) (**Figure 4.3A**) were excluded in the analysis due to the small sample size of responders. The overall functional profile showed no significant differences between seropositive children and seropositive mothers (**Figure 4.3F** pie charts). Despite this, SARS-CoV-2-responding CD4+ cells exhibited a polyfunctional profile enriched with cells co-expressing of all three cytokines (IFN- γ +TNF- α +IL-2+) in seropositive mothers compared to seropositive children (p=0.0332, **Figure 4.3F**). Whereas seropositive children had a 2.5-fold higher proportion of SARS-CoV-2-specific CD4+ T cells producing TNF- α only compared to seropositive mothers (median: 10% vs 4%, respectively, **Figure 4.3F**). We were particularly interested in analysing the seronegative children sharing a household with a seropositive mother since they are likely to have been exposed. There were no significant differences in the overall functional profile between seronegative children (n=7) and seropositive mothers (n=18) (**Figure 4.3G**), although a trend (p=0.053) was observed, whereby seropositive mothers (n=18) had 2.5-fold increase in the proportion of CD4+ T cells producing all three cytokines (IFN- γ +TNF- α +IL-2+) compared to seronegative children (median: 28% vs 11% respectively, **Figure 4.3G**).

Despite the lower magnitude of CD8+ responses and subsequently CD8 responders, we compared the polyfunctional profiles between children and mothers (**Figure 4.3H-I**). We performed this analysis for all participants with detectable CD8 responses, in both the seropositive children and mothers' group and for the seronegative children and seropositive mothers' group. No significant differences were detected in the overall polyfunctional profile of SARS-CoV-2-responding CD8 cells in seropositive (n=11) children compared to seropositive mothers (n=18), (**Figure 4.3H**). Meanwhile in seronegative children (n=5) and seropositive mothers (n=13), the overall

polyfunctional profile of SARS-CoV-2-responding CD8 cells was distinctive between groups ($p=0.0389$, **Figure 4.3I**). Seronegative children had 3.9-fold higher proportion of SARS-CoV-2-responding CD8+ cells producing TNF- α only, compared to seropositive mothers (median: 55% vs 14% respectively, $p=0.055$, **Figure 4.3I**). Overall, we confirm our previous findings with an entirely separate cohort, that the lower SARS-CoV-2-specific T cell responses observed in children, were not influenced by differences in the timing of SARS-CoV-2 infection, as this cohort consists of matched mother-child pairs.



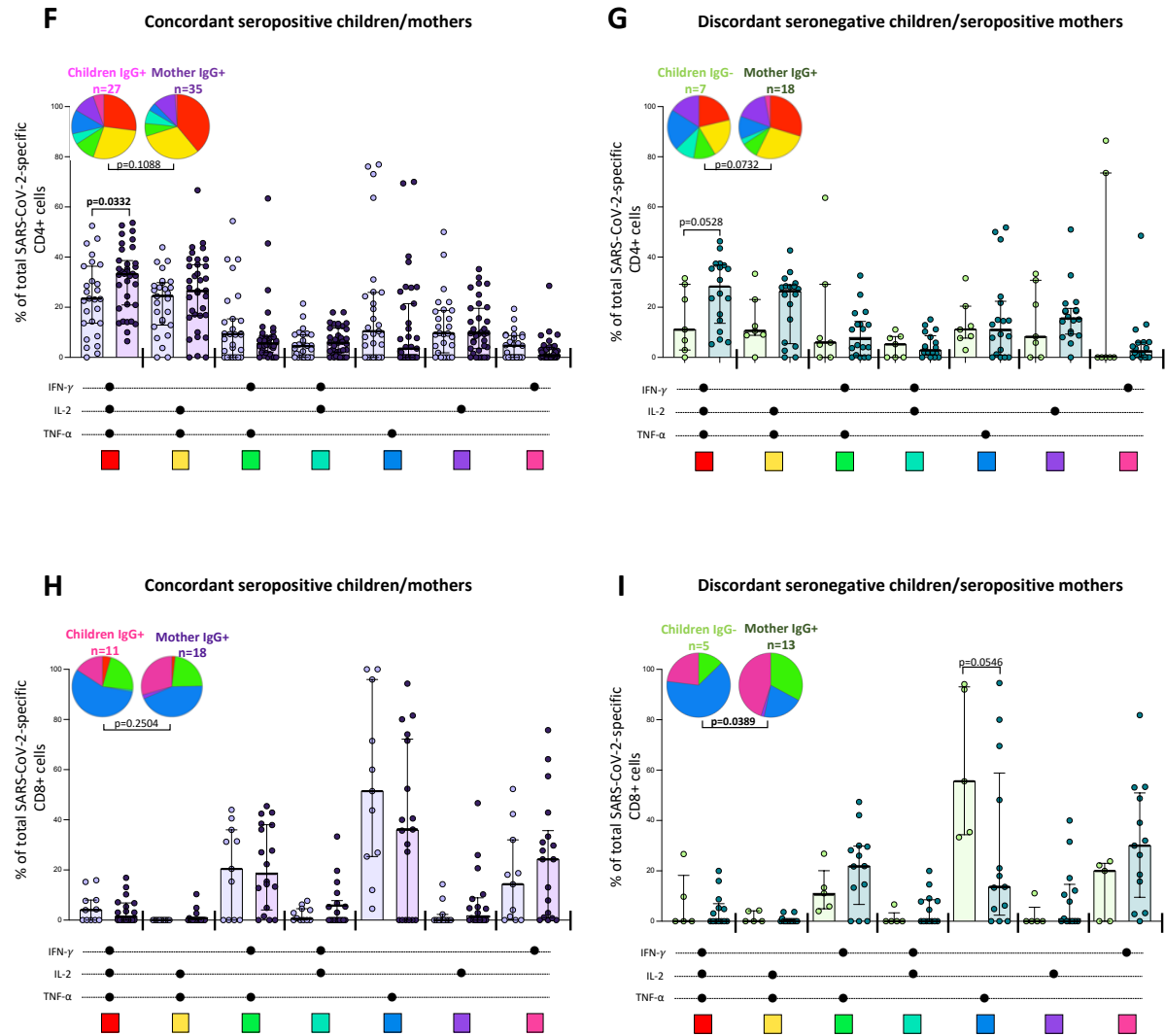


Figure 4.3: T cell responses and polyfunctional prolife of SARS-CoV-2-specific T cells in children compared to mothers at BL.

(A) The proportion of child-mother pairs grouped according to SARS-CoV-2 seroprevalence. **(B)** Representative flow cytometry plots of IFN- γ , TNF- α and IL-2 production by CD4+ T cells of an adult participant. NS: no stimulation; SNM: combined peptide pools of SARS-CoV-2-spike, nucleocapsid and membrane proteins. **(C)** Frequency of SARS-CoV-2-specific CD4+ T cells producing any of the measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. Children and mother pairs (n=73) were grouped based on SARS-CoV-2-spike serology. Bars represent median and median values are indicated below the graph. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders in each group are indicated. The graph shows seropositive matched children (n=41; light purple) and mothers (n=41; dark purple) pairs, seropositive children (n=3; light blue) and seronegative mother (n=3; dark blue) pairs, seronegative children (n=23; light green) and seropositive mother (n=23; dark green) pairs and lastly seronegative matched children (n=6;

light orange) and mother (n=6; dark orange) pairs. **(D)** Representative flow cytometry plots of IFN- γ , TNF- α and IL-2 production in CD8+ T cells of an adult participant. NS: no stimulation; SNM: combined peptide pools of SARS-CoV-2-spike, nucleocapsid and membrane proteins. **(E)** Frequency of SARS-CoV-2-specific CD8+ T cells producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. Children and mother pairs (n=73) were grouped based on SARS-CoV-2-spike serology. Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders in each group are indicated. **(F)** The polyfunctional profile of SARS-CoV-2-specific CD4+ T cells in all participants with detectable T cell responses; in concordant seropositive children (n=27, light purple) and mother (n=35, dark purple) and **(G)** discordant seronegative children (n=7, light green) and seropositive mothers (n=18, dark green). **(H)** The polyfunctional profile of SARS-CoV-2-specific CD8+ T cells in all participants with detectable T cell responses; concordant seropositive children (n=11, light purple) and mother (n=18, dark purple) and **(I)** discordant seronegative children (n=5, light green) and seropositive mothers (n=13, dark green). The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. Statistical comparisons in **(C)** and **(E)** were performed using the Mann-Whitney test between children and mothers and the Chi-square test compare the percentage of responders. Statistical comparisons in **(F) - (I)** were performed using the SPICE software with the permutation test for pie charts and the Wilcoxon unpaired t test to compare response patterns between seropositive children and mother pairs; *p* values <0.05 were considered statistically significant and are bolded.

4.3.4 SARS-CoV-2-specific CD4+ cells exhibit predominantly early differentiated memory profile in both children and mothers at BL visit.

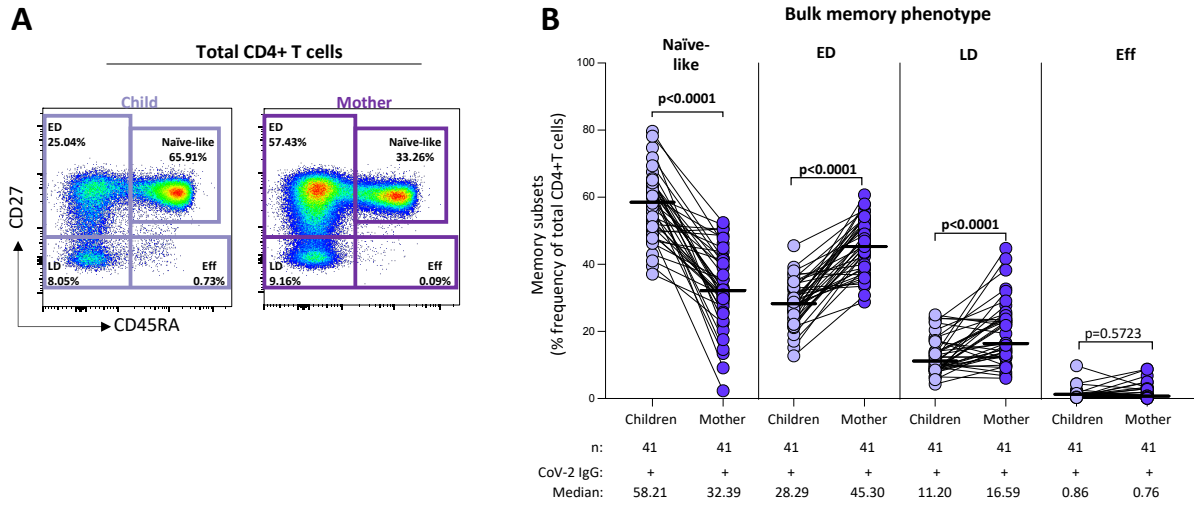
An alternative hypothesis is that the differences in magnitude of responses between children and adults may be due to age-related differences in maturation of the adaptive immune system. We therefore performed phenotypic characterization of SARS-CoV-2-specific T cells in seropositive children and seropositive mothers and seronegative children and seropositive mothers with detectable T cell responses, by the measurement of memory differentiation markers CD27 and CD45RA. To phenotype the cytokine-producing cells we applied a cutoff of 20 events. Firstly, we looked at the total CD4+ memory subsets in children and compared to mothers (**Figure 4.4A-D**). Unsurprisingly children, despite the SARS-CoV-2 serostatus, had significantly increased naïve-like memory subsets compared to seropositive mothers ($p \leq 0.0001$, **Figure 4.4A-D**). Meanwhile the early differentiated (ED) and late differentiated (LD) memory subsets were significantly increased in mothers compared to children ($p \leq 0.0001$, **Figure 4.4B & D**). Lastly the effector (Eff) memory

subsets were comparable between children and mothers (**Figure 4.4B&D**). The dominant subset in SARS-CoV-2-responding CD4 cells (**Figure 4.4E-H**), in both mothers and children was ED memory cells although this was still significantly lower in seropositive children (median: 75.47% and 65.79% for seropositive mother and children, respectively, $p=0.0231$, **Figure 4.4F**). In contrast, seropositive children ($n=25$) had a significantly increased proportion of naïve-like memory subsets in comparison to seropositive mothers ($n=34$) (median: 13.16% for seropositive children vs 6.11% for seropositive mothers, $p=0.0041$, **Figure 4.4F**). The LD memory subsets were comparable between seropositive children and seropositive mothers (median: 15.25% vs 14.81%, respectively), and no Eff memory cells were detected (**Figure 4.4F**).

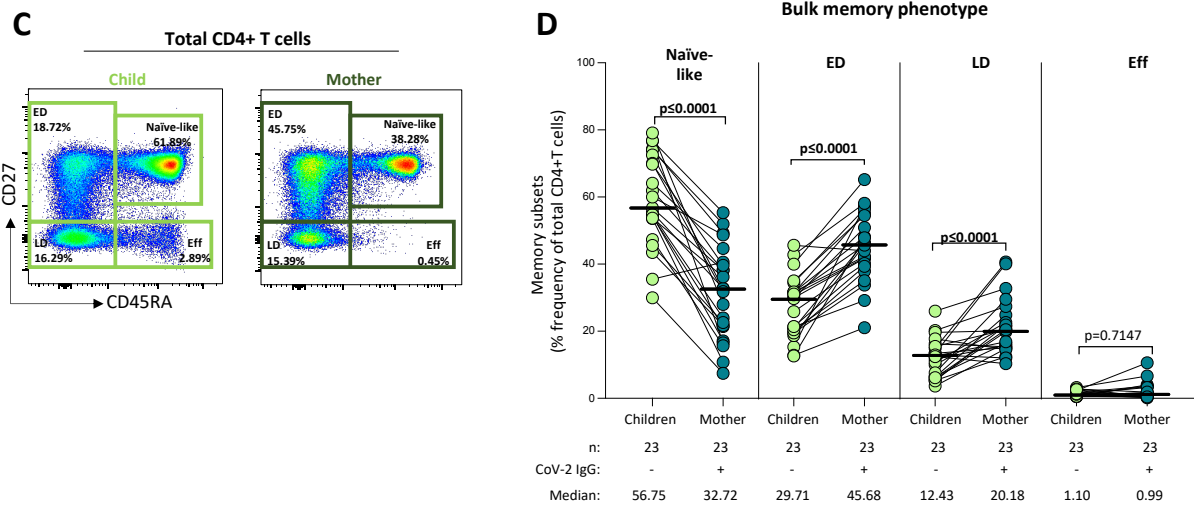
We also assessed the memory differentiation profile between seronegative children ($n=6$) and seropositive mother ($n=18$) CD4 T cell responders (**Figure 4.4G**). A similar pattern of memory profile distribution was observed with the ED subset being the dominant subset. We observed that seropositive mothers had increased proportions of ED memory cells compared to seronegative children (median: 78.88% vs 48.54%, respectively, $p=0.0044$, **Figure 4.4H**), while in seronegative children ($n=6$), we found a significantly increased proportion of naïve-like memory subsets compared to seropositive mothers (median: 35% vs 6.69%, respectively, $p=0.0177$, **Figure 4.4H**). The total memory subsets on bulk CD8+ T cells (**Figure 4.4I-L**), was distributed similarly to total CD4+ memory subsets, with seropositive ($n=41$) children having significantly higher proportions of naïve-like memory CD8+ cells (median: 47.67% vs 32.30%, respectively, $p<0.0001$, **Figure 4.4J**), with reduced ED (median: 13.65% vs 18.31% respectively, $p=0.0284$) and LD (median: 8.47% vs 15.89%, respectively, $p=0.0002$) but similar eff (median: 21.18% vs 25.48% respectively) compared to seropositive mothers ($n=41$) pairs (**Figure 4.4J**). Similarly, the CD8 memory profile in seronegative children ($n=23$) compared to seropositive mothers ($n=23$) exhibited significantly higher naïve-like (median: 47.69% vs 38.28%, respectively, $p=0.0101$, **Figure 4.4L**) and reduced ED (median: 11.10% vs 18.03%, respectively, $p=0.0010$) memory cells. LD (median: 9.93% vs 18.85% for children and mothers, respectively) and eff (median: 21.59% vs 23.98% for children and mothers, respectively) memory profiles were comparable in both seronegative children and seropositive mothers ($n=23$; paired) (**Figure 4.4L**). The phenotypic analysis of SARS-CoV-2-specific

CD8+ T cells was not performed due to the low number of events to characterize the memory profiles. Overall, these data, show that SARS-CoV-2-specific CD4+ T cells exhibited predominantly early differentiated memory phenotypes in both children and mothers.

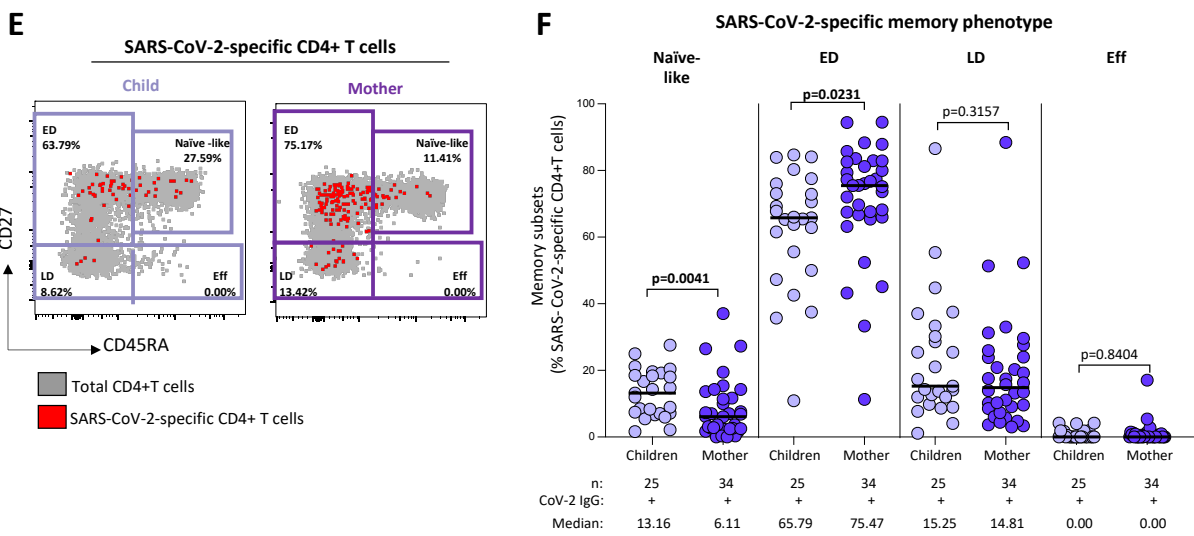
Concordant seropositive children/mothers



Discordant seronegative children/seropositive mothers

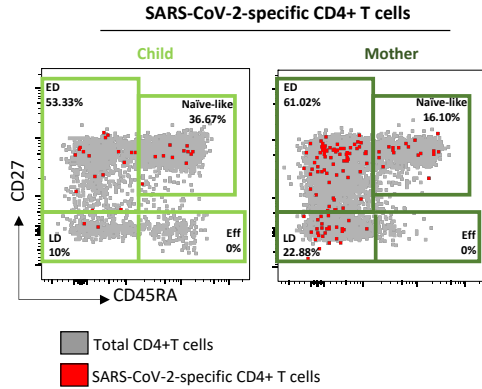


Concordant seropositive children/mothers

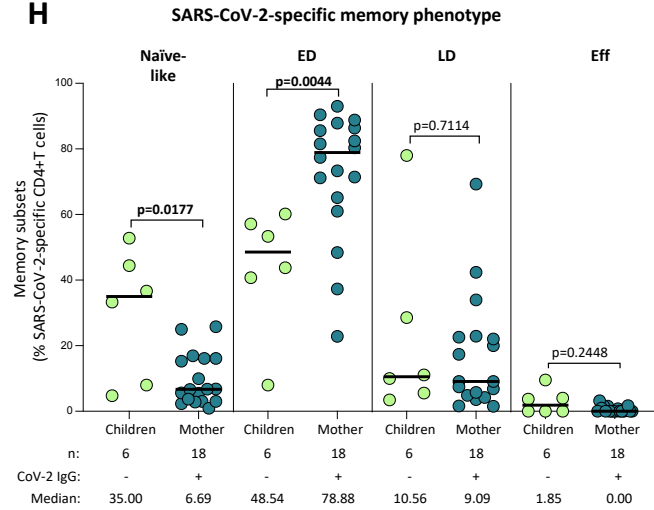


Discordant seronegative children/seropositive mothers

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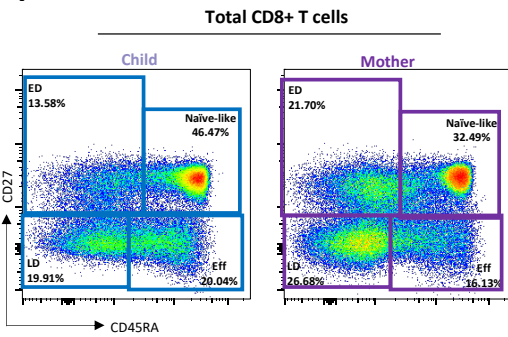


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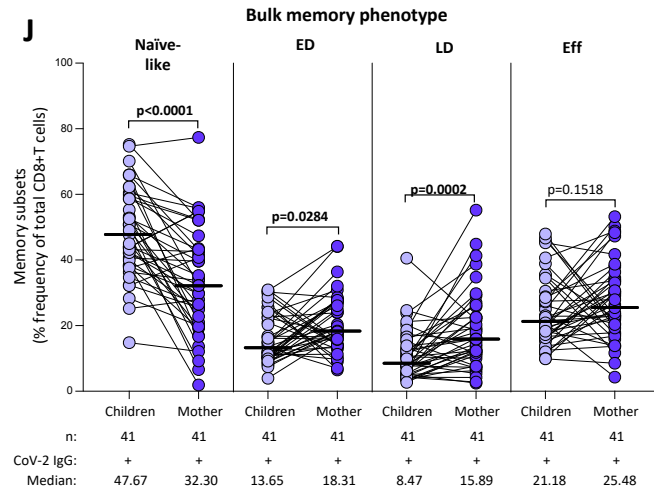


Concordant seropositive children/mothers

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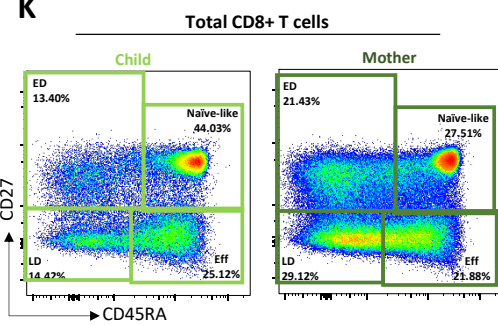


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Discordant seronegative children/ seropositive mothers

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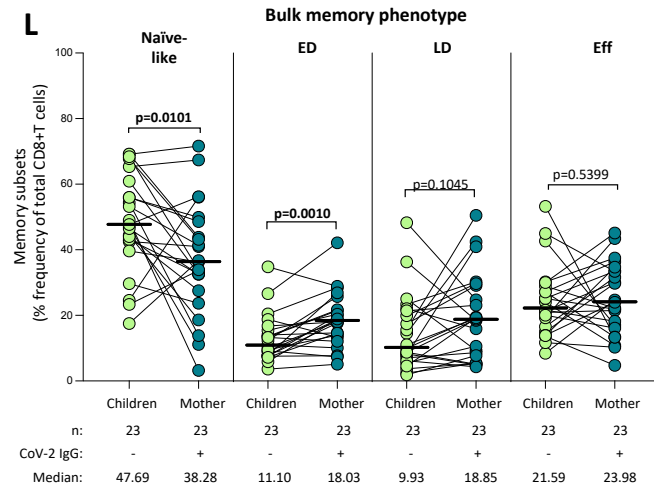


Figure 4.4: Memory differentiation profile of SARS-CoV-2-specific T cells in children and mothers at BL.

(A) Representative flow plots of the memory differentiation profile in total CD4⁺ T cells of concordant seropositive child and seropositive mothers. Based on expression of CD27 and CD45RA, the memory phenotypic subsets are subdivided into naïve-like (CD45RA⁺CD27⁺), early differentiated (ED;CD45RA⁺CD27⁺), late differentiated (LD;CD45RA⁺CD27⁺) and effector (Eff;CD45RA⁺CD27⁺) cells. **(B)** The frequency of total bulk CD4⁺ naïve-like, ED, LD and Eff memory T cells in SARS-CoV-2 seropositive children and mother (n=41, pairs). Bars represent median and median values are indicated. **(C)** Representative flow plots of the memory differentiation profile in total CD4⁺ T cells of discordant seronegative child and seropositive mother. **(D)** The frequency of total bulk CD4⁺ naïve-like, ED, LD and Eff memory T cells in SARS-CoV-2 seronegative children and seropositive mother (n=23, pairs). Bars represent median and median values are indicated. **(E)** Representative overlay flow plots showing memory differentiation profiles of SARS-CoV-2-specific CD4⁺ T cells in concordant seropositive child and seropositive mother; red dots illustrate SARS-CoV-2-specific CD4⁺ T cells and grey dots represent total CD4⁺ T cells. **(F)** The proportion of each memory subsets in SARS-CoV-2-specific CD4⁺ T cells in SARS-CoV-2 seropositive children (25) and mother (n=34) with detectable responses. Bars represent median and median values are indicated. **(G)** Representative overlay flow plot of memory differentiation profile of SARS-CoV-2-specific CD4⁺ T cells in discordant seronegative child and seropositive mother. **(H)** The proportion of the memory subsets in SARS-CoV-2-specific CD4⁺ T cells in seronegative children (n=6) and seropositive mother (n=18) with detectable responses. Bars represent median and median values are indicated. **(I)** Representative flow plots of the memory differentiation profile in total CD8⁺ T cells of concordant seropositive child and seropositive mother. **(J)** The frequency of total CD8⁺ T cells of each memory subsets in seropositive children (n=41) and mother (n=41) pairs. Bars represent medians and median values are indicated. **(K)** Representative flow plots of the memory differentiation profile in total CD8⁺ T cells of discordant seronegative child and seropositive mother. **(L)** The frequency of total CD8⁺ T cells of each memory subsets in seronegative children (n=23) and mother (n=23) pairs. Bars represent medians and median values are indicated below the graph. Statistical comparisons in **B**, **D**, **J** and **L** were performed using Wilcoxon matched-pairs signed rank test between children-mother pairs. Statistical comparisons in **(F)** and **(H)** were performed using Mann-Whitney test between children and mothers; *p* values <0.05 were considered statistically significant and are bolded.

4.3.5 Maintenance and *de novo* SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive and seronegative children over a 16-month period (V2).

We measured the maintenance and *de novo* SARS-CoV-2-specific T cell responses in children by comparing SARS-CoV-2-specific T cell responses at BL which was the first visit to a second visit ≈16 months (V2) after BL visit (**Figure 4.1B**). Since children were initially grouped according to SARS-CoV-2 serostatus at BL, we were able to evaluate the maintenance of T cell responses in

children who were SARS-CoV-2 seropositive at BL (n=60) and *de novo* responses in those seronegative at BL (n=38) (**Figure 4.5**).

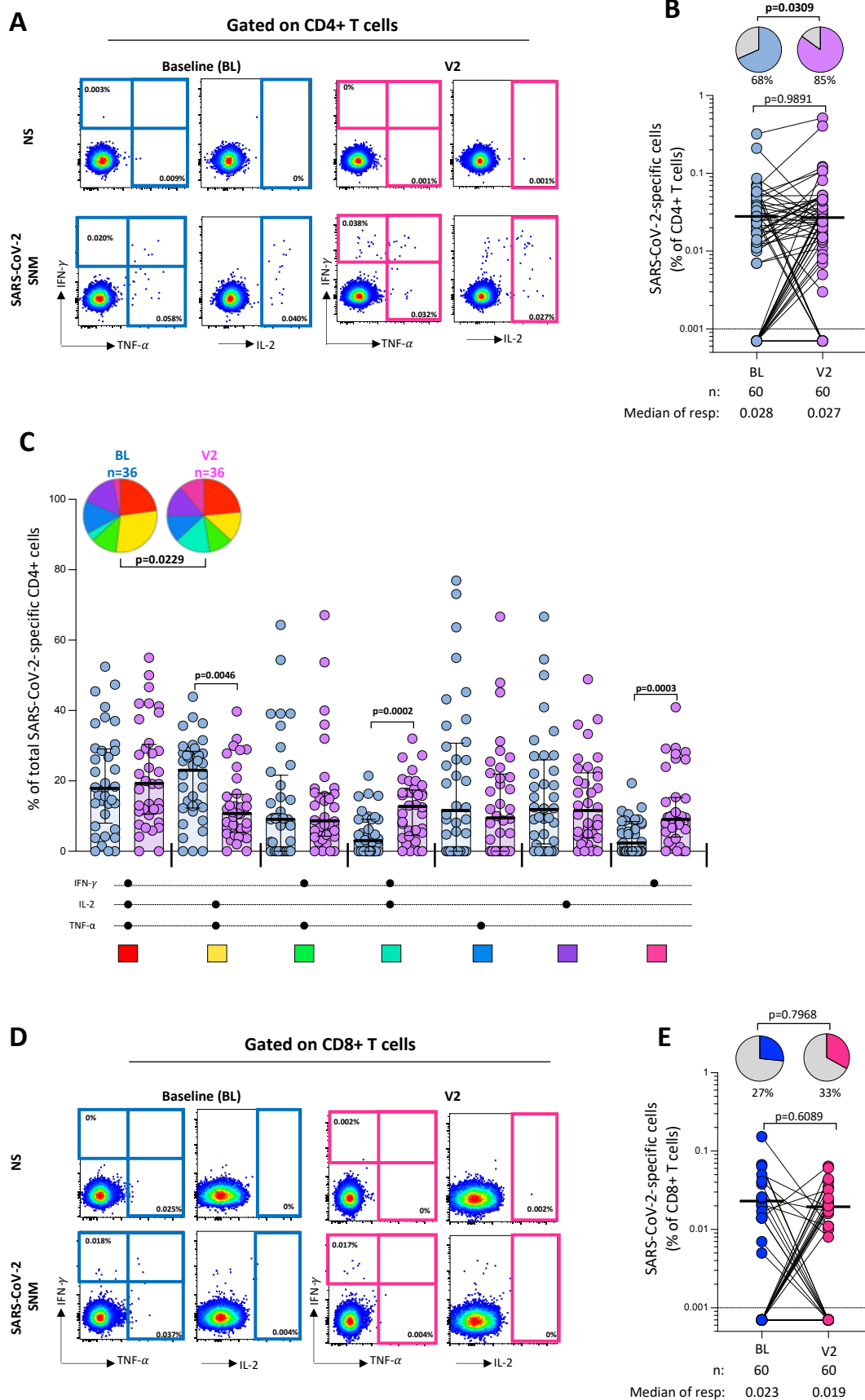
Representative plots of SARS-CoV-2-specific CD4+ and CD8+ T cells (producing IFN- γ , IL-2 or TNF- α) in SARS-CoV-2 seropositive children (n=60) at BL and V2, is illustrated in **Figure 4.5A & D**. SARS-CoV-2-specific CD4+ T cells at BL, were comparable to V2 (median of responders: 0.028% vs 0.027%, respectively) in children who were seropositive at BL (**Figure 4.5B**). Moreover, the proportion of CD4 responders significantly increased by V2 (median: 68% at BL vs 85% at V2, $p=0.0309$, **Figure 4.5B**) indicative of repeat or new exposures over the 5 infection waves in SA. The polyfunctional profile of SARS-CoV-2-specific CD4+ T cells was distinctive in BL vs V2 in seropositive children (n=36) with detectable response at both time points ($p=0.0229$, **Figure 4.5C**). Indeed, by V2 seropositive children (n=36), had a higher proportion of SARS-CoV-2-specific CD4+ cells co-expressing IFN- γ +IL-2+ (median: 3% for BL and 12% for V2, $p=0.0002$) and IFN- γ producing cells (median: 2%, for BL and 9% for V2, respectively, $p=0.0003$) compared to BL. In contrast, a decreased proportion of IL-2+TNF- α + producing cells was observed at V2 when compared to BL (median: 28% at BL vs 11% at V2; $p=0.0046$, **Figure 4.5C**). In the CD8 compartment, we found no significant differences in the proportion of responders or magnitude of SARS-CoV-2-specific CD8+ T cell responses between BL and V2 in children who were seropositive at BL (**Figure 4.5E**; n=60). The polyfunctional analysis of SARS-CoV-2-specific CD8+ T cells analysis was not performed due to low number of responders at both time-points.

Subsequently, we assessed if T cell responses in children who were seronegative at BL (n=38) in comparison to V2 (**Figure 4.5F-H**). We found a significant increase in the proportion of responders at V2 in comparison to BL (84% vs 26%, respectively, $p\leq 0.0001$, **Figure 4.5F**). We also found the frequency of SARS-CoV-2-specific CD4+ T cells was significantly higher at V2 in comparison to BL (median of responders: 0.035% vs 0.018%, respectively, $p=0.0199$) in seronegative children (**Figure 4.5F**). The polyfunctional analysis included paired samples from seronegative children with detectable responses at BL and V2 (n=10). We found no significant differences in the overall functional profile of CD4+ T cell responses (**Figure 4.5G**), although we found a significantly

increased proportion of SARS-CoV-2-specific CD4⁺ T cells co-expressing IFN- γ and IL-2, at V2 compared to BL (median: 8% vs 0% respectively, $p=0.0211$, **Figure 4.5G**). In contrast to CD4, we found no significant differences in the proportion of CD8 responders or the frequency of SARS-CoV-2-specific CD8⁺ T cells at BL and V2 (18% vs 21%, respectively, **Figure 4.5H**). The polyfunctional analysis for the CD8 compartment for seronegative children, was also not conducted due to limited number of responders at both BL and V2 timepoints.

The fold change in the frequency of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses at V2 relative to BL (**Figure 4.5I-J**) was calculated for seropositive ($n=60$) and seronegative ($n=38$) children. The fold change was determined by dividing the frequency of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells at V2 to the frequency of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells at BL. An increase of response was any value ≥ 1.2 and a gain of response was determined if there were undetectable responses at BL and detectable responses at V2. We found in seropositive children ($n=60$), approximately 60% gained and/or had increased CD4 responses, 26% had decreased or lost responses, lastly 7% had unchanged SARS-CoV-2-specific CD4⁺ T cell responses over 16-month period between BL and V2, and 7% had undetectable responses at both time points (**Figure 4.5I**). While the majority of seronegative children ($\approx 76\%$) gained and/or had increased responses, 5% remained unchanged, 3% lost responses over the 16-month period between BL and V2 and 16% had undetectable responses at both visits (**Figure 4.5I**). Fold change of SARS-CoV-2-specific CD8⁺ T cell responses over time illustrated in **Figure 4.5J**, shows that approximately 26% of seropositive children gained (23%) and/or had increased (3%) responses, while 8% had unchanged responses, 15% lost responses over the 16-month period and 50% remained undetectable at BL and V2 (**Figure 4.5J**). A small proportion (16%) of seronegative children gained (13%) or had increased (3%) CD8 responses, while responses remained undetectable in the majority (68%) of seronegative children at both time points and 10% lost responses over time (**Figure 4.5J**). Despite the lower magnitude of responses reported in children, these data show that T cell responses are maintained for over 16 months in the context of an ongoing pandemic, and more than 60% of children had an increase or gained CD4⁺ T cell responses over the 16-

month period, whether they were seropositive or seronegative at BL. This suggests that new or repeated exposures to SARS-CoV-2 occurred during 3 infection waves since the initial visit.



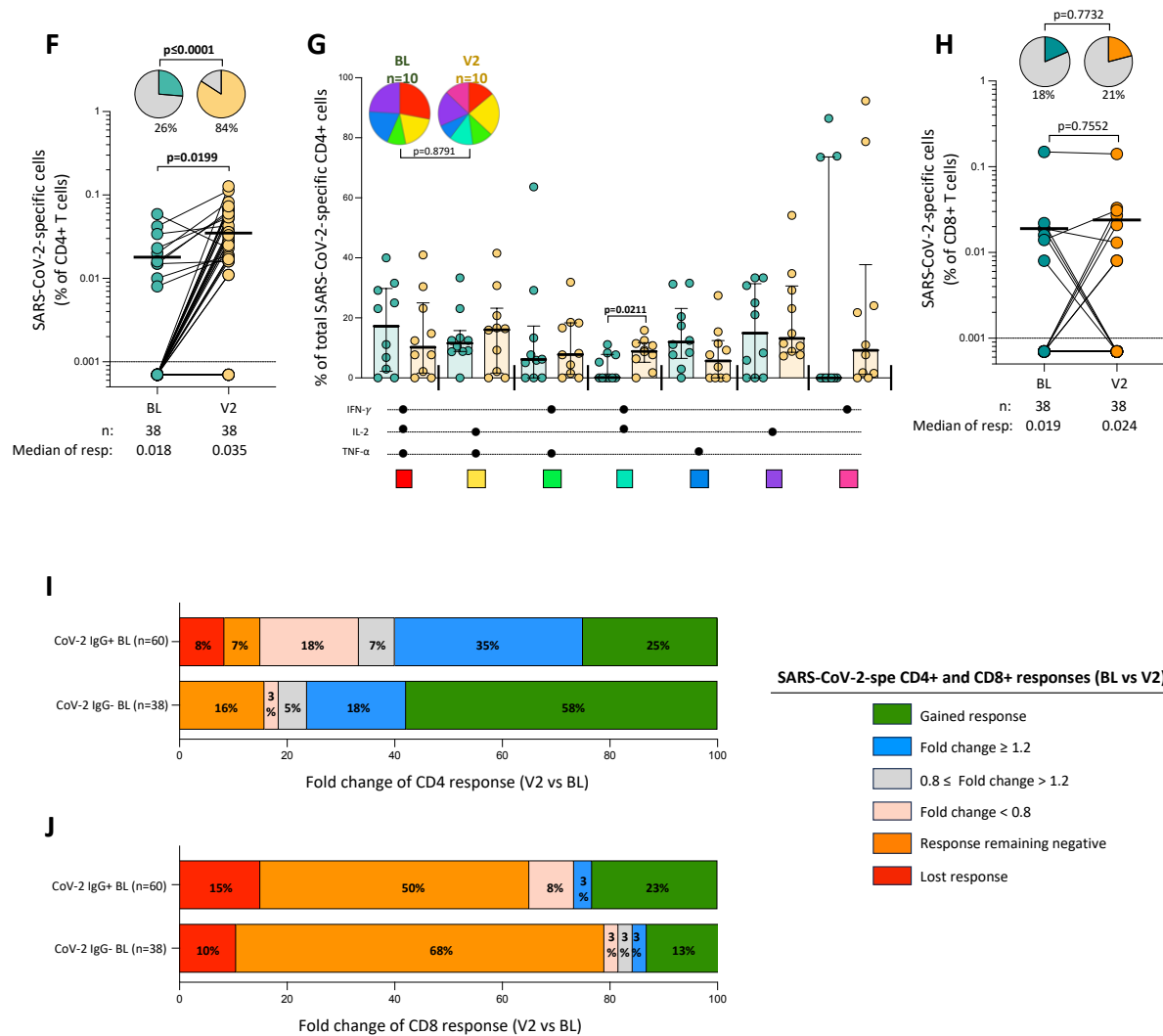


Figure 4.5: Maintenance and *de novo* SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive (blue and pink) and seronegative (green and yellow) children over a 16-month period (V2).

(A) Representative flow cytometry plots of IFN- γ , TNF- α and IL-2 production in CD4+ T cells at baseline (BL) and V2 in a child participant. NS: no stimulation; SNM: combined peptide pools of SARS-CoV-2-spike, nucleocapsid and membrane proteins. **(B)** Frequency of SARS-CoV-2-specific CD4+ T cells producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. SARS-CoV-2 seropositive children at baseline (BL; light blue, n=60) and visit 2 (V2; light purple; n=60). Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders are indicated. **(C)** The polyfunctional profile of SARS-CoV-2-specific CD4+ T cells in seropositive children (n=36) with detectable T cell responses at both BL and V2 time points. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. **(D)** Representative flow cytometry plots of IFN- γ , TNF- α and IL-2 production in CD8+ T cells at baseline (BL) and V2 in a child participant. **(E)** Frequency of SARS-CoV-2-specific CD8+ T cells

producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. SARS-CoV-2 seropositive children at baseline (BL; dark blue, n=60) and visit 2 (V2; dark purple; n=60) are shown. Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders in each group are indicated. **(F)** Frequency of SARS-CoV-2-specific CD4+ T cells producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in SARS-CoV-2 seronegative children at baseline (BL; green n=38) and V2 (yellow; n=38). Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders in each group are indicated. **(G)** The polyfunctional profile of SARS-CoV-2-specific CD4+ T cells in seronegative children at BL and V2 (n=10) with detectable T cell responses at both time points, BL and V2. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. **(H)** The frequency of SARS-CoV-2-specific CD8+ T cells producing any of measured cytokines (IFN- γ , TNF- α or IL-2) in response to SARS-CoV-2 SNM peptide pools. SARS-CoV-2 seronegative children at baseline (BL; dark green n=38) and V2 (dark yellow; n=38). Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders are indicated. **(I)** The fold change in the frequency of SARS-CoV-2-specific CD4+ and **(J)** CD8+ T cell responses at V2 relative to baseline (BL) in seropositive (n=60) and seronegative (n=38) children. Statistical comparisons in **B**, **D**, **F** and **H** were performed using Mann-Whitney test and the Chi-square test compare the percentage of responders. Statistical analysis of **C** and **G** were performed using the SPICE software with the permutation test for pie charts and the Wilcoxon unpaired t test to compare response patterns between BL and V2; *p values* <0.05 were considered statistically significant and are bolded.

4.3.6 Dominant early differentiated memory profile in SARS-CoV-2-specific CD4+ T cells after 16 months (V2).

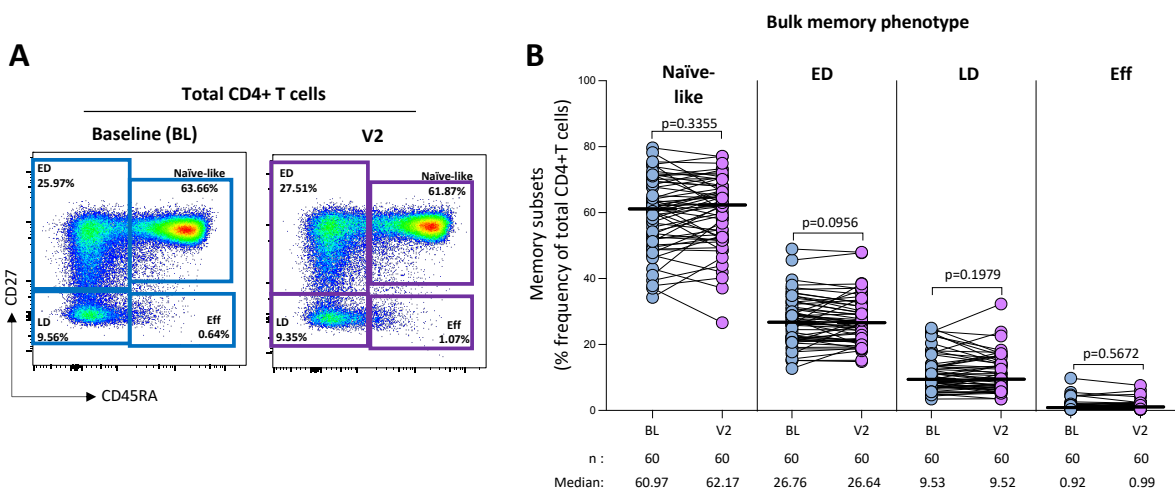
We next performed phenotypic characterization of SARS-CoV-2-specific CD4+ T cells at BL and V2 in children who were seropositive or seronegative at BL but had detectable CD4+ T cell responses at both timepoints (BL and V2), with a minimum of 20 cytokine events as a cut-off (**Figure 4.6**). The memory phenotypic profile of SARS-CoV-2-specific CD4+ T cell responses was identified based on the expression of CD27 and CD45RA to identify four memory subsets, namely naïve-like (CD45RA+CD27+), early differentiated (ED:CD45RA-CD27+), late differentiated (LD:CD45RA-CD27-) and effector (Eff:CD45RA+CD27-) memory subsets (**Figure 4.6I & K**).

The total bulk CD4+ T cell memory subsets in seropositive (n=60, **Figure 4.6A-B**) and seronegative (n=38, **Figure 4.6C-D**) children showed no differences for any of the memory subsets at BL

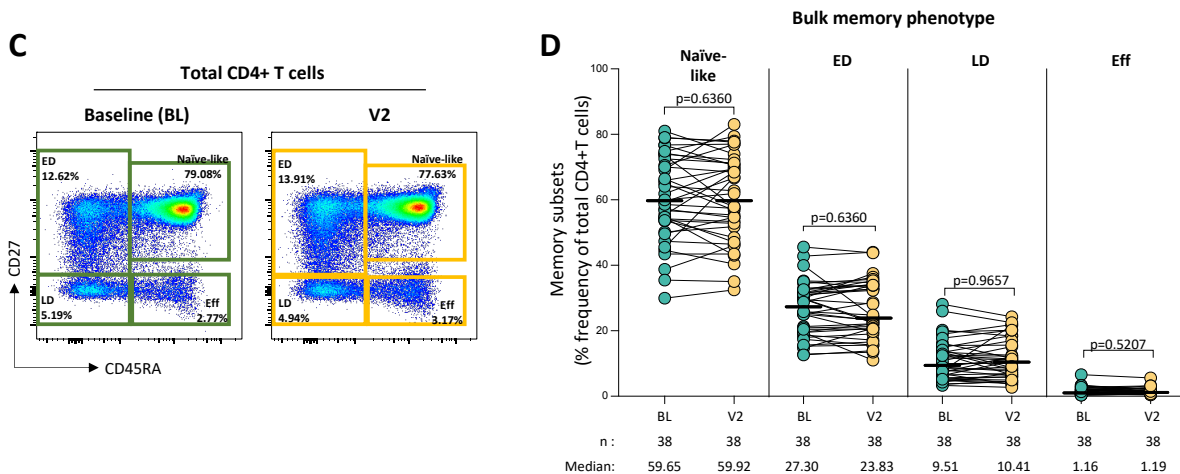
compared to V2, which was also similar for total bulk CD8⁺ T cell memory subsets (**Figure 4.6E-H**). The memory phenotypic analysis of SARS-CoV-2-specific CD8⁺ T cells was not performed due to limited event numbers in children with detectable T cell responses.

The dominant memory subset at BL and V2 in seropositive children (n=32) was ED cells, although a significant increase in the proportion of ED subsets was detected at V2 compared to BL (median: 75.31% and 65.26%, respectively, p=0.0013, **Figure 4.6J**). At BL, we found significantly higher proportion of naïve-like memory subsets compared to V2 (median: 13.04% for BL and 5.97% for V2, p=0.0048, **Figure 4.6J**). The LD memory subset was comparable at BL and V2 (median: 16.76% vs 15.95%, respectively) and negligible Eff memory cells were detected in seropositive children (n=32) (**Figure 4.6J**). Similarly, the dominant memory subset in seronegative children (n=7) was ED memory T cells (median: 46.15% for BL and 55.17% for V2, **Figure 4.6K-L**). Unlike the seropositive children, we found a significantly higher proportion of LD memory subset in seronegative children at BL compared to the V2 follow-up (median: 11.11% vs 5.17%, respectively, p=0.0156, **Figure 4.6L**). The naïve-like memory subsets were comparable at BL and V2 (median: 36.67% vs 28.57% respectively), while negligible Eff memory cells were detected at either time points (**Figure 4.6L**). Overall, these results show SARS-CoV-2-specific CD4⁺ T cell responses exhibited a predominantly ED memory phenotype over 16 months in children, despite SARS-CoV-2 serostatus at baseline.

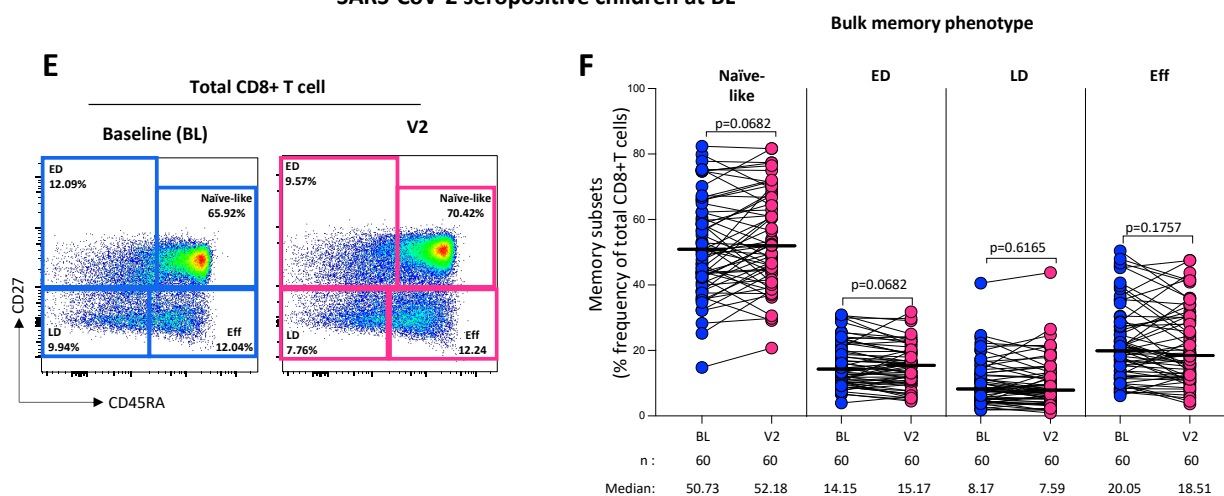
SARS-CoV-2 seropositive children at BL



SARS-CoV-2 seronegative children at BL

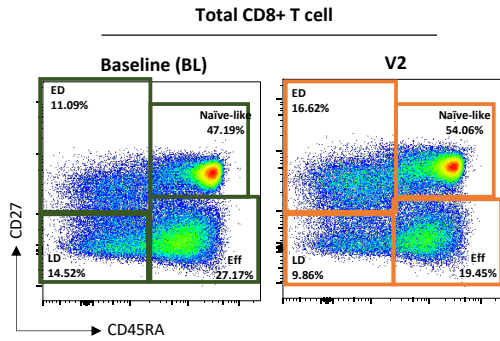


SARS-CoV-2 seropositive children at BL

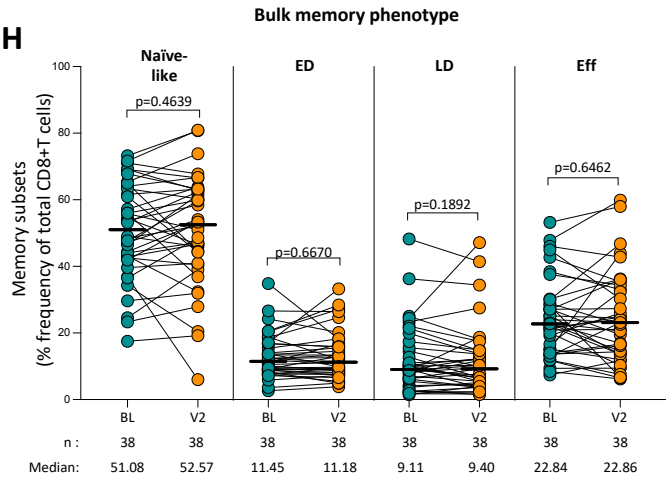


SARS-CoV-2 seronegative children at BL

G

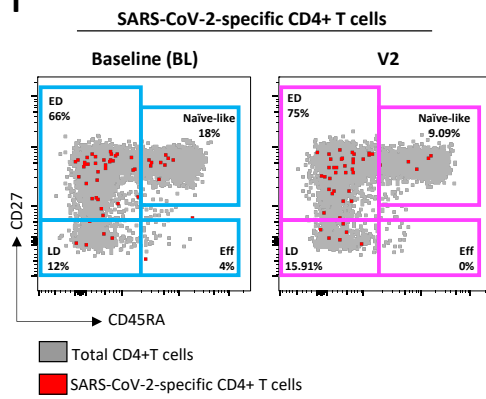


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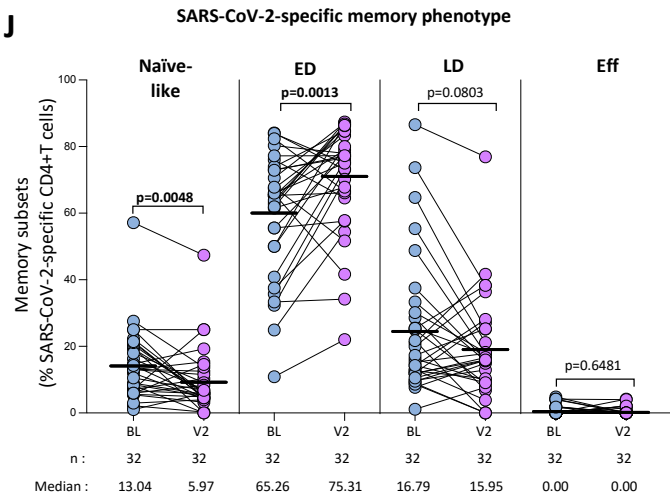


SARS-CoV-2 seropositive children at BL

I

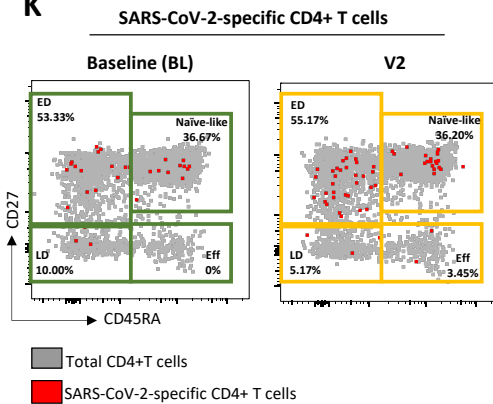


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SARS-CoV-2 seronegative children at BL

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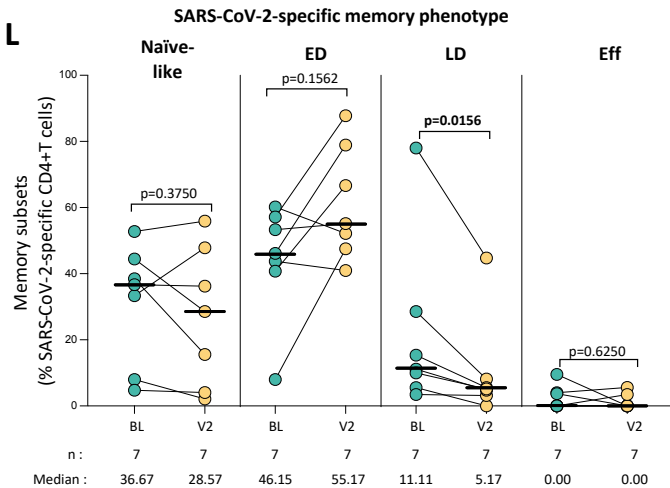


Figure 4.6: Memory differentiation phenotype of SARS-CoV-2-specific T cells after 16 months (V2) in SARS-CoV-2 seropositive (blue and purple) and seronegative (green and yellow) children.

(A) Representative flow plot examples of the memory differentiation profile in total CD4⁺ T cells of seropositive child at BL and V2. Memory phenotypic subsets were delineated based on the expression of CD45RA and CD27, namely naïve-like (CD45RA⁺CD27⁺), early differentiated (ED:CD45RA⁺CD27⁺), late differentiated (LD:CD45RA⁺CD27⁻) and effector (Eff:CD45RA⁺CD27⁻) cells. **(B)** The proportion of total CD4⁺ naïve-like, ED, LD and Eff memory subsets in seropositive children (n=60) at BL and V2. Bars represent median and median values are indicated. **(C)** Representative flow plot examples of the memory differentiation profile in total CD4⁺ T cells of seronegative child at BL and V2. **(D)** The proportion of total CD4⁺ cells in seronegative children (n=38) at BL and V2. Bars represent median and median values are indicated. **(E)** Representative flow plot examples of the memory differentiation profile in total CD8⁺ T cells of a seropositive child at BL and V2. **(F)** The proportion of each memory subset of total CD8⁺ T cells in seropositive children (n=60) at BL and V2. Bars represent median and median values are indicated. **(G)** Representative flow plot examples of the memory differentiation profile in total CD8⁺ T cells of a seronegative child at BL and V2. **(H)** The proportion of each memory subset of total CD8⁺ in seronegative children (n=38) at BL and V2. **(I)** Representative overlay flow plots of memory differentiation profile in seropositive child at BL and V2, red dots illustrate SARS-CoV-2-specific CD4⁺ T cells and grey dots represent total CD4⁺ T cells. **(J)** The proportion of naïve-like, ED, LD and Eff memory subsets of SARS-CoV-2-specific CD4⁺ T cells in seropositive children (n=32) with detectable responses at BL and V2. Bars represent medians and median values are indicated. **(K)** Overlay flow plots of memory differentiation profile; red dots illustrate SARS-CoV-2-specific CD4⁺ T cells and grey dots represent total CD4⁺ T cells in seronegative child at BL and V2. **(L)** The proportion of each memory subset of SARS-CoV-2-specific CD4⁺ T cells in seronegative children (n=7) with detectable responses at baseline and V2. Bars represent median and median values are indicated. Statistical comparisons in **B, D, F, H, J** and **L** were performed using Wilcoxon matched-pairs signed rank test between paired samples; *p* values <0.05 were considered statistically significant and are bolded.

4.3.7 T cell responses are preserved across Delta and Omicron BA.1 variants in children after 16 months (V2).

The initial (BL) visit for the participants was dominated by the ancestral D614G strain and Beta variant after 2 epidemic waves, thereafter SA experienced 3 additional waves of infection dominated by Delta, Omicron subvariants BA.1/2 and BA.4/5 before the second visit approximately 16 months after BL (**Figure 4.1B**). We measured the magnitude of T cell responses to Delta (DEL) and Omicron BA.1 (BA.1) variant S in comparison to ancestral strain (ANC) in a subset of children (n=50) at the V2 time-point. Due the timing of the sampling (**Figure 4.1**), children who were seropositive at BL (n=31) would have had their primary infection with either

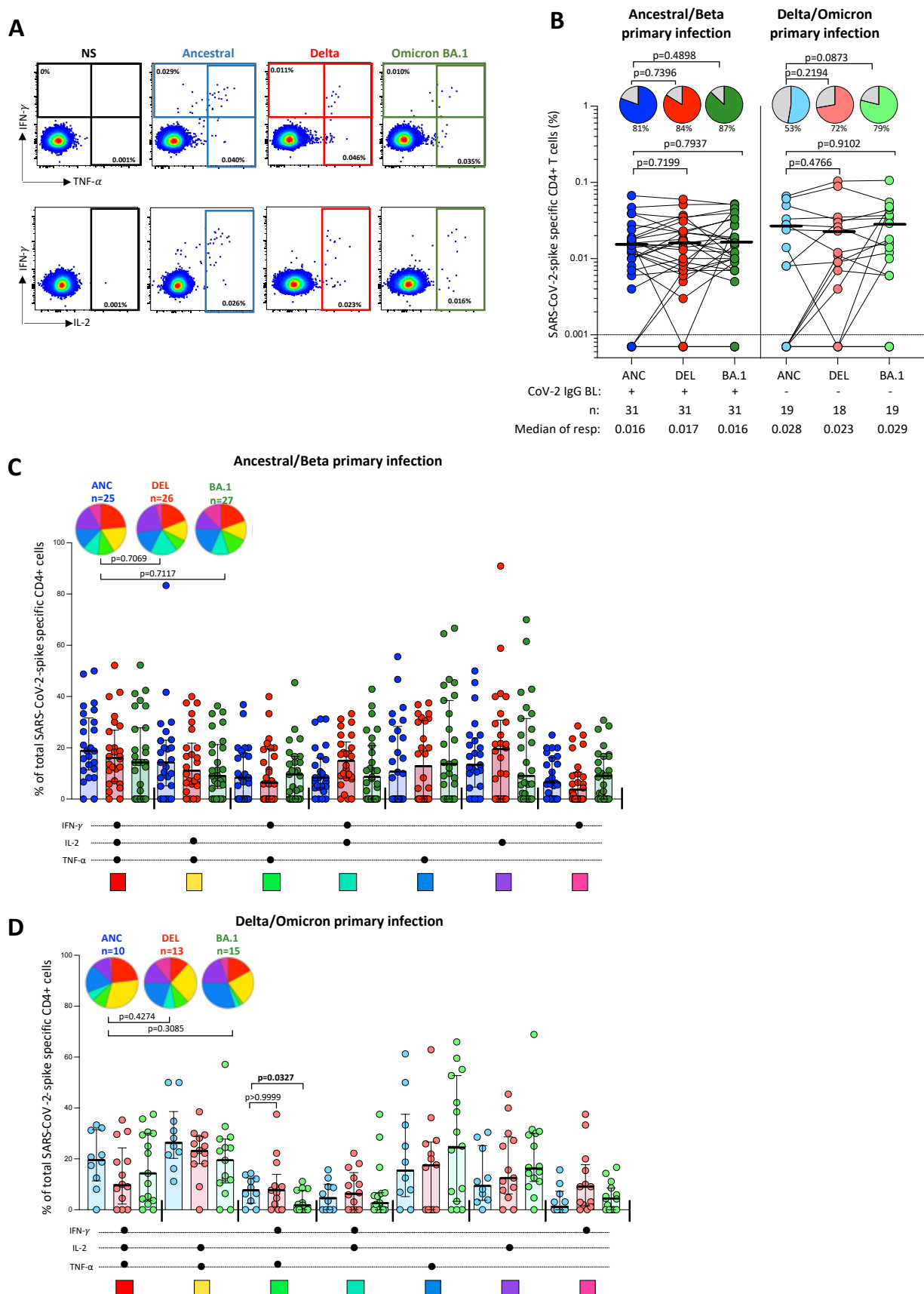
ancestral or Beta variant, while those who were seronegative at BL (n=19) would likely have had their first exposure to Delta or Omicron variants.

Figure 4.7A illustrates the representative flow plots of CD4⁺ T cells (producing IFN- γ , IL-2 or TNF- α) following stimulation with SARS-CoV-2 ANC, DEL or BA.1 variant S peptide pools. We measured SARS-CoV-2-S specific CD4⁺ T cell responses to ANC, DEL and BA.1 variant S and we found a comparable proportion of CD4⁺ T cell responders to ANC (81%), DEL (84%) and BA.1 (87%) variant S in children who had first exposure to ANC/Beta (**Figure 4.7B**). Here we report that of the 81% (25/31) of children that had detectable CD4⁺ T cell responses against ANC S, 96% (24/25) maintained CD4⁺ T cell responses against DEL and BA.1 variants, whereas 4% (1/25) lost responses to DEL and BA.1 variants. Of the 19% (6/31) children that were non-responders to ANC S, 33% (2/6) gained a response to DEL and 17% (1/6) gained a response to BA.1. No differences were detected in the magnitude of responses to ANC, DEL or BA.1 (median of responders: 0.016%, 0.017% and 0.016%, respectively, **Figure 4.7B**). It is noteworthy, that although this group likely had a primary infection with ANC/Beta, there is the possibility that they may have been reinfected with DEL or BA.1.

Notably, in children with a likely primary infection with DEL/OMI, we found that a lower proportion of 53% (10/19) had CD4⁺ T cell responses to ANC compared to Delta (72%) and BA.1 (79%) variant S, although within the responders the magnitude of the response was similar (median of responders: ANC: 0.028% vs DEL: 0.023% and BA.1: 0.029%, respectively **Figure 4.7B**). Of interest we found that of the 47% (9/19) of children had no detectable CD4⁺ T cell responses to ANC S, 4 gained responses to both DEL and BA.1 variants, while a further 2 gained responses to BA.1 S variant only (**Figure 4.7B**).

We also investigated the polyfunctional profile of SARS-CoV-2-S specific responding cells by their capacity to co-express IFN- γ , IL-2 or TNF- α in children with detectable T cell responses (**Figure 4.7C-D**). Notably, the overall polyfunctional profiles showed no significant differences between CD4⁺ T cell responses to ANC, DEL or BA.1 variant S in children with ANC/Beta primary infection

(**Figure 4.7C**) or children with possible DEL/OMI primary infection (**Figure 4.7D**). The proportion of CD8 responders were relatively low ($\leq 29\%$) across variants tested in both seropositive and seronegative children (**Figure 4.7E**). We also found no differences in the magnitude of CD8+ T cell responses across variants (**Figure 4.7E**) or the polyfunctional profile of CD8+ T cell responses in children with ANC/Beta primary infection (**Figure 4.7F**). Polyfunctional analysis was not performed on children with DEL/OMI primary infection due to the low responses. Overall, these results show that T cell responses generated after 5 waves of the COVID-19 pandemic in SA, were equally robust against ANC, DEL and BA.1 variant S, thus demonstrating that despite the lower magnitude of T cell responses in children compared to adults, these responses were ultimately preserved across variants in children as has been previously shown in adults (Keeton et al., 2022, 2023; Nesamari et al., 2024).



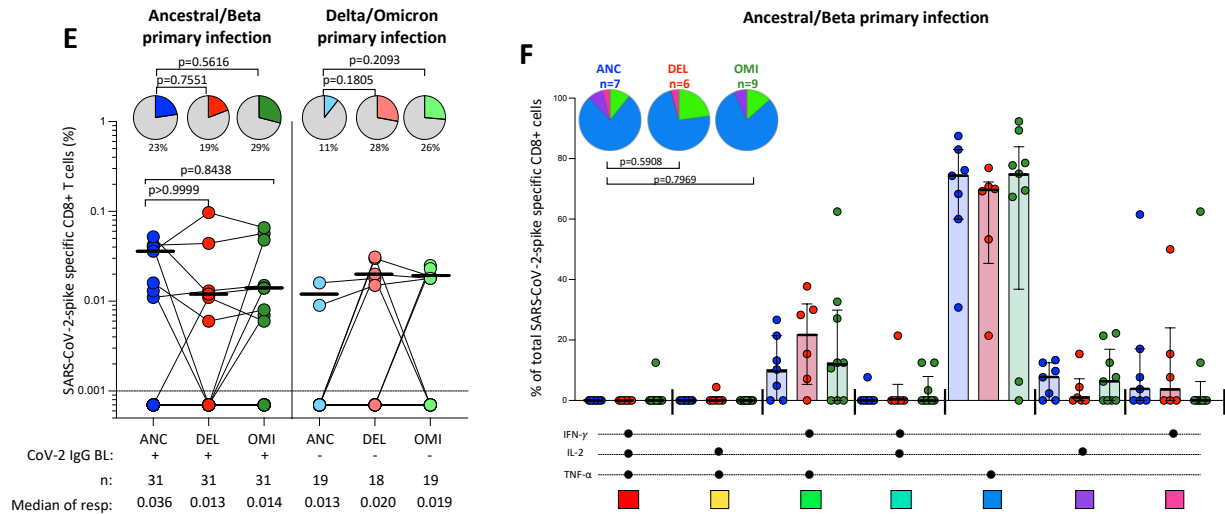


Figure 4.7: T cell responses to SARS-CoV-2 Ancestral, Delta and Omicron BA.1 variants in children after 16-month period (V2).

(A) Representative flow cytometry plots showing IFN- γ , TNF- α and IL-2 production in response to SARS-CoV-2 Ancestral, Delta and Omicron BA.1 spike peptide stimulation in children. NS: no stimulation, ANC: Ancestral, DEL: Delta, BA.1: Omicron BA.1. **(B)** Frequency of SARS-CoV-2-spike specific CD4+ T cells producing IFN- γ , TNF α or IL-2 in response to SARS-CoV-2 ANC, DEL and BA.1 variants spike peptide pools in children with Ancestral/Beta primary infection (n=31) and children with possible Delta/Omicron primary infection (n=19). Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders in each group are indicated. **(C)** Polyfunctional profile comparison of SARS-CoV-2 ANC (n=25), DEL (n=26) and BA.1 (n=27) spike-specific CD4+ T cells in children with Ancestral/Beta primary infection. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. **(D)** Polyfunctional profile comparison of SARS-CoV-2 ANC (n=10), DEL (n=13) and OMI (n=15) spike-specific CD4+ T cells in children with possible Delta/Omicron primary infection. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. **(E)** Frequency of SARS-CoV-2-spike specific CD8+ T cells producing IFN- γ , TNF- α or IL-2 in response to SARS-CoV-2 ANC, DEL and BA.1 variants spike peptide pools in children with Ancestral/Beta primary infection (n=31) and children with possible Delta/Omicron primary infection (n=19). Bars represent median and median values are indicated. The pie charts represent the proportion of participants with detectable T cell response to SARS-CoV-2 and the percentage of responders in each group are indicated. **(F)** Polyfunctional profile comparison of SARS-CoV-2 ANC (n=7), DEL (n=6) and OMI BA.1 (n=9) spike-specific CD8+ T cells in children with Ancestral/Beta primary infection. The median and IQR are shown. Each pie slice represents the median contribution of IFN- γ , TNF- α or IL-2 expression (colour coded on the x-axis) to the total SARS-CoV-2 responses. Statistical comparisons in **(B)** and **(E)** were performed using the Friedman test with multiple comparisons and the Chi-square test compare the percentage of responders. Statistical comparisons in **C**, **D** and **F** were performed using the SPICE software, the permutation test for the pie charts and the Wilcoxon unpaired t test to compare response patterns

between baseline; *p values* <0.05 were considered statistically significant. ANC: Ancestral, DEL: Delta, OMI: Omicron.

4.4 Discussion

It is well established that SARS-CoV-2-specific memory T cells persist for long periods of over 1 year in comparison to antibody responses that wane after 6 - 31 weeks (Anand et al., 2021; Kedzierska & Thomas, 2022; Perreault et al., 2020; Robbani et al., 2020), and cross-recognition occurs between SARS-CoV-2 VOC, although this has been reported primarily in adults (Kedzierska & Thomas, 2022). Similar profiling in children has been understudied. Children remain the largest unvaccinated population against SARS-CoV-2 (Naeimi et al., 2023; Ni et al., 2024). Therefore, it is imperative to investigate the immune responses to SARS-CoV-2 infection that could provide relevant data on the immunological memory for protection against subsequent re-infections. This study expands on our previous findings presented in Chapter 3, with a separate longitudinal cohort with additional characterization of polyfunctional and memory differentiation profiles of SARS-CoV-2-specific T cells between children and mothers, durability and variant cross-reactivity in children.

In this study, we reported that children have lower SARS-CoV-2-specific T cell responses compared to adults, which is concordant with our previous cohort and studies by several other research groups (C. A. Cohen et al., 2021; Goenka et al., 2021; Jacobsen et al., 2022; Kaaijk et al., 2022; Lima et al., 2022; Moratto et al., 2020; Nattrass et al., 2022; Pierce et al., 2020; Rowntree et al., 2022). However, there is some discordance in the literature. A study by Dowell et al. reported that children have higher SARS-CoV-2-specific cellular immune responses compared to adults (Dowell et al., 2022a), while a study by Tosif et al. reported no differences between children and adults (Tosif et al., 2020). Four factors contributing to the differences in the magnitude of SARS-CoV-2-specific T cell responses between children and adults may be the timing of SARS-CoV-2 infection, disease severity, age-related differences in adaptive T cell immunity and/or robust innate immunity (Chou et al., 2022; Kloc et al., 2020; B. V. Kumar et al., 2018; Loske et al., 2022; Simon et al., 2015; Yoshida et al., 2021). These factors may contribute to the discordance in the literature

regarding the magnitude of T cell responses in children and adults depending on cohort design. To control for timing of infection, in this study, we analyzed SARS-CoV-2-specific T cell responses between child-mother pairs (n=73) living in a shared household in the BL sample. In SA, during the time-period these samples were collected, lockdown was still in place and children were not attending school (C. Cohen et al., 2022; D. Shepherd & Mohohlwane, 2021; Spaull & Van der Berg, 2020), increasing the likelihood of children being exposed/infected by their mothers thus resulting in similar timing of infection. To exclude disease severity as a variable, all children and mothers recruited into this cohort had had either asymptomatic or mild COVID-19, with no hospitalisations.

We reported that 36% (23/64) of children potentially exposed to SARS-CoV-2 at the same time as the seropositive mothers, remained SARS-CoV-2 seronegative suggesting resistance to productive infection. Of these, 30% (7/23) had detectable SARS-CoV-2-specific T cell responses which could be due to cross-reactive immunity from endemic HCoVs, while the remaining 70% of these seronegative children had no detectable virus-specific T cell responses, despite the similar exposure with matched seropositive mothers. The lack of virus-specific adaptive immune responses in the seronegative children may be a result of early viral clearance by the innate immunity and/or early abortive SARS-CoV-2 infection in seronegative children. Early termination of viral infection in highly exposed seronegative individuals has previously been reported for herpes simplex virus (HSV)-2 (Posavad et al., 2010) and SARS-CoV-2 (Jay et al., 2023; Swadling et al., 2021) infection. Indeed, a study by Rowntree and colleagues reported that SARS-CoV-2 exposed seronegative children had detectable SARS-CoV-2-specific tetrameric T cells with a predominant naïve phenotype and more diverse TCR $\alpha\beta$ repertoires, suggesting that the virus-specific T cell responses were present early, following SARS-CoV-2 infection and likely resulting in early abortive infection or non-infection with SARS-CoV-2 (Nguyen et al., 2021; Rowntree et al., 2022). Although, we did not measure T cells against SARS-CoV-2 non-structural proteins (NSP) that make up the replication-transcription complex (RTC) (Swadling et al., 2021) or epitope-specific responses (Habel et al., 2020; Nguyen et al., 2021; Rowntree et al., 2022), we hypothesise that in our study, SARS-CoV-2 seronegative children living with seropositive mothers may have had early

terminated SARS-CoV-2 infection, potentially explaining the lack of SARS-CoV-2 S-specific antibody and SNM-specific T cell responses in this group.

The T cell qualitative differences measured as the polyfunctional profile of SARS-CoV-2-specific T cells was comparable between mothers and children. Consistent with our findings, a study by Cohen et al. reported that SARS-CoV-2 infected children had significantly lower magnitude of SARS-CoV-2 T cell responses than infected adults and the polyfunctionality of SARS-CoV-2-structural protein specific T cells was comparable between children and adults infected with SARS-CoV-2 at acute phase, 60 days recovery phase and 180 days memory phase (C. A. Cohen et al., 2021). Altogether with our findings, this suggests that age is associated with a lower magnitude of cellular immunity to SARS-CoV-2 but similar polyfunctionality of cytokine-producing T cells in children, which may contribute to reduced proinflammatory damage in children while still providing adequate SARS-CoV-2 viral control in comparison to adults (C. A. Cohen et al., 2021; Natrass et al., 2022). The memory differentiation profile of SARS-CoV-2-specific CD4⁺ T cells revealed a predominant early differentiated (ED:CD45RA-CD27⁺) memory subset in both children and mothers, although this was lower in children compared to the seropositive mothers. In our study, we delineated the memory subsets based on CD45RA and CD27 expression, therefore the ED memory cells could be central memory (TCM: CD45RA-CD27⁺CCR7⁺) or effector memory type 1 (TEM1: CD45RA-CD27⁺CCR7⁻) subsets depending on the expression of CCR7 marker (Mathew et al., 2020; Painter et al., 2021). Our findings are concordant with several studies that have found that children exhibit reduced SARS-CoV-2-specific TCM and TEM cells (C. A. Cohen et al., 2021; Jacobsen et al., 2022; Khoo et al., 2023; Neeland et al., 2021b; Rowntree et al., 2022), highlighting that the generation of long-term memory pools following SARS-CoV-2 infection or exposure may be equivalent in children and adults.

SARS-CoV-specific memory T cells have been shown to persist up to 17 years after SARS infection (Le Bert et al., 2020; O. W. Ng et al., 2016) and several studies have shown that SARS-CoV-2-specific T cells are durable for long periods after infection and/or vaccination in adults (Adamo et al., 2022; Bonifacius et al., 2021; K. W. Cohen et al., 2021; Dan et al., 2021; Geers et al., 2021;

Goel et al., 2021a; Guerrero et al., 2021; Z. Lu et al., 2021; Mak et al., 2022; Y. Peng et al., 2020). Since the emergence of SARS-CoV-2 variants, early in the pandemic, SA experienced five infection waves dominated by the Ancestral, Beta, Delta, Omicron BA.1/BA.2 and BA.4/5 variants. By the second visit in our study, the majority of adults had received COVID-19 vaccines. We, therefore, only considered the maintenance of SARS-CoV-2-specific T cell responses after 16 months, in children who were seropositive at BL and assessed *de novo* responses in those that were seronegative at the BL visit. We found that SARS-CoV-2-specific T cell responses were detectable in a higher proportion of children and at a higher magnitude after 16 months. This might be due to the likelihood of new or multiple SARS-CoV-2 infections which occurred during the 3 infection waves dominated by Delta and Omicron subvariants, between the first and the second visit. This is concordant with the proportion of children who were seronegative at BL, gaining a response. In contrast to the increased T cell responses in children we found in our study, several studies have shown that SARS-CoV-2-specific cellular immunity persists for up to 12 months after infection in children despite a reduced magnitude at the second timepoints (Dowell et al., 2022a; Jacobsen et al., 2022; Seidel et al., 2023; Sieber et al., 2022). Indeed, findings reported by Dowell et al. show that SARS-CoV-2 S- and N-specific responses were reduced after 12 months post infection in children (Dowell et al., 2022a). Differences between our study and Dowell et al. might be different experimental approaches and/or the number of possible exposures between sampling and the timing of sampling in relation to the stage of the pandemic which may explain the decrease in responses after 12 months in the Dowell et al. (Dowell et al., 2022a) study and an increase of responses after 16 months in our study. We also showed that the polyfunctional profile of SARS-CoV-2-specific CD4⁺ T cells in seropositive children changed over the 16-month period, driven by the acquisition of dual (IFN- γ +IL-2+) and single (IFN- γ +) producing cells at the second visit. The change in T cell quality over time could be attributed to multiple exposures to SARS-CoV-2, which had been reported to result in qualitative changes in the functional CD4⁺ profile to promote expansions of memory T cells by production of IL-2 in an autocrine manner (Dooms et al., 2004, 2007; Keeton et al., 2023; Z. Lu et al., 2021; McKinstry et al., 2014; Natrass et al., 2022; Seder et al., 2008; M. A. Williams et al., 2006).

The phenotypic analysis showed that there was an increase of the proportion of ED memory subsets in children after 16-months. In contrast to our findings, Jacobsen and colleagues reported that children had reduced TEM (defined as CCR7-CD45RO+) and small increase of TCM (defined as CCR7+CD45RO+) CD4+ T cells after 12 months (Jacobsen et al., 2022). It is important to note the assay differences, particularly memory markers used in the two studies. Notwithstanding, the dissimilarities between the studies could be the timing or number of exposures/infections prior to the second visit. In our study, we assessed T cell responses after 16 months compared to 12 months as reported by Jacobsen et al (Jacobsen et al., 2022). In addition, three waves occurred in SA between our sampling timepoints compared to one infection wave dominated by the Alpha variant in Germany during the sampling timeline in the Jacobsen study. The increase of SARS-CoV-2-specific ED memory cells after 16 months could be related to new or multiple exposures to SARS-CoV-2 or the expansion or contraction of cross-reactive T cell memory pools as a result of previous or new exposure to other endemic HCoVs in seronegative children. It is important to emphasize that the analysis of qualitative changes for CD8+ T cells was not performed in our study, due to the limited number of participants with detectable CD8+ T cell responses. Nevertheless, our findings highlight that children develop long-term memory T cells, that are maintained after 16 months and potentially providing long-lasting memory responses to subsequent re-infections in the absence of SARS-CoV-2 vaccination.

The Omicron variant has been reported to evade antibody neutralizing activity induced by previous infection and vaccination (Carabelli et al., 2023; C. Chen et al., 2023; Hoffmann et al., 2022; Shrestha et al., 2022), and several reports have demonstrated that children and adolescents have poorer cross-neutralization capacity against VOC, including Omicron subvariants than adults (Bellusci et al., 2023; Paniskaki et al., 2023; W. Peng et al., 2023; Seidel et al., 2023; Sieber et al., 2022; J. Tang et al., 2022; Toh et al., 2022). However, T cell responses to SARS-CoV-2 VOC including Omicron subvariants have been widely reported to be largely unaffected, following SARS-CoV-2 infection and/or vaccination in adults (Gao et al., 2022; Garcia-Beltran et al., 2021; Keeton et al., 2022, 2023; Naranbhai et al., 2022; Nesamari et al., 2024; Tarke et al., 2022a). Since we have serology data on these children, we could theorize that children who were seropositive at BL had

their first infection with ancestral or Beta variant, while those that were seronegative were infected with Delta or Omicron. It is important to note that our variant analysis was done at the V2 time-point and therefore we cannot exclude the possibility that those children first infected by ancestral or Beta, were not reinfected with Delta or Omicron. In this study, we reported that children had comparable SARS-CoV-2-S specific T cell responses against ancestral, Delta and Omicron BA.1 variants. Our findings are supported by previous studies that have shown cross-reactive T cell responses against Omicron BA.1 variants are preserved in children as reported in adults (Paniskaki et al., 2023; Saggau et al., 2022; Seidel et al., 2023; Sieber et al., 2022). Indeed, a recent study by Paniskaki et al. reported that unvaccinated children generate SARS-CoV-2 cross-reactive T cell responses that can cross-recognize Delta and Omicron BA.1 variants, comparable to adults (Paniskaki et al., 2023). In our study, we also reported that 81% of children with ancestral/Beta variant primary infection had robust CD4+ T cell responses against ancestral S and of that, more than 90% maintained CD4+ T cell responses to Delta and BA.1 S, suggesting that dominant T cell epitopes from prior SARS-CoV-2 variants were unaffected by SARS-CoV-2 viral mutations. Children in whom the first exposure was to Delta or Omicron showed a slight increase in the proportion CD4+ T cell responders against Delta and BA.1 variants compared to ancestral S, we found that 47% (9/19) of children had undetectable CD4+ T cell responses to ancestral S, and of that 66% (6/9) gained responses to Delta and/or BA.1 variants, further suggesting Delta and Omicron variant infection as the first potential SARS-CoV-2 exposure in these children. Moreover, the T cell quality of SARS-CoV-2-responding CD4+ T cells was comparable across SARS-CoV-2 S variants for both children infected primarily with ancestral or Beta and children infected with Delta or Omicron, suggesting that viral mutations had no impact on the functionality of cytokine production by CD4+ Th1 cells in this population.

In conclusion, we reported that although the magnitude of SARS-CoV-2-specific T cell responses is lower in children compared to adults, the quality of this response as measured by the polyfunctional T cell profile is similar and maintained in an early differentiated phenotype known to be associated with durability. Despite the lower initial magnitude of T cell response this virus-specific T cell response was maintained after 16 months in unvaccinated children and cross-

recognized Delta and BA.1 variants albeit in the context of an ongoing pandemic. The cross-recognition potential of T cells against SARS-CoV-2 Delta and BA.1 variants in children, may provide protective immunity against severe COVID-19 outcomes during subsequent re-infections in the absence of SARS-CoV-2 vaccination. This study provides important data on the longevity of SARS-CoV-2-specific T cell responses and cross-reactivity to VOC in unvaccinated children that may be applicable to current and future vaccine strategies in the paediatric population.

Limitations of the study

This study had a few caveats. These include the lack of PCR-confirmed SARS-CoV-2 infection in our cohort as previously mentioned in Chapter 3, and the adult participants included in this study are all females as they were recruited as part of a birth-cohort study. We measured T cell responses against structural proteins (S, N, M), which excludes non-structural proteins of the viral proteome which are involved in early viral replication and contribute to abortive infection. We also collected T cell samples at two time points between which three infection waves occurred, which may have resulted in new or repeated exposure to SARS-CoV-2 during Delta, BA.1/2 and BA.4/5 waves in SA. Serological data after each infection wave since the initial visit are being generated by our collaborators to control for this. Finally, we measured virus-specific T cell responses in the periphery, which does not represent the magnitude or specificity of responses in the respiratory tract, the primary site of infection.

CHAPTER 5: COMPARING THE IMMUNE ABNORMALITIES IN MIS-C TO HEALTHY CHILDREN AND THOSE WITH INFLAMMATORY DISEASE REVEALS DISTINCT INFLAMMATORY CYTOKINE PRODUCTION AND A MONOFUNCTIONAL T CELL RESPONSE.

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5.1 Abstract

Multisystem inflammatory syndrome in children (MIS-C) is a severe, hyperinflammatory disease that occurs after exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The underlying immune pathology of MIS-C is incompletely understood, with limited data comparing MIS-C to clinically similar paediatric febrile diseases at presentation. SARS-CoV-2-specific T cell responses have not been compared in these groups to assess whether there is a T cell profile unique to MIS-C. In this study, we measured inflammatory cytokine concentration and SARS-CoV-2-specific humoral immunity and T cell responses in children with fever and suspected MIS-C at presentation (n=83) where MIS-C was ultimately confirmed (n=58) or another diagnosis was made (n=25) and healthy children (n=91). Children with confirmed MIS-C exhibited distinctly elevated serum IL-10, IL-6, and CRP at presentation. No differences were detected in SARS-CoV-2 spike (S) IgG serum concentration, neutralisation capacity, antibody dependant cellular phagocytosis, antibody dependant cellular cytotoxicity or SARS-CoV-2-specific T cell frequency between the groups. Healthy SARS-CoV-2 seropositive children had a higher proportion of polyfunctional SARS-CoV-2-specific CD4+ T cells compared to children with MIS-C and those with other inflammatory or infectious diagnoses, who both presented a largely monofunctional SARS-CoV-2-specific CD4+ T cell profile. Treatment with steroids and/or intravenous immunoglobulins resulted in rapid reduction of inflammatory cytokines but did not affect the SARS-CoV-2-specific IgG or CD4+ T cell responses in MIS-C. In these data, MIS-C had a unique cytokine profile but not a unique SARS-CoV-2 specific humoral or T cell cytokine response.

5.2 Introduction

Multisystem inflammatory syndrome in children (MIS-C) is a novel and severe hyperinflammatory disease associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Riphagen et al., 2020). MIS-C generally occurs 2-4 weeks after primary infection (Lazova et al., 2022; Rowley,

2020a), with an estimated incidence of 31.6 cases per 100 000 paediatric SARS-CoV-2 cases in the USA (Payne et al., 2021), and 22 cases per 100 000 paediatric SARS-CoV-2 infections in Cape Town, South Africa (SA) (Butters et al., 2022). As children are relatively spared from acute severe SARS-CoV-2, MIS-C represents the most severe manifestation of SARS-CoV-2 infection in children; with approximately 40% of children with MIS-C requiring intensive care unit admission (Payne et al., 2021) and a 2% mortality rate, which is markedly higher than primary coronavirus disease 2019 (COVID-19) in children (Holm et al., 2021; Weatherhead et al., 2020).

MIS-C is widely reported throughout the world, and affects black children disproportionately (Feldstein et al., 2020; Whittaker et al., 2020). Despite this, MIS-C has been poorly reported in Africa, with scattered clinical case series (Van Der Zalm et al., 2023). In Cape Town, SA, we have documented and described the clinical phenotype of a large cohort of children with MIS-C throughout the COVID-19 pandemic at the two major paediatric referral hospitals in the city (Abraham et al., 2022; Butters et al., 2022; Webb et al., 2022). These data show that MIS-C does affect children in Africa and the dearth of reported cases likely reflects poor recognition of the disease in children from less resourced areas. Children with MIS-C present acutely unwell with fever, rash, multi-organ disease and shock (Butters et al., 2022; Davies et al., 2020; Whittaker et al., 2020). This is not an uncommon clinical presentation in children, especially in SA and other African countries where sepsis and gastro-intestinal shock are common (Kwizera et al., 2021). The unique immune phenotype in children with MIS-C as compared to clinically similar diseases at presentation in this setting is unknown. MIS-C is treated with intravenous immunoglobulins (IVIg) and/or steroids, with recent evidence showing that steroid therapy alone may be sufficient (McArdle et al., 2021; Ouldali et al., 2021). These treatments may be contra-indicated in sepsis, infections and other diseases that present similarly. Immunological markers may therefore be useful in identifying MIS-C at presentation and thereby initiating therapy sooner.

The underlying immune pathology and cause of MIS-C is incompletely understood. There is a mature Ig response in MIS-C as compared to acute SARS-CoV-2 infection, confirming the post-infectious nature of the disease (Consiglio et al., 2020), with no unique production of SARS-CoV-

2 specific antibodies (Consiglio et al., 2020; Conway et al., 2022; Gruber et al., 2020; Pierce et al., 2020; Rostad et al., 2022; Sananez et al., 2021; Singh et al., 2022; Talarico et al., 2022; Thiriard et al., 2023; Weisberg et al., 2021; Yonker et al., 2020), however the function of these antibodies has been incompletely assessed. Multiple cytokines, including interleukin (IL)-1 β , IL-6, IFN- γ , IL-10 and clinical markers of inflammation such as C-reactive protein (CRP) are markedly elevated compared to children with acute SARS-CoV-2 infection, previous SARS-CoV-2 infection or Kawasaki Disease (KD) (Abo-Haded et al., 2022; Butters et al., 2022; Carter et al., 2020; Consiglio et al., 2020; de Cevins et al., 2021; Druzak et al., 2023; Esteve-Sole et al., 2021; Gruber et al., 2020; Hoste et al., 2021; N. P. Kumar et al., 2022; Lapp et al., 2022; P. Y. Lee et al., 2020; Mostafa et al., 2022; Ramaswamy et al., 2021; Rey-Jurado et al., 2022; Sacco et al., 2022; Whittaker et al., 2020). These comparator groups are less representative of the clinical setting, where clinicians must distinguish MIS-C from other common paediatric febrile diseases at presentation. Fewer data compare the cytokine production at presentation, prior to therapy, between MIS-C and similar inflammatory or infectious childhood diseases (Diaz et al., 2021; N. P. Kumar et al., 2022; Rey-Jurado et al., 2022).

Children with MIS-C have low T cell frequencies, but increased T cell activation and expansion of non-specific TCR V β 21.3+ cells (Carter et al., 2020; Consiglio et al., 2020; Diaz et al., 2021; Moreews et al., 2021; Sacco et al., 2022; Vella et al., 2021). This expansion bears the hallmark of a superantigen response (Kappler et al., 1989); indeed, a superantigen-like motif has been identified in S using in silico modelling (Porritt et al., 2021b) but it remains to be confirmed experimentally, and it is unclear whether T cells respond differently to SARS-CoV-2 in patients with MIS-C with conflicting published data available. Hsieh et. al. report that SARS-CoV-2-specific T cell responses were similar in children with MIS-C compared to children with previous SARS-CoV-2 infection (Hsieh et al., 2022a), while Singh et. al. report that SARS-CoV-2-specific T cell responses in children with MIS-C were reduced compared to COVID-19 convalescent children although comparable to healthy children (Singh et al., 2022). Both studies included children who had received steroids which may have affected T cell function, and neither quantified the virus-specific T cell response in MIS-C. In addition, SARS-CoV-2-specific T cell responses in MIS-C have

not been compared to other inflammatory conditions to assess whether the T cell response is unique in MIS-C or rather reflects non-specific inflammation.

In this study we aimed to discover the unique immune phenotype of MIS-C in treatment-naïve SA children with MIS-C, SARS-CoV-2 exposed healthy children and children with diseases that are clinically similar to MIS-C at presentation within a similar time. We investigated the cytokine production, SARS-CoV-2 antibody responses and function, and SARS-CoV-2-specific T cell responses in this cohort and measured the change in immune variables with time and treatment in children with MIS-C.

5.3 Materials and methods

5.3.1 Patient cohort and sample collection

From 26 June 2020 to 9 February 2022, children presenting to the emergency room with suspected MIS-C and at least 3 days of fever at the Red Cross War Memorial Children's Hospital in Cape Town, SA were recruited. Clinical data and samples were collected at presentation and throughout hospitalisation. Patients were ultimately diagnosed by attending clinicians as having confirmed MIS-C (WHO criteria (WHO working group, 2020) or another final diagnosis and these children were categorised as 'inflammatory controls'. Children with KD (classified as meeting AHA 2017 criteria (McCrindle et al., 2017) and SARS-CoV-2 antibody negative) were excluded from this study. During the same period, healthy children without chronic or inflammatory diseases who were hospitalised for elective surgeries were recruited and classified as healthy controls. The detailed clinical features and whole blood RNA expression of this cohort has been published (Butters et al., 2022; Spracklen et al., 2022).

This protocol was approved by the relevant ethics committees at both the University of Cape Town (UCT HREC 112/2012; 599/2020) and Red Cross War Memorial Children's Hospital (RXH RCC240/WC_202008_113). Consent, and assent where appropriate, were provided prior to recruitment as per ethical requirements.

5.3.2 Serum cytokines and chemokines

Patient serum was used to measure inflammatory cytokine concentrations in a subset of children with MIS-C, inflammatory diseases and healthy controls. A customised Milliplex kit (Millipore, St Charles, Missouri) was used to quantify the serum cytokines IL-1 receptor antagonist (RA), IL-1 β , IL-10, IL-6, IL-27, monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor alpha (TNF- α) on the Bio-Plex platform (Bio-Rad Laboratories, Hercules, California), according to manufacturer instructions. Briefly filter plates were coated with wash buffer and assay buffer thereafter internal standards, controls and serum samples were added to respective wells. Pre-mixed beads (antibody immobilized) of the markers of interest were added to all wells and incubated overnight at 4°C. Plates were washed and detection antibody was added and incubated for 1 hour. Thereafter Streptavidin-Phycoerythrin was added and incubated for 30 minutes in the dark. Following this, Sheath Fluid was added to each well and sheath and results were read on the Luminex® instrument plate reader.

5.3.3 Serum CRP

Patient CRP tests were performed for clinical indications at the local clinical laboratory.

5.3.4 Antibody Enzyme-Linked Immunosorbent Assay

Antibody titres were measured in serum from children with MIS-C, seropositive inflammatory controls, and seropositive healthy controls. Briefly, two $\mu\text{g/mL}$ of SARS-CoV-2 spike (S) protein was used to coat 96-well, high-binding plates and incubated overnight at 4°C. The plates were incubated in a blocking buffer consisting of 5% skimmed milk powder, 0.05% Tween 20, 1xPBS. Plasma samples were diluted to a 1:100 dilution and added to plates followed by the addition of secondary antibody diluted to 1:3000 in blocking buffer. TMB substrate (ThermoFisher Scientific) was then added and upon stopping the reaction with 1 M H_2SO_4 , absorbance was measured at a 450 nm wavelength. In all instances, monoclonal antibody CR3022 was used as a positive control and Palivizumab was used as a negative control.

5.3.5 Neutralisation assays

Methods for neutralisation assay were performed as previously described (Kitchin et al., 2022). Briefly, SARS-CoV-2 pseudotyped lentiviruses were prepared by co-transfecting the HEK 293T cell line with either the SARS-CoV-2 614G S (D614G), Beta (L18F, D80A, D215G, 242-244del, K417N, E484K, N501Y, D614G, and A701V), Delta (T19R, 156-157del, R158G, L452R, T478K, D614G, P681R, and D950N) and Omicron (BA.1) (A67V, D69-70, T95I, G142D, D143-145, D211, L212I, 214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, and L981F) plasmids, in conjunction with a firefly luciferase encoding lentivirus backbone plasmid and a murine leukaemia virus backbone plasmid. The parental plasmids were kindly provided by Dr Elise Landais and Dr Devin Sok (International AIDS Vaccine Initiative, New York, NY USA). Heat-inactivated plasma samples from MIS-C patients were incubated with the SARS-CoV-2 pseudotyped virus for 1 hour at 37°C, 5% CO₂. Subsequently, 1 x 10⁴ HEK293T cells engineered to over-express ACE-2, kindly provided by Dr Michael Farzan (Scripps Research, La Jolla, CA USA), were added and incubated at 37°C, 5% CO₂ for 72 hours, upon which the luminescence was measured. Both neutralisation ID₅₀ values for the D614G SARS-CoV-2 variant and the neutralisation ID₅₀ values for the SARS-CoV-2 variant that the patient was likely exposed to (autologous) was assessed. This autologous variant was assigned using the NICD definition of 30 cases per 100 000 population in the province (National Institute of Communicable Diseases, 2021). Additionally, the cross reactivity of anti-SARS-CoV-2 S antibodies was assessed.

5.3.6 Antibody-dependent cellular phagocytosis (ADCP)

The ADCP assay measures the level at which antibody-opsonised target beads are engulfed by THP-1 cells. ADCP assays were performed as previously described (Richardson et al., 2022). Briefly, fluorescent neutravidin beads were coated with biotinylated SARS-CoV-2 S protein and incubated for 2 hours with either monoclonal antibodies at a starting concentration of 50 µg/mL titrated fivefold or participant plasma samples at a dilution of 1:100. CR3022 and a plasma pool

of SARS-CoV-2 positive individuals were used as a positive control while Palivizumab and a plasma pool of negative individuals were used as a negative control. The beads were then incubated with THP-1 cells per well overnight, fixed with 4% paraformaldehyde and interrogated on the FACS Aria II. Phagocytosis score was calculated as the percentage of THP-1 cells that engulfed fluorescent beads multiplied by the geometric mean fluorescence intensity of the population in the FITC channel with background (no antibody control) subtraction.

5.3.7 Antibody-dependant cellular cytotoxicity (ADCC)

The ADCC assay measures the ability of antibodies to cross link S-expressing target cells and the FcγRIIIa receptor present on Jurkat cells (Kitchin et al., 2022). Briefly, HEK293T cells were transfected with 5 µg SARS-CoV-2 D614G S variant plasmid and were incubated at 37°C for 2 days. Subsequently, 1×10^5 S transfected cells per well were incubated with heat inactivated plasma (1:100 final dilution) or monoclonal antibodies (final concentration of 100 µg/mL) in RPMI 1640 media supplemented with 10% FBS 1% Pen/Strep (Gibco, Gaithersburg, MD) for 1 hour at 37°C. Jurkat-Lucia NFAT-CD16 cells (2×10^5 cells/well; Invivogen) were added and were incubated at 37°C for 24 hours. Twenty microlitres supernatant was removed and transferred to a white 96-well plate and luciferase activity was measured following the addition of 50 µL reconstituted QUANTI-Luc secreted luciferase reagent with a one second integration time. Relative light units (RLU) of a no antibody control were subtracted as background. Palivizumab was used as a negative control, while CR3022 was used as a positive control. To induce the transgene 1× cell stimulation cocktail (ThermoFisher Scientific, Oslo, Norway) and 2 µg/mL ionomycin in R10 was added as a positive control to confirm sufficient expression of the Fc receptor. RLUs for S were normalised to each other and between runs using CR3022.

5.3.8 SARS-CoV-2 antigens

Commercially available peptide pools (15-mer sequences with 11 amino acid overlap) covering the immunodominant regions of the Wuhan-1 SARS-CoV-2 S, (PepTivator, Miltenyi Biotech), nucleocapsid (N) and membrane (M) (17-mer with 10 amino acid overlap; BEI resources) proteins

were used. The S peptide pool was prepared by resuspending in distilled water at a concentration of 50 µg/mL and used at a concentration of 1 µg/mL. N and M peptide pools were prepared by resuspending in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a concentration of 100 µg/mL and used at a final concentration of 1 µg/mL.

5.3.9 Whole blood-based T cell detection assay

Whole blood was collected in sodium heparin tubes and processed within 4-8 hours of collection. Blood (500 µL) was stimulated for 24 hours, at 37°C with SARS-CoV-2 S, N and M peptide pools in the presence of Brefeldin-A (10 µg/mL, Sigma-Aldrich) and co-stimulatory antibodies against CD28 (clone 28.2) and CD49d (clone L25) (1 µg/mL each; BD Biosciences). Unstimulated blood was incubated with an equimolar amount of dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a background control. After 24 hours, blood was treated with 2 mM EDTA (Sigma-Aldrich) and followed by red blood cell lysis and cell fixation using BD FACS lysing solution (BD Biosciences). Cells were cryopreserved in freezing media (90% foetal bovine serum [FBS] and 10% DMSO) and stored at -80°C until batch analysis.

Fixed, cryopreserved cells were subsequently thawed, washed in FACS washing buffer (1% FBS and 99% PBS) and transferred to a 96-well V-bottom plate prior to surface staining with CD4 ECD (SFC112T4D11, Beckman Coulter) and CD8 BV510 (RPA-8, Biolegend). Cells were permeabilized using BD Perm/wash buffer (BD Bioscience) and stained with CD3 BV650 (OKT3, Biolegend) IFN-γ AlexaFluor® 700 (B27, Biolegend), TNF-α BV786 (Mab11, Biolegend) and IL-2 APC (MQ1-17H12, Biolegend). Stained cells were washed and fixed with BD CellFIX buffer (BD Bioscience). Samples were acquired on a BD LSR-II flow cytometer and analysed using FlowJo (v10). Cells were gated on singlets, lymphocytes and CD3 cells. Results are expressed as the frequency of CD4+ or CD8+ T cells expressing IFN-γ, IL-2 or TNF-α. Cytokine responses are presented as background subtracted values (from the frequency of cytokine produced in unstimulated cells). The gating strategy used to identify populations of interested are depicted in **Chapter 2, Figure 2.2.A-K**.

5.3.10 Quantification and statistical analysis

Statistical analyses of serum inflammatory markers, antibody titres and antibody-dependent processes were performed in SPSS Statistics (v28, IBM Corp., Armonk, NY) while statistical analyses of T cell responses were performed in Prism (v9.4.1; GraphPad Software Inc). One-way analysis of variants (ANOVA) was used for comparing three or more cohorts. Inflammatory marker concentrations were log transformed and therefore a parametric ANOVA was used, and non-parametric tests were used for all other comparisons. The Mann-Whitney and Kruskal-Wallis test with Dunn's multiple comparisons post-test were used for unmatched samples. Fisher's exact tests were used for comparisons between the proportion of responders represented as pie charts, and Chi-square test was used to compare the statistical differences between the median contributions of the polyfunctional profiles. *P values* less than 0.05 were considered statistically significant. Details of analyses performed for each experiment are described in the figure legends.

5.4 Results

5.4.1 Study cohort description.

A total of 83 children with suspected MIS-C were enrolled: 58 had ultimately confirmed MIS-C and 25 had an alternate diagnosis (inflammatory controls). Ninety-one healthy children (healthy controls) were recruited (**Table 5.1**). The clinical phenotype of these patients has been previously described in larger cohort studies (Abraham et al., 2022; Butters et al., 2022; Spracklen et al., 2022; Webb et al., 2022), but the basic clinical details and treatments are provided in **Table 5.2**. The inflammatory controls had a range of diagnoses which are further outlined in **Table 5.3**. Briefly, the median age amongst the children recruited in the study was similar; children with MIS-C had a median age of 5.07 years, and the median age of both the inflammatory controls and healthy controls was 5.32 years. There was no sex bias in the MIS-C and inflammatory control cohort, with 51% and 48% of children being female respectively, while 29% of healthy controls were female. The racial and ethnic distribution of the cohort consisted mostly of black and mixed-race children (**Table 5.1**). Samples were collected prior to any MIS-C treatment (methylprednisolone or IVIG) in 74% (43/58) of children with MIS-C (**Table 5.2**). Based on the

SARS-CoV-2-S specific serology, 98% (57/58) of children with MIS-C were SARS-CoV-2 seropositive, whilst 56% (14/25) of inflammatory controls and 37% (34/91) of the healthy controls had detectable SARS-CoV-2-S specific antibodies (**Table 5.1**). No patients had been vaccinated at the time of sampling and none of the seropositive healthy children or inflammatory controls had a history of confirmed SARS-CoV-2 infection.

Table 5.1: Study cohort demographic features and assay sample sizes.

	MIS-C	Inflammatory controls	Healthy
n	58	25	91
Age, median (IQR)	5.07 (2.45-8.75)	5.32 (2.39-8.73)	5.32 (2.38-9.33)
Female, n (%)	30 (51.7%)	12 (48.0%)	27 (29.7%)
Black African, n (%)	31 (53.4%)	10 (40.0%)	37 (40.7%)
SARS-CoV-2 antibody positive, n (%)	57 (98.3%)	14 (56.0%)	34 (37.3%)
Sample analysed, n			
Serum cytokine	35	13	41
Antibody response	58	14	33
T cell response	8	6	24

Table 5.2: MIS-C and inflammatory controls cohort clinical description.

	MIS-C (n=58)	Inflammatory controls (n=25)
Age (years), median (IQR)	5.28 (2.81-8.73)	5.31 (2.39-8.73)
Female, n (%) 29	29 (50.0)	13 (52.0)
Days of fever (IQR)	6 (4-7)	6 (3-8)
Tachycardia (%)	57 (98.3)	19 (76.0)
Rash (%)	54 (93.1)	10 (40.0)
Conjunctivitis (%)	49 (84.5)	6 (24.0)
Received IVIG, n (%)	54 (93.1)	7 (28.0)
Received methylprednisolone, n (%)	33 (56.9)	3 (12.0)
Admitted to ICU, n (%)	17 (29.3)	4 (16.0)
Received Inotropes, n (%)	16 (27.6)	4 (16.0)

Ventilated, n (%)	2 (3.4)	4 (16.0)
Highest CRP (mmol/L), median (IQR)	193 (129, 300)	145 (44, 230)

MIS-C - multisystem inflammatory syndrome in children; IQR - interquartile range; IVIG - intravenous immunoglobulin; ICU - intensive care unit; CRP - C reactive protein.

Table 5.3: Inflammatory control description diagnosis (n = 24).

	Number of patients
Adenovirus	1
Tuberculosis	2
Typhoid	2
Pneumonia	1
Dysentery	4
Appendicitis	1
Abdominal sepsis	2
Systemic JIA with MAS	1
Gastroenteritis	2
Kukuchi Disease	1
Salmonella	1
Pyrexia of unknown origin	2
Viral upper RTI	3
Viral lower RTI	1

Inflammatory control description diagnosis (n = 24). JIA - juvenile idiopathic arthritis, MAS - macrophage activation syndrome; RTI - respiratory tract infection.

5.4.2 Cytokines and CRP are upregulated in MIS-C compared to other inflammatory conditions.

Of the consented patients, 28 MIS-C, 13 inflammatory controls and 41 healthy controls had pre-treatment serum inflammatory cytokine concentrations measured (**Table 5.1**). Compared to inflammatory controls, children with MIS-C had significantly increased concentrations of IL-10 ($p=0.033$, **Figure 5.1.C**), IL-6 ($p=0.01$, **Figure 5.1.D**) and CRP ($p=0.008$, **Figure 5.1.H**). IL-1RA ($p=0.054$, **Figure 5.1.A**) and MCP-1 ($p=0.059$, **Figure 5.1.E**) were elevated in MIS-C, although this did not reach statistical significance. Compared to healthy controls, children with MIS-C had increased concentrations of all cytokines measured (**Figure 5.1.A-G**).

Following treatment with IVIG and/or methylprednisolone, there was a rapid decrease in the serum concentration of all measured cytokines and CRP in children with MIS-C (**Figure 5.2**).

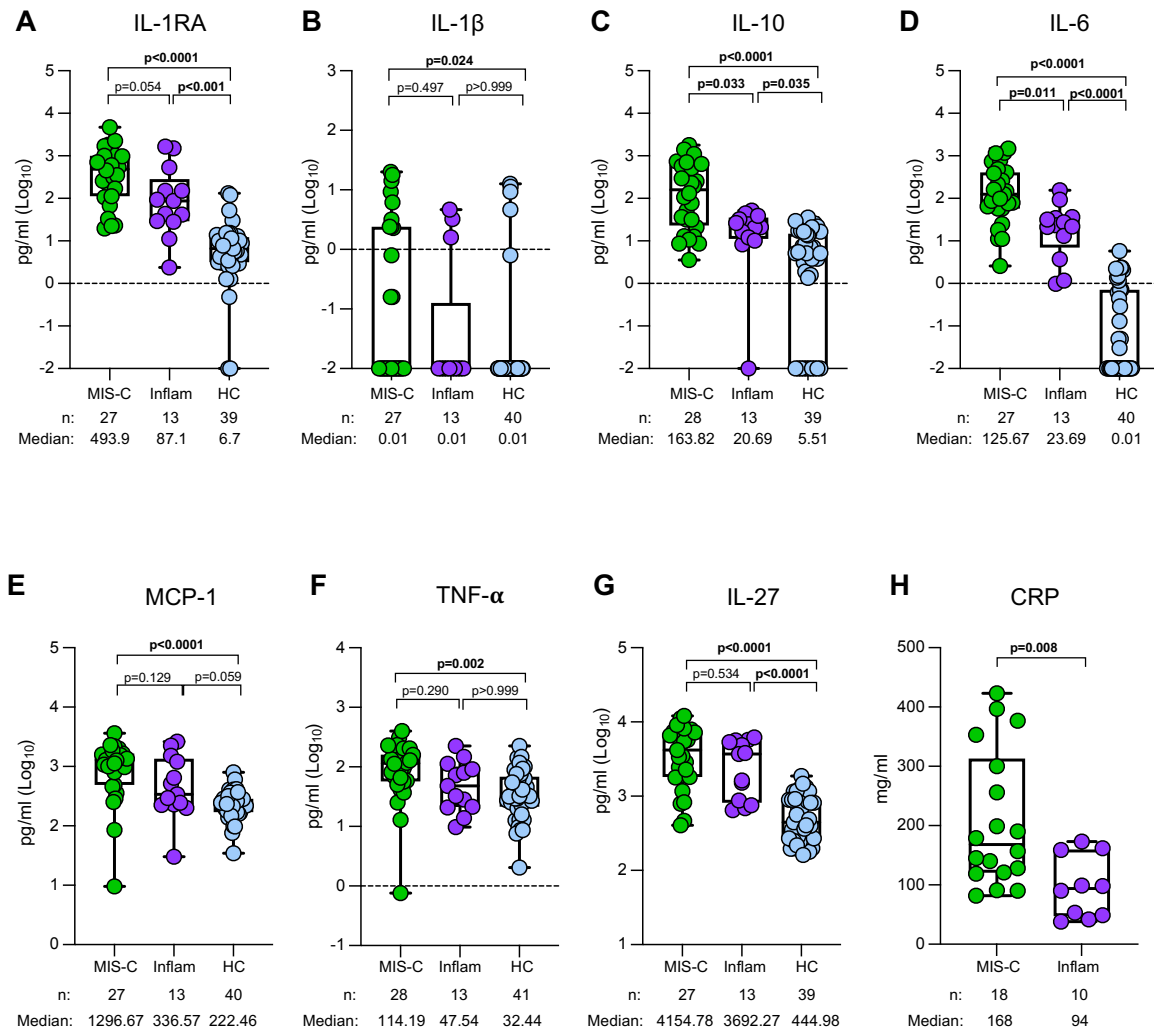


Figure 5.1: Serum cytokine levels in pre-treatment samples from MIS-C patients, inflammatory controls, and healthy children.

Box and whisker plots demonstrate the median Log₁₀ concentration, range, and interquartile range for (A) IL-1RA, (B) IL-1 β , (C) IL-10, (D) IL-6, (E) MCP-1, (F) TNF- α , (G) IL-27 and (H) CRP in each group (Green - MIS-C: multisystem inflammatory syndrome in children, purple - Inflam: inflammatory controls, blue - HC: healthy controls). Detection limits were 1.29 pg/mL for IL-1RA, 0.52 pg/mL for IL-1 β , 0.91 pg/mL for IL-10, 0.14 pg/mL for IL-6, 3.05 pg/mL for MCP-1, 5.39 pg/mL for TNF- α and 50.78 pg/mL for IL-27. The median

concentrations are shown below the graphs. Statistical comparisons were assessed using a one-way analysis of variants (ANOVA) with Bonferroni correction for multiple testing. *p* values <0.05 were considered statistically significant and are bolded.

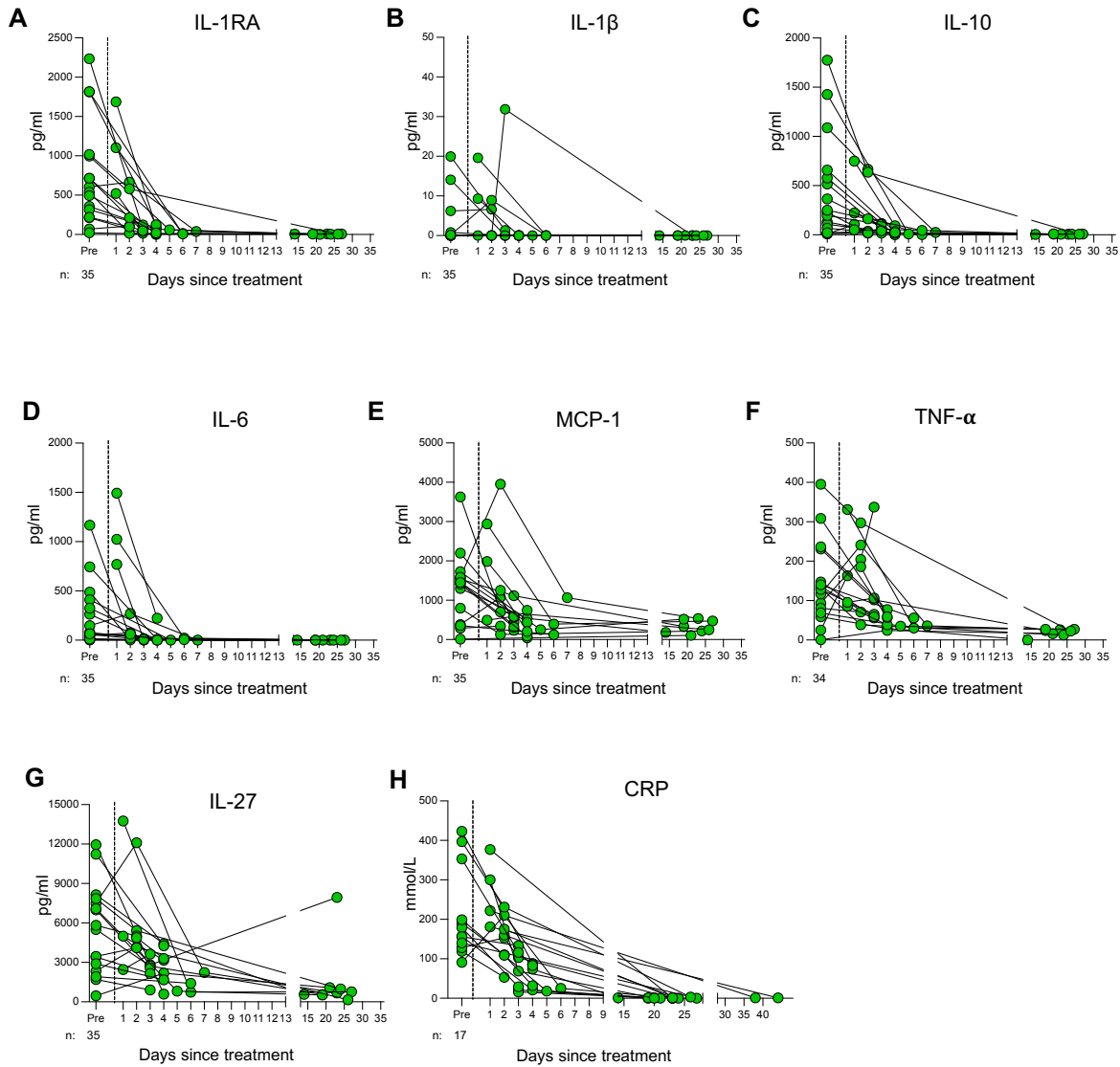


Figure 5.2: Effect of treatment on the inflammatory cytokine profile in MIS-C cases.

Longitudinal profile of serum inflammatory marker concentrations in children with MIS-C pre- and post-treatment. **(A)** IL-1RA, **(B)** IL-1 β , **(C)** IL-10, **(D)** IL-6, **(E)** MCP-1, **(F)** TNF- α , **(G)** IL-27 and **(H)** CRP.

5.4.3 Anti-SARS-CoV-2 IgG binding antibody titres, neutralising activity and Fc effector function do not differ between groups but are affected by epidemiological wave.

There was no difference in the wild-type D614G variant S IgG titres in patients with MIS-C compared to SARS-CoV-2-seropositive inflammatory controls or SARS-CoV-2-seropositive healthy controls (**Figure 5.3.A**). There was no difference in the 'autologous' neutralizing ability (as measured towards the SARS-CoV-2 variant that the patient was likely exposed to in a specific infection wave), ADCP or ADCC between groups (**Figure 5.3.B-D**). Children with MIS-C did not have altered neutralising cross-reactivity of SARS-CoV-2 antibodies across the SARS-CoV-2 waves compared to SARS-CoV-2-seropositive inflammatory controls or SARS-CoV-2-seropositive healthy controls (**Figure 5.3.E**). S IgG responses were unaffected by treatment in children with MIS-C (**Figure 5.3.F**).

Children with MIS-C who presented in the first SARS-CoV-2 infection wave driven by the D614G variant had significantly higher S-specific IgG antibody titres compared to those who presented in the waves driven by the Beta ($p=0.032$), Delta ($p=0.013$) and Omicron (BA.1) ($p<0.0001$) variants (**Figure 5.4.A**). Overall, there were no significant differences in the autologous neutralization activity in MIS-C patients who were infected with any SARS-CoV-2 variant (**Figure 5.4.B**).

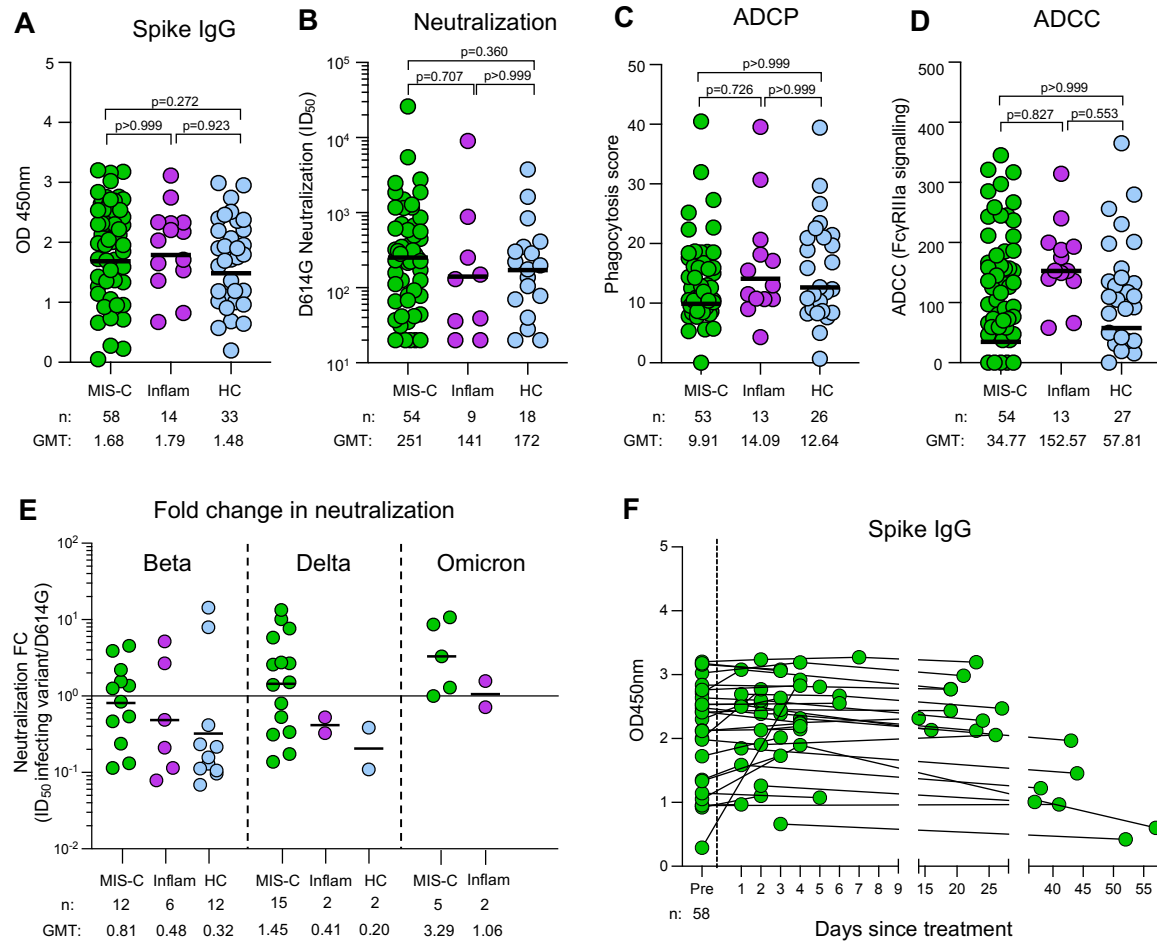


Figure 5.3: Comparison of SARS-CoV-2 spike IgG binding antibody titre, neutralisation activity and antibody-dependent processes between groups.

Serum samples were analysed to measure **(A)** SARS-CoV-2 D614G spike IgG binding antibodies quantified by OD450nm values, **(B)** Neutralisation activity of the SARS-CoV-2 autologous pseudovirus indicated by 50% inhibitory dilution (ID₅₀) values, **(C)** Antibody-dependent cellular phagocytosis (ADCP) activity, **(D)** Antibody dependent cellular cytotoxicity (ADCC) activity measured in FcγRIIIa signalling. **(E)** Fold change in D614G neutralising activity relative to SARS-CoV-2 Beta, Delta, and Omicron (BA.1) variants in children with MIS-C (green), inflammatory controls (purple) and healthy controls (blue). The cross-reactivity of anti-SARS-CoV-2-S antibodies was assessed by dividing the neutralisation ID₅₀ of the autologous SARS-CoV-2 variant to the neutralisation ID₅₀ of D614G SARS-CoV-2 variant. Geometric mean titre (GMT) is shown below the graphs. **(F)** longitudinal profile of SARS-CoV-2 D614G S IgG pre- and post-treatment in children with MIS-C. Statistical comparisons were assessed using a one-way analysis of variants (ANOVA) test with multiple comparisons.

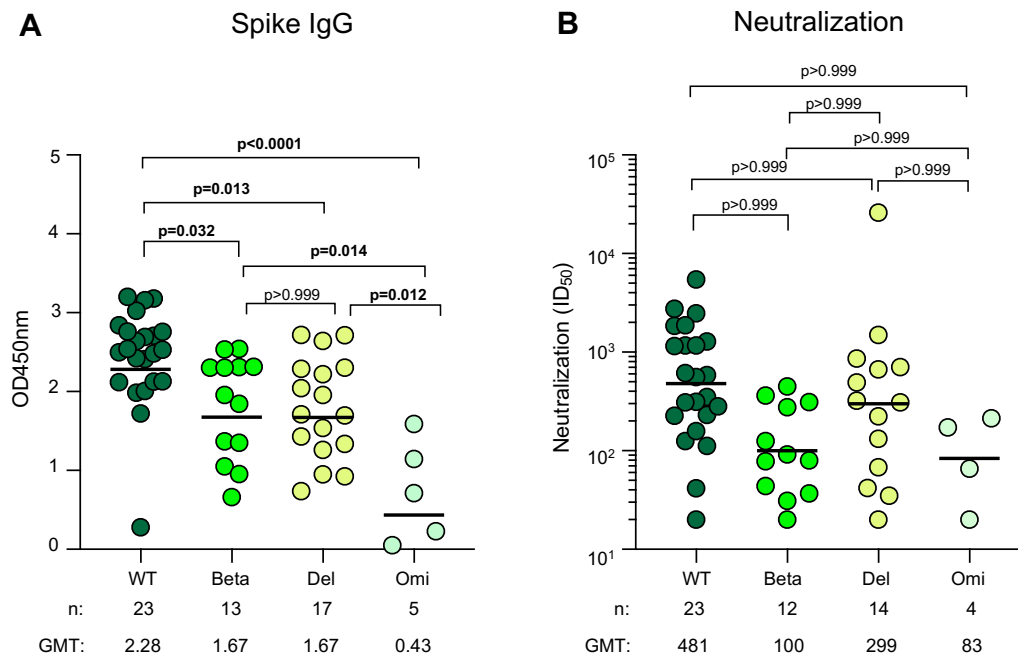


Figure 5.4: Antibody titre and neutralisation in patients with MIS-C across four SARS-CoV-2 waves.

(A) Anti-SARS-CoV-2 D614G S IgG titres in children with MIS-C presenting in the waves driven by SARS-CoV-2 D614G, Beta, Delta, and Omicron (BA.1) variants. **(B)** Neutralisation ID₅₀ tested against the autologous SARS-CoV-2 variant in children with MIS-C presenting in the waves driven by D614G, Beta, Delta, and Omicron (BA.1) SARS-CoV-2 variants. Bars indicate the geometric mean titre (GMT) ID₅₀. The Kruskal-Wallis test with Dunn's multiple comparison tests was used. *p* values <0.05 were considered statistically significant and are bolded.

5.4.4 SARS-CoV-2-specific T cell profiling reveals a largely monofunctional CD4⁺ T cell profile in MIS-C patients and inflammatory controls compared to healthy controls.

T cell responses were measured pre-treatment in children with MIS-C (n=8), seropositive inflammatory controls (n=6) and seropositive healthy controls (n=24) (**Figure 5.5.A & E**). Children with MIS-C had lower frequencies of CD3⁺ T cells than SARS-CoV-2-seropositive healthy controls (*p*=0.0320) but similar to SARS-CoV-2 seropositive inflammatory controls (*p*>0.9999, **Figure 5.5.B**). No difference in CD3⁺ T cell frequency was observed between SARS-CoV-2-seropositive

healthy controls and inflammatory controls ($p=0.0814$, **Figure 5.5.B**). There was no difference in the frequency of CD4⁺ and CD8⁺ T cells between all the groups (**Figures 5.5.C-D**).

There was no difference in the proportion of SARS-CoV-2-specific CD4⁺ T cell responders between all groups, with all (8/8) children with MIS-C (pre-treatment), all inflammatory controls (6/6) and 83% (20/24) of the healthy controls having detectable SARS-CoV-2-specific CD4⁺ T cell responses against SARS-CoV-2 S, N, and M combined peptide pools. Similarly, no differences were observed in the proportion of responders ($p=0.5569$) and the magnitude of responses between inflammatory and healthy controls ($p=0.4490$, **Figure 5.5.F**).

Overall, 25% (2/8) of children with MIS-C, 50% (3/6) of inflammatory controls and 42% (10/24) of healthy controls had detectable SARS-CoV-2-specific CD8⁺ T cell responses, with no differences in the proportion of responders or magnitude of CD8⁺ T cell responses between each of the groups (**Figure 5.6.A**). Furthermore, no differences were observed between the proportion of responders ($p=0.2918$) or the magnitude of SARS-CoV-2-specific CD4⁺ T cell responses ($p=0.1875$) pre- and post-treatment in children with MIS-C (**Figure 5.5.G**).

The functional profile of the responding T cells was measured by their capacity to produce different combinations of IFN- γ , TNF- α or IL-2 after stimulation with SARS-CoV-2 S, N and M peptide pools (**Figure 5.5.H**). The overall CD4⁺ T cell functional profile in children with MIS-C and inflammatory controls was similar ($p=0.0558$), whereas in children with MIS-C this was found to be different compared to healthy controls ($p<0.0001$, **Figure 5.5.H**). Indeed, in healthy controls a higher proportion of SARS-CoV-2-specific CD4⁺ T cells were polyfunctional, characterized by the production all three measured cytokines ($p=0.0058$), and dual-functional cells producing any two of the measured cytokines ($p=0.0221$) compared to children with MIS-C, who presented a largely monofunctional SARS-CoV-2-specific CD4⁺ T cell profile compared to healthy controls ($p=0.0033$, **Figure 5.5.H**). The predominant cytokines produced by monofunctional T cells in MIS-C and inflammatory controls were TNF- α (50.57% and 28.39%, respectively; $p=0.439$) or IL-2 (25.25% and 29.17%, respectively) compared to 12.7% and 21.52%, respectively, for TNF- α and IL-2 in

healthy controls. The proportion of TNF- α monofunctional T cells among MIS-C patients was highly variable (IQR: 5.19%-92.42%).

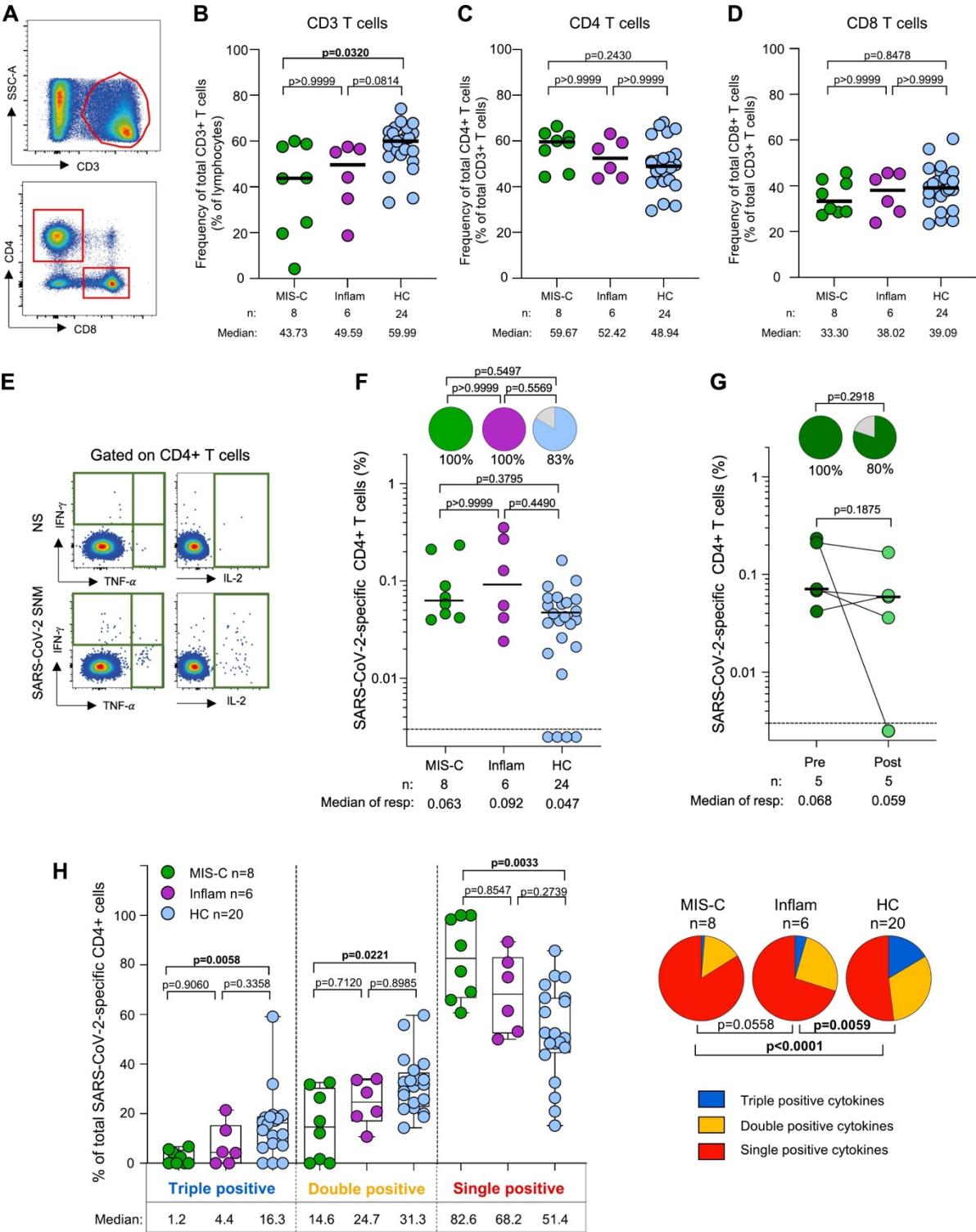


Figure 5.5: T cell frequency, SARS-CoV-2-specific T cell responses and functional profile in children presenting MIS-C, SARS-CoV-2 seropositive inflammatory controls and healthy controls.

A) Representative examples of CD4 and CD8 T cell distribution. The top plot shows the gating of total CD3+ T cells and the bottom plot shows CD4+ and CD8+ T cells. Frequency of CD3+ T cells **(B)**, CD8+ T cells **(C)** and CD4+ T cells **(D)** in each study group. The bars represent the median. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's correction. **(E)** Representative flow cytometry plots of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) and interleukin-2 (IL-2) cytokine production by CD4+ T cells in response to SARS-CoV-2 peptide pools in one child with MIS-C. NS: no stimulation, SNM: SARS-CoV-2-S, N and M combined peptide pools. **(F)** The magnitude of SARS-CoV-2-specific CD4+ T cell responses producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). The pie charts represent the proportion of responders with a detectable SARS-CoV-2-specific CD4 T cell response. Bars represent the median of the responders. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's correction and Fisher's exact test was used to compare the percentage of responders. **(G)** The magnitude of SARS-CoV-2-specific CD4+ T cell responses in children with MIS-C (n=5) pre- and post-treatment (steroids and/or intravenous immunoglobulins). The pies represent the proportion of responders with a detectable SARS-CoV-2-specific CD4 response. Bars represent the median of the responders. Statistical comparison was performed using the Wilcoxon matched pairs signed rank test and Fisher's exact test was used to compare the percentage of responders. **(H)** Polyfunctional profile of SARS-CoV-2-specific CD4+ T cells. The pie charts represent the proportions of triple (IFN- γ +IL-2+TNF- α +), double (IFN- γ +IL-2+ or IFN- γ +TNF- α + or IL-2+TNF- α +) and single (IFN- γ + or IL-2+ or TNF- α +) cytokine producing cells. Each pie slice represents the median contribution of produced cytokines to the total SARS-CoV-2 responses. Bars represent the median and interquartile range. The Kruskal-Wallis test with Dunn's correction was used to compare the response patterns between the groups and Chi-square test was used to compare the statistical differences between the pie charts. *p values* <0.05 were considered statistically significant and are bolded.

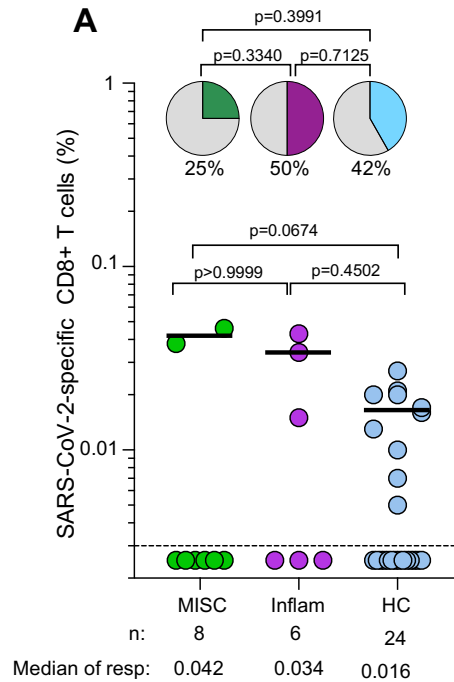


Figure 5.6: Magnitude of SARS-CoV-2-specific CD8+ T cell responses.

A) Frequency of SARS-CoV-2-specific CD8+ T cells producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). Green: MIS-C n=8; purple: inflammatory controls n=6 and blue: seropositive healthy controls n=24. The pie charts represent the proportion of responders with detectable T cell response to SARS-CoV-2 SNM combined peptide pools. Bars represent the median of the responders. The Kruskal-Wallis test with Dunn's multiple comparison tests was used to compare the response patterns between the groups and Chi-square test was used to compare the statistical differences between the pie charts.

5.5 Discussion

In this study we observed that serum IL-6, IL-10 and CRP were distinctly raised in MIS-C, suggesting a unique MIS-C cytokine response. This inflammatory cytokine response resolved quickly with treatment in MIS-C, mirroring the clinical phenotype (Butters et al., 2022). Intriguingly for a disease related to SARS-CoV-2 infection, the SARS-CoV-2 specific antibody and T cell responses were not unique to MIS-C.

It has been documented that patients with MIS-C have an overproduction of IL-6, IL-10, IL-1 β , IL-27, IFN- γ , MCP-1, IL-1RA, TNF- α and CRP (Carter et al., 2020; Consiglio et al., 2020; de Cevins et al., 2021; Druzak et al., 2023; Esteve-Sole et al., 2021; Gruber et al., 2020; Hoste et al., 2021; Lapp et al., 2022; P. Y. Lee et al., 2020; Ramaswamy et al., 2021; Rey-Jurado et al., 2022; Sacco et al., 2022), which is mirrored in our data when comparing the serum levels of these inflammatory cytokines to healthy controls. The normalization of cytokines with disease improvement that we have observed matches other data (Abo-Haded et al., 2022; Sacco et al., 2022). A unique strength of this study is that it agnostically recruited patients with fever and suspected MIS-C at presentation and pre-therapy. These groups represent the true conundrum that clinicians face in identifying cases with true MIS-C vs other clinically similar, severe diseases in the acute setting, especially in a region where shock, sepsis and infectious diseases are common. In this setting, we have shown that IL-6, IL-10, and CRP, are elevated in MIS-C compared to children with similar clinical diseases and may be useful diagnostic markers for further investigation. A previous study reported that IL-6 and CRP concentrations were increased in ICU-admitted children with sepsis compared to children with MIS-C (Diaz et al., 2021). We argue that ICU admitted children with sepsis are not an optimal comparator group, as only about 38% of children with MIS-C require ICU admission (Butters et al., 2022) and sepsis is probably the most severe of the mimics of MIS-C. This may skew these findings resulting in higher inflammatory cytokines in the ICU group. It is noteworthy that IL-27 has previously been shown to have increased concentrations in 8 children with MIS-C pre-treatment compared to 14 febrile controls who were recruited in a similar manner to this study (J. J. Huang et al., 2022). This is of particular interest to us as we have recently shown that the gene expression of *IL-27* was the best discriminator between MIS-C and inflammatory controls in a panel of 84 immune genes (Spracklen et al., 2022). The current data, however, did not support this, with no significant difference in IL-27 concentrations between MIS-C and inflammatory controls. These data further enforce that MIS-C is a hyperinflammatory disease that has its own distinct inflammatory signature.

Our data complement other studies that have reported that children with MIS-C have comparable titres of SARS-CoV-2 antibodies compared to healthy children exposed to SARS-CoV-2 who did not

develop MIS-C (Conway et al., 2022; Gruber et al., 2020; Rostad et al., 2022; Sananez et al., 2021; Weisberg et al., 2021). These antibodies have been shown to neutralize the SARS-CoV-2 to the same degree as COVID-19 convalescent children (Gruber et al., 2020; Rostad et al., 2022; Weisberg et al., 2021), similar to our reported findings. There is less published data on the SARS-CoV-2 antibody Fc effector function in children with MIS-C. In this study we investigated ADCP and ADCC function in children with MIS-C, inflammatory and healthy controls. Rostad et al. found that healthy exposed children had comparable ADCC function compared to patients with MIS-C (Rostad et al., 2022), which was also found in the current study. Therefore, accumulating evidence demonstrates no difference in the levels or function of SARS-CoV-2 antibodies in MIS-C, suggesting that these antibodies are unlikely to be driving the unique pathology of MIS-C.

Multiple studies have analysed SARS-CoV-2-specific CD4+ and CD8+ T cell responses in COVID-19 convalescent, mild and severely ill adults, confirming a role for T cells in ameliorating disease severity (Rydzynski Moderbacher et al., 2020; Sekine et al., 2020; Weiskopf et al., 2020). Similar to severe COVID-19 illness, lymphopenia has been reported in MIS-C (Filippatos et al., 2023; Vella et al., 2021). In this study we found that the frequency of CD3+ T cells was lower in children with MIS-C compared to healthy controls, although the CD3+ T cell frequency was measured as the proportion of lymphocytes using flow cytometry. We can therefore not conclude that this reduction indicates lymphopenia and could be as a result of expansion of CD3- populations. To this end, a study by Vella et al. showed a decreased proportion of CD3+ T cells and increased proportion of B cells in MIS-C compared to COVID-19 in children, demonstrating lymphocyte dysregulation (Vella et al., 2021).

Numerous studies have analysed SARS-CoV-2-specific T cell responses in MIS-C, compared to acute or convalescent COVID-19 in children and adults (Conway et al., 2022; Lam et al., 2022; Moreews et al., 2021; Pierce et al., 2020; Rybkina et al., 2023; Singh et al., 2022). The data are largely conflicting, with some reports showing increased antigen-specific T cell responses in children with MIS-C (Conway et al., 2022; Rybkina et al., 2023), some showing equivalent responses (Lam et al., 2022; Pierce et al., 2020) and yet others demonstrating a reduced

magnitude T cell response (Moreews et al., 2021; Singh et al., 2022) compared to either acute or convalescent children or adults. Several factors could account for the differences between studies, such as: 1) the different types of T cell assays employed; 2) antigens used to quantify responses; and 3) cohort differences, including timing of sampling during acute or convalescent MIS-C, differing comparator groups, as well as the severity of disease in MIS-C or convalescent groups. It is important to note that we, and others (Benede et al., 2024; C. A. Cohen et al., 2021; Kaaijk et al., 2022; Pierce et al., 2020) have previously shown that the SARS-CoV-2-specific T cell responses in adults are higher than in children, rendering comparisons to adults difficult to interpret. Our data showing a similar magnitude of responses in children with MIS-C and healthy children exposed to SARS-CoV-2 are concordant with that of Pierce et. al. and Lam et. al (Lam et al., 2022; Pierce et al., 2020). Our data also extend these studies to demonstrate that the magnitude of SARS-CoV-2-specific T cell responses in children with MIS-C is comparable to SARS-CoV-2 seropositive children with non-MIS-C inflammatory conditions, suggesting that generalized inflammation does not impair the capacity of T cells to produce a SARS-CoV-2-specific T cell response.

We observed that in children with MIS-C, SARS-CoV-2-specific responding cells were predominantly monofunctional, producing a single cytokine (either IL-2 or TNF- α), while SARS-CoV-2 seropositive healthy controls exhibited a polyfunctional CD4+ T cell profile. In contrast, the study by Rybkina et al showed an increased frequency of IL-2 producing CD4+ T cells, as well as IFN- γ and TNF- α CD8+ T cells in convalescent MIS-C compared convalescent COVID-19 patients (Rybkina et al., 2023) from which they concluded that MIS-C T cells had increased functionality. However, it is important to note that polyfunctional analysis to determine the capacity of these T cells to co-produce 2 or more cytokines was not undertaken. In our study, a similar monofunctional profile was observed in SARS-CoV-2 seropositive inflammatory controls as was seen in MIS-C patients. Two possible explanations for the observed shift towards a monofunctional response are: 1) the superantigen-like properties of the S protein that have been described; and 2) T cell exhaustion due to sustained systemic inflammation. In support of the first, it is plausible that non-specific superantigen-TCR binding (Hoffman, 1990) does not fully engage the TCR,

resulting in suboptimal T cell activation and a skewing towards monofunctional cells producing TNF- α or IL-2. Interestingly, there is a precedent for TNF- α or IL-2 dominance in the T cell responses to other known superantigens (F. R. Shepherd et al., 2023). The highly variable proportions of monofunctional TNF-producing T cells could be explained by different frequencies of TCR V β 21.3⁺ T cells in individual patients, although this requires experimental confirmation. In contrast, it is possible that the monofunctional profile, shared by MIS-C patients and inflammatory controls, results from sustained systemic inflammation that could drive excessive proliferation, differentiation, and possible exhaustion of antigen-specific T cells, leading to a skewed T cell response enriched with monofunctional cells. Indeed, studies have reported increased expression of PD-1 on SARS-CoV-2-specific T cells in MIS-C, possibly indicative of T cell exhaustion (Day et al., 2006; Vella et al., 2021). In this scenario, the highly variable proportions of monofunctional TNF-producing T cells could be explained by different degrees of T cell exhaustion in individual patients.

5.5.1 Conclusion

Our data provide an immunological comparison between children with MIS-C, other inflammatory diseases, and healthy children. At presentation, serum IL-6, IL-10 and CRP were raised in children with MIS-C, and rapidly returned to normal following treatment. SARS-CoV-2-specific antibody titres, antibody-dependent functions, and the magnitude of the SARS-CoV-2-specific T cell responses, however, were not unique in MIS-C. The quality of the T cell response to SARS-CoV-2, however, was compromised in children with MIS-C displaying decreased T cell polyfunctionality which was similar to that observed in non-MIS-C inflammatory controls. These results suggest that the monofunctional T cell cytokine production may be due to non-specific inflammation and not unique to MIS-C.

5.6 Appendix: TCR V β 21.3 expression in children with MIS-C compared to non-MIS-C inflammatory conditions or healthy controls.

This section covers the additional data and analysis that were not included in the publication or supplemental materials.

5.6.1 Introduction

SARS-CoV-2 S protein has been proposed to behave as a superantigen and the protein was reported to encode a high affinity superantigen-like sequence motif near S1/S2 sites. The region containing this motif exhibits high affinity to bind to T cell receptors (TCR) which is similar to *Staphylococcus aureus* enterotoxin B (SEB) (Cheng et al., 2020; Rivas et al., 2021). Superantigens bypass the normal antigen specificity of TCR through direct binding to the invariant region of major histocompatibility class II (MHC-II) molecules and specific TCR variable domain β chains to induce polyclonal expansion and activation of T cells (Amormino et al., 2022; Brodin, 2023; Cheng et al., 2020; Filippatos et al., 2023). Earlier studies have reported on superantigen activity by bacterial proteins, enterotoxins and exotoxin C resulting in expansion of T cells expressing TCR V β and the development of a cytokine storm (Choi et al., 1990; Filippatos et al., 2023; Kum et al., 2000; F. R. Shepherd et al., 2023). It has been reported that SARS-CoV-2 S may cause superantigen-like T cell activation demonstrated by the expansion of four TCRs with distinct variable genes enriched in adults with severe/hyperinflammatory COVID-19 (Cheng et al., 2020). Moreews et al. showed TCR V β 21.3+ T cell expansion in a proportion of children with MIS-C and the expansion of these T cells was considered a discriminating feature of patients with MIS-C compared with patients with KD, TSS and COVID-19 (Moreews et al., 2021). The development of MIS-C is suggested to be attributed by the superantigen-like structure of SARS-CoV-2 S which results in cytokines storm and robust T cell activation and lastly expansion of TCR V β 21.3 cells which is comparable to characteristics of TSS. In this study, we analysed the expression of TCR V β 21.3 on SARS-CoV-2-specific CD4+ and CD8+ T cells in children with MIS-C compared to non-MIS-C groups in a SA cohort.

5.6.2 Methods

All materials and methods used in this study are described in **Chapter 2 section 2.2.3** and **section 2.3**. Study participants included in this study are reported in **Table 5.1** under sample analyzed for T cell response i.e. MIS-C n=8, inflammatory controls n=6 and healthy controls n=24.

5.6.3 Results

5.6.3.1 Flow cytometry - Panel optimization

Multicolor flow cytometry measures multiple fluorescent parameters in single sample including cell surface markers and intracellular markers. Such multiple parameters require optimization of experimental procedures to obtain good resolution of signal. The addition of fluorophores to a flow cytometry panel can potentially influence another fluorophore which can cause loss in resolution.

5.6.3.2 TCR V β 21.3 antibody titration

Fluorochrome-conjugated antibodies are used to detect and quantify cell markers of interest on single cells (Cossarizza et al., 2019). Antibody titration is a crucial recommended step of flow cytometry panel optimization. It is performed to determine the optimal antibody amount and concentration that results in separation of positive and negative populations. It is also essential to minimize background from non-specific binding and ensure staining is done at a saturating concentration to have good resolution of the results (Mair & Tyznik, 2019). Excess antibody staining can result in reduced resolution by binding at low affinity leading to false positive responses. Despite the manufacture's recommendations of the antibody dilution for usage, it is important to consider there are differences in each assay such as cell types, staining conditions, and the flow cytometry instruments to be used (Adan et al., 2017; Mair & Tyznik, 2019).

The fluorochrome-conjugated antibodies were titrated by two-fold serial dilution starting from 2 μ l – 0 μ l, along with T cell lineage as anchor markers. Whole blood processing and cell staining were performed as described in **Chapter 2, section 2.3.1**.

Figure 6.1 illustrates the titration of TCR V β 21.3 antibody. Cells were stained with decreasing volume starting from 2 μ l to 0 μ l of the antibody. The concatenated flow plot examples show TCR V β 21.3 staining against the volume of antibody titrated on whole blood samples (**Figure 6.1**). To select the optimal staining volume for TCR V β 21.3 antibody staining, we compared three parameters, namely frequency of positive population, the MFI and the staining index, calculated as the separation ratio between the positive and negative population over two times the standard deviation of the negative population ($(\text{MFI pos} - \text{MFI neg}) / 2 * \text{SD MFI neg}$). The frequency of TCR V β 21.3+ cells gated on CD4+ and CD8+ T cells were highest at the highest volume (2 μ l) (frequency: 0.919% of CD4+ T cells, **Figure 6.1.A (i)** and 0.288% of CD8+ T cells, **Figure 6.1.B (i)**). The highest MFI of TCR V β 21.3 positive population was observed at 2 μ l for CD4+ (1908.62 MFI units, **Figure 6.1.A (ii)**) and CD8+ (1870.18 MFI units, **Figure 6.1.B (ii)**) T cells. The staining index was found to be highest at 2 μ l volume when gated on CD8+ T cells (5.29; **Figure 6.1.B (iii)**) compared to CD4+ T cells (5.22; **Figure 6.1.A (iii)**) where 1 μ l was the highest staining index at this volume (5.95). The optimal staining volume for TCR V β 21.3 staining was selected as 2 μ l because of the increased frequency and MFI of CD4+ and CD8+ T cells and staining index which provides the brightest staining with minimal background.

Table 6.1: Fluorescence Minus One (FMO) controls. Summary of frequencies of full panel, samples stained with all antibodies in Panel 1 (Chapter 2: Table 2.2) and TCR $V\beta 21.3$ FMO, samples stained with all antibodies besides the TCR $V\beta 21.3$ -FITC.

	CD3+ cells	CD4+ T cells					CD8+ T cells				
	CD3- BV650	CD4- ECD	TCR $V\beta 21.3$ -FITC	IFN- γ - AF700	TNF α - BV786	IL-2- APC	CD8- BV510	TCR $V\beta 21.3$ -FITC	IFN- γ - AF700	TNF α - BV786 6	IL-2- APC
Full panel	38.09	66.46	0.16	12.24	35.49	39.36	24.68	0.09	20.21	21.50	11.34
TCR $V\beta 21.3$ FMO	36.73	65.22	0.002	11.17	35.62	37.96	26.38	0	19.51	21.09	10.54
Fold change	0.96	0.98	0.013	0.91	1.00	0.96	1.06	0	0.96	0.98	0.93
% change	1.36	1.24	0.16	1.07	0	1.40	0	0.09	0.71	0.42	0.81

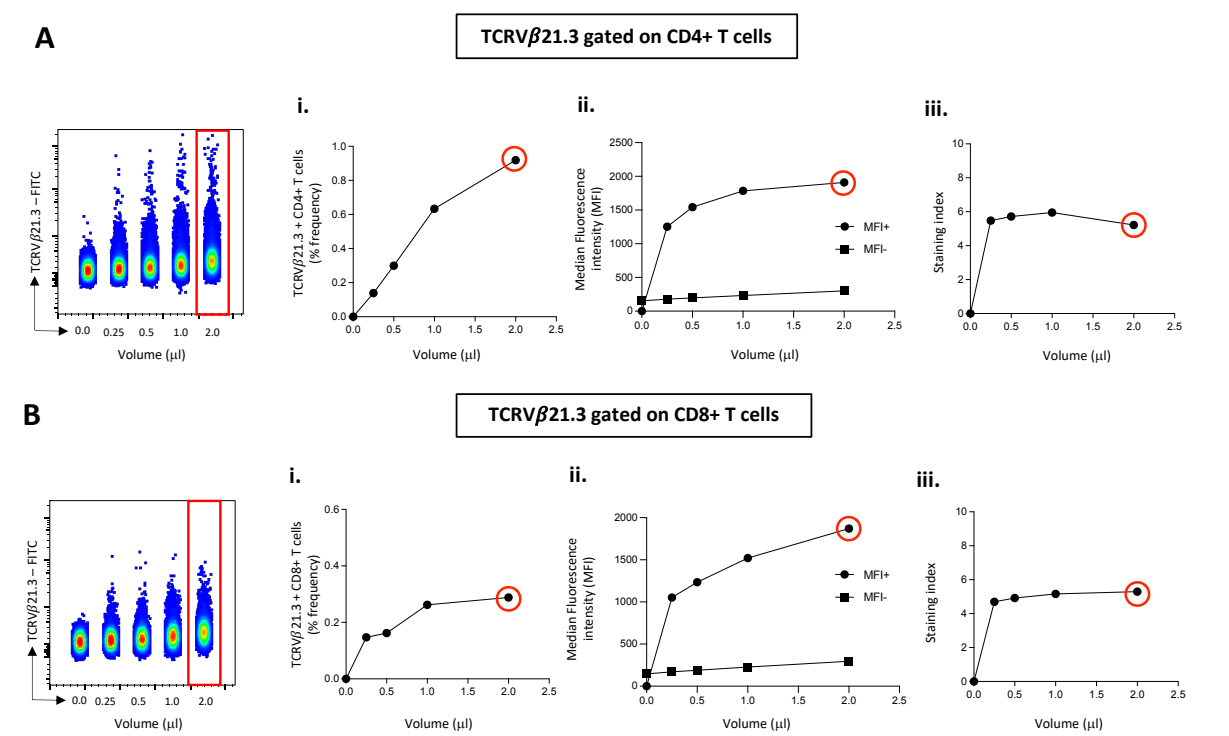


Figure 6.1: Antibody titration of TCR V β 21.3-FITC marker.

(A) Concatenated representative flow cytometry plots of TCR V β 21.3+ CD4+ T cells and **(B)** CD8+ T cells in whole blood samples. **(i)** shows the frequency of positive populations **(ii)** the MFI of positive and negative population and **(iii)** staining index against titration volumes. MFI: Median fluorescence intensity.

5.6.3.3 Fluorescence minus one control of TCR V β 21.3-FITC

Following antibody titration, the flow cytometry panel was optimized using fluorescence minus one (FMO) controls to evaluate any fluorescence spill-over of between channels or unexpected interaction between fluorochromes and for setting gates for separating the negative and positive populations. FMO controls are cells stained with full antibody-conjugated fluorochromes mix expect one antibody/fluorochrome conjugate.

FMO controls were performed for TCR V β 21.3-FITC to further optimize the addition of the marker to the antibody panel to determine if adding TCR V β 21.3-FITC marker into existing panel that contains T cell lineage markers (CD3, CD4 and CD8) and cytokine markers (IFN- γ , TNF- α and IL-2) (shown in **Table 6.1**) does not cause spectral overlap between channels causing false positive results. Additionally TCR V β 21.3 FMO was performed to set gates to identify the positive population (**Figure 6.2**). **Table 6.1** shows the frequency of each target marker in the full panel which includes CD3 BV650 (OKT3, Biolegend), CD4 ECD (SFC112T4D11, Beckman Coulter), CD8 BV510 (RPA-8, Biolegend), TCR-V β 21.3 FITC (REA894, Biolegend), IFN- γ Alexa 700 (B27, Biolegend), TNF- α BV786 (Mab11, Biolegend) and IL-2 APC (MQ1-17H12, Biolegend), while the TCR FMO consists of all the markers besides TCR-V β 21.3 FITC. The fold change represents the change of frequency in the FMO panel relative to the full panel. This shows that the addition of TCR-V β 21.3 FITC had a negligible effect on the frequency of existing markers with fold changes ranging from 0.91 to 1.06 for CD3, CD4, CD8, IFN- γ , TNF- α and IL-2 (**Table 6.1**). **Figure 6.2** shows the flow cytometry plot examples of full panel and for TCR V β 21.3 FMO controls gated on CD4+ and CD8+ T cells. The flow plots visually illustrates the exclusion of TCR V β 21.3 markers from the full antibody panel results in no positive population while inclusion of this marker results in clear

positive population. The panel without TCR V β 21.3 (identified as TCR V β 21.3 FMO) showed only background levels of CD4+ TCR V β 21.3 (0.003%, **Figure 6.2.A**) and CD8+ TCR V β 21.3 (0%, **Figure 6.2.B**) whereas the full panel which included this marker, showed an increase in the frequency of positive populations (0.554% for CD4+ and 0.655% for CD8+ T cells). Overall, this data shows that the TCR signal being detected is a true signal and not an artifact due to spectral overlap from another fluorochrome/antibody conjugate.

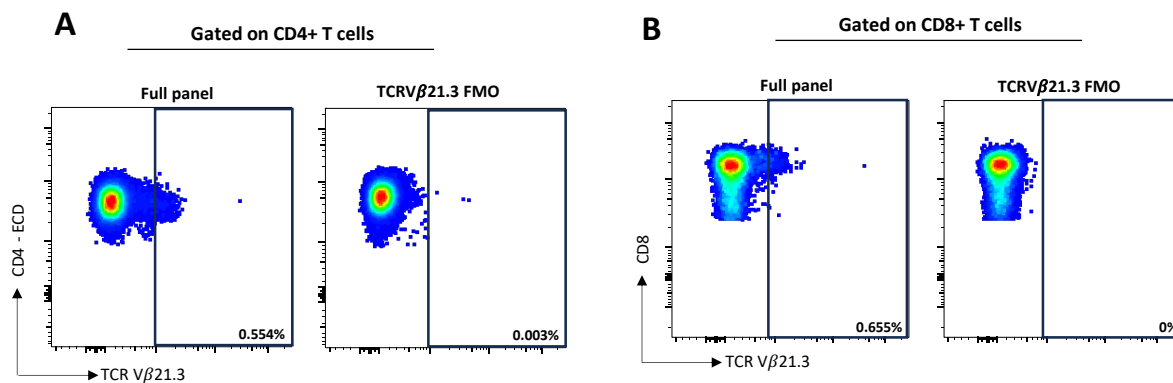


Figure 6.2: Fluorescence minus one (FMO) controls of TCR V β 21.3 on CD4+ (A) and CD8+ (B) T cells on unstimulated cells.

The detection of TCR V β 21.3-FITC on the full panel shown on the (left) and FMO control (right).

5.3.6.4 TCR V β 21.3 expression on CD4+ and CD8+ T cells

TCR V β 21.3 expression on total CD4+ and CD8+ T cells is illustrated in **Figure 6.3.A & Figure 6.3.B**. Total TCR V β 21.3+CD4+ T cells were comparable in MIS-C group when compared to inflammatory controls (median: 0.98% and 1.48%, $p=0.9624$, **Figure 6.3.B**). The frequency of total TCR V β 21.3+CD4+ T cells were significantly reduced in MIS-C group compared to seropositive healthy controls (HC) (median: 0.98% and 2.17%, respectively, $p=0.0065$, **Figure 6.3.B**). We found the frequency of TCR V β 21.3 on total CD8+ T cells was comparable across all groups (MIS-C median: 0.74%, inflammatory control median: 0.78% and HC median: 1.35%, **Figure 6.3.C**). **Figure 6.3.D** shows the representative overlay plot examples of TCR V β 21.3 expression in SARS-CoV-2-specific CD4+ and CD8+ T cells. We detected the expression of TCR V β 21.3 in SARS-CoV-2-specific CD4+ T cells in 100% (8/8) of MIS-C children compared to 83% (20/24) in HC.

The expression of TCR V β 21.3 in SARS-CoV-2-responding CD4+ T cells was comparable between MIS-C and inflammatory groups (median: 27.07% for and 22.39% for MIS-C and inflammatory group, respectively, $p>0.9999$, **Figure 6.3.E**). In contrast, HC showed a decreased proportion of TCR V β 21.3 in SARS-CoV-specific CD4+ T cells when compared to the MIS-C group (median: 4.06% and 22.07%, respectively, $p=0.0004$, **Figure 6.3.E**). In contrast, we found no significant differences in the proportion of TCR V β 21.3 expression in SARS-CoV-2-specific CD8+ T cells between the MIS-C group and the inflammatory or healthy control group, although the sample size is small (**Figure 6.3.F**).

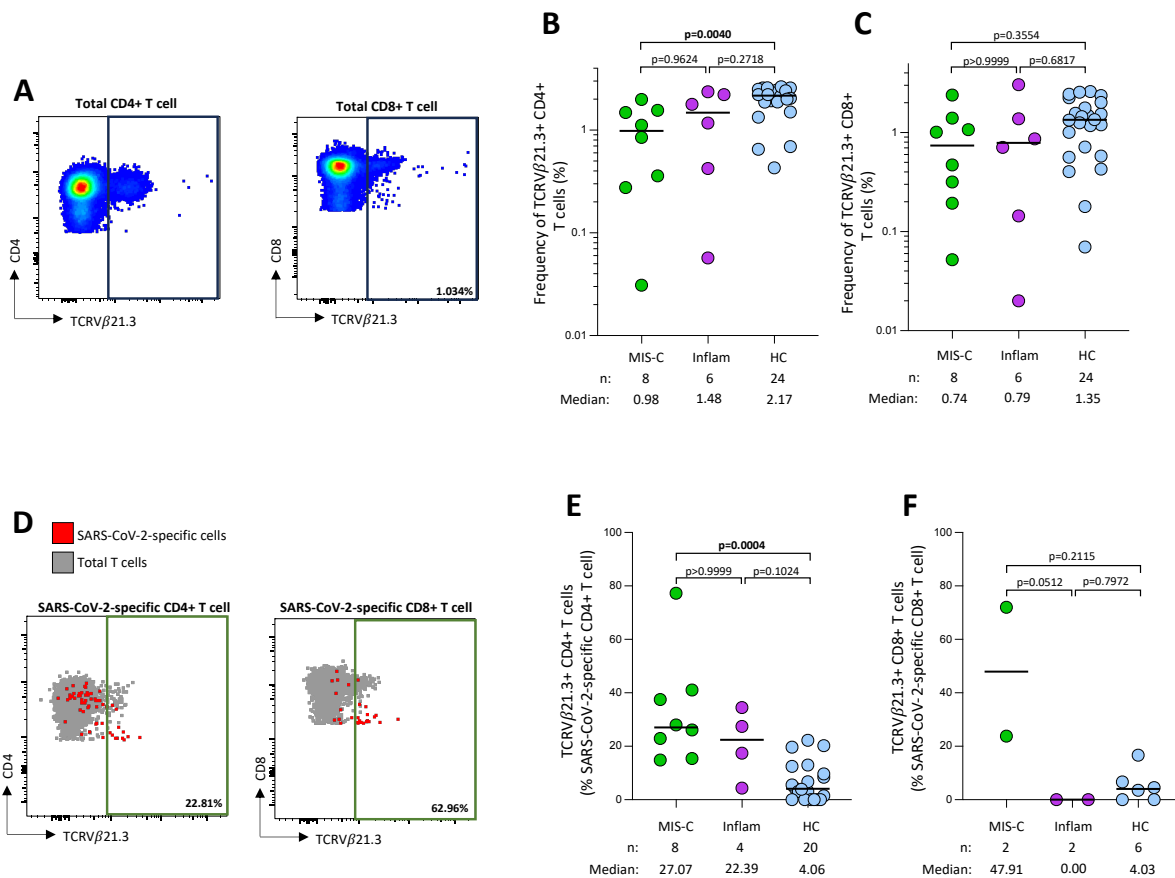


Figure 6.3: TCR V β 21.3 expression on total and SARS-CoV-2-specific T cells.

(A) Representative examples of CD4+ (left) and CD8+ (right) T cells based on expression of TCR V β 21.3 marker. **(B)** Frequency of total TCR V β 21.3+ CD4+ and **(C)** CD8+ T cells in children with MIS-C (n=8), inflammatory

controls (n=6) and seropositive HC (n=24). **(D)** Overlay flow plot examples of TCR V β 21.3 profile in SARS-CoV-2-specific CD4+ (left) and CD8+ (right) T cells. The grey flow plot represents total T cells and red dots depict SARS-CoV-2-specific T cells. **(E)** The proportion of TCR V β 21.3 profiles in SARS-CoV-2-specific CD4+ and **(F)** CD8+ T cells in children with detectable T cell response. Bars represent median and median values are indicated. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's multiple comparisons test; *p* values <0.05 were considered statistically significant and are bolded.

5.6.4 Discussion

To better understand the superantigen-like activity of SARS-CoV-2 S and determine if V β 21.3+ T cells are a distinguishing feature of MIS-C, we compared the expression of TCR V β 21.3 in SARS-CoV-2-specific T cells in SA children. We found TCR V β 21.3+ SARS-CoV-2-responding CD4 T cells in children with MIS-C were comparable to a febrile inflammatory group, although significantly increased in comparison to seropositive HC.

The expansion of distinct T cells with V β TCR chains in response to superantigens is commonly reported in TSS, triggered by bacterial proteins or antigen leading to up to 30% of T cell activation and an uncontrolled proinflammatory cytokine storm (Fraser & Proft, 2008; Kappler et al., 1989; Krakauer, 2019; Rivas et al., 2022; F. R. Shepherd et al., 2023). The overlapping clinical features with TSS and MIS-C namely gastrointestinal symptoms, neurological symptoms, myocardial cardiogenic shock, along with hyperinflammatory syndrome, multisystem manifestations, acute fever and skin rash, had led to the hypothesis that SARS-CoV-2 may possess superantigen activity resulting in the development of MIS-C (Riphagen et al., 2020; Rivas et al., 2021, 2022). A study by Cheng et al. showed adults with severe COVID-19 exhibited a skewing and expansion of TCR V β repertoires characterised by *TRBV5-6*, *TRBV13*, *TRBV14* and *TRBV24-1* genes which was consistent with superantigen activity of SARS-CoV-2 S protein (Cheng et al., 2020). The expansion and skewing of TCR repertoire in adults were associated with hyperinflammatory clinical course and cytokine storms resulting in multiple organ damage similar to MIS-C features (Cheng et al., 2020; Rivas et al., 2022).

Superantigen-like activity mediated by SARS-CoV-2 S protein during MIS-C pathogenesis has been reported by several studies, showing some patients with MIS-C have expansion of *TRBV11-2+* (gene encoding TCR V β 21.3 chain) polyclonal T cells (Hoste et al., 2021; Hsieh et al., 2022b; Lam et al., 2022; D. Lee et al., 2023; Moreews et al., 2021; Porritt et al., 2021a; Porritt et al., 2021b; Ramaswamy et al., 2021; Sacco et al., 2022). Lam et al. reported that children with COVID-19 or hyperinflammatory syndromes and children who met the CDC or WHO criteria and those suspected to have MIS-C by their treating providers, but were negative for SARS-CoV-2 by PCR and serology, all had no expansion of *V β 11-2+* cells, whereas, MIS-C patients positive for SARS-CoV-2 by either PCR or serology had expansion of V β 21.3+ polyclonal cells (Lam et al., 2022). These studies have commonly concluded that expansion of TCR V β 21.3+ cells could be a defining characteristic of the T cell repertoire for children with MIS-C and not patients with paediatric hyperinflammatory syndromes (Lam et al., 2022; Moreews et al., 2021).

In our study, we found 100% of children with MIS-C (8/8) had expression of TCR V β 21.3 in SARS-CoV-2-specific CD4+ T cells, compared with 83% in seropositive HC (20/24) and 67% in the inflammatory group (4/6). Notably, the frequency of TCR V β 21.3 expressing SARS-CoV-2-specific CD4+ T cells was approximately 6-fold higher in children with MIS-C than seropositive HC. Our findings are concordant with several studies that have shown V β 21.3+ cells is a common feature in children diagnosed with MIS-C, although in our study we found no differences in the frequencies of V β 21.3+ SARS-CoV-2-specific T cells between children with MIS-C and inflammatory groups. In contrast to our findings, Lam et al. found patients with MIS-C had significant expression of *TRBV11-2+* T cells compared to children with paediatric hyperinflammatory conditions, KD, systemic juvenile idiopathic arthritis (sJIA) and macrophage activation syndrome (MAS) (Lam et al., 2022). The differences between our study and Lam et al., could be the study cohort, sample size and the techniques used to measure V β 21.3+ cells. Lam et al. applied TCR sequencing to measure the *TRBV11-2* gene encoding V β 21.3 cells, whereas we used antibody-conjugated flow cytometry technique to determine the expression of TCR V β 21.3 in SARS-CoV-2-responding cells (Lam et al., 2022). Secondly, within the inflammatory group in our

study (n=4) children were diagnosed with dysentery, gastroenteritis (n=2) and abdominal sepsis, while Lam 2022 only consisted of sJIA (n=7) and MAS (n=3) as inflammatory controls.

Overall, in this study we found an increase of TCR V β 21.3 expression in SARS-CoV-2-responding T cells in the MIS-C group in comparison to seropositive HC but not inflammatory controls. Thus, in our cohort TCR V β 21.3+ alone is not a good differential for MIS-C but could be used in conjunction with other markers. It is noteworthy to point out, this conclusion is based on small sample size and requires validation in a larger cohort or TCR sequencing to strengthen the conclusion.

CHAPTER 6: DISCUSSION AND CONCLUDING REMARKS

Since the start of the Coronavirus disease 2019 (COVID-19) pandemic, there has been extensive research on the immune responses to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, which has provided useful scientific and real-time data for understanding and controlling SARS-CoV-2 infection, for better management of the COVID-19 global health emergency (Bertoletti, 2024). Early studies reported that the risk factors for susceptibility to SARS-CoV-2 infection include age, biological sex and pre-existing comorbidities (J. jin Zhang et al., 2023; F. Zhou et al., 2020). Children typically presented with milder COVID-19 disease than adults. Milder illness following Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) infection have also been reported to be common in children (Aleebrahim-Dehkordi et al., 2021; Bartenfeld et al., 2017; Iannarella et al., 2020; Stockman et al., 2007). This resulted in the underreporting of incident cases due to fewer SARS-CoV-2 testing practices in this population (Amodio et al., 2022). Of note, early in the COVID-19 pandemic in April 2020, a hyperinflammatory syndrome as a notable COVID-19 complication in children, was reported and later described as multisystem inflammatory syndrome in children (MIS-C) (Amodio et al., 2022; Brodin, 2021; Riphagen et al., 2020; Rotulo & Palma, 2023). In contrast to the relatively mild course of COVID-19 in children, it is widely known that children are more susceptible to acute respiratory infections caused by a range of respiratory viruses including endemic human coronaviruses (HCoVs), which contributes to the mortality rates in children under 5 years of age (Nair et al., 2011; Tregoning & Schwarze, 2010). Although, specific mechanisms driving lower COVID-19 severity in children and young adults remain undefined, several theories have been proposed which may contribute to resistance to SARS-CoV-2 infection in this population namely 1) less chronic conditions, 2) differences in expression levels of viral entry factors, 3) more robust innate immune responses, 4) pre-existing immunity to HCoVs, and 5) differences in T cell homeostasis and activation (Chou et al., 2022; Kang et al., 2022; Pierce et al., 2022; Zimmermann & Curtis, 2021). This thesis specifically investigated factors 4 and 5.

Here, this study aimed to characterize SARS-CoV-2-specific immune responses and cross-reactivity to HCoVs in pediatric cohorts compared to adults. The study was accomplished by using three different cohorts, 1) a cross-sectional cohort (Red Cross MIS-C sub-study) that included

children diagnosed with MIS-C, non-MIS-C inflammatory diseases and healthy controls, 2) a longitudinal cohort of child-mother pairs recruited from a cohort established in 2015 (Drakenstein Child Health Study: DCHS), which follows children and mothers every year since birth and 3) a cross-sectional adult cohort consisting of healthcare workers (HCW) who were COVID-19 convalescent, based at the Groote Schuur hospital (GSH) in the Western Cape. Using these cohorts, we evaluated SARS-CoV-2-specific T cells responses in South African (SA) children, including SARS-CoV-2 seropositive and seronegative healthy children as well as those diagnosed with MIS-C and febrile paediatric inflammatory diseases. This study measured the quantitative and qualitative changes (polyfunctional and memory differentiation profiles) of SARS-CoV-2-specific T cells in children and compared them to adults. The study also assessed the maintenance of the T cell responses over a 16-month period and cross-reactive immunity to SARS-CoV-2 variants of concern (VOC) in children. Finally, in a small cohort of children diagnosed with MIS-C, we examined the immune determinants of MIS-C on SARS-CoV-2-specific T cell responses and the expression of the TCR V β 21.3 receptor on SARS-CoV-2-responding T cells.

Cross-reactive immunity can be beneficial by providing protection against secondary infections, or it can be detrimental by causing immunopathology due to ineffective immune responses (Jarjour et al., 2021; Lipsitch et al., 2020; Murray et al., 2023; Sette & Crotty, 2020). SARS-CoV-2 and common cold endemic HCoVs have been reported to share over 65% of sequence homology, suggesting that pre-existing immunity from prior infections with endemic HCoVs may play an important role in disease outcome (C. H. Lee et al., 2020; Mateus et al., 2020; Murray et al., 2023) and has been postulated to confer protection against severe COVID-19 illness. However, this may be difficult to confirm as both children and adults may have some degree of pre-existing immune memory to HCoVs (Amodio et al., 2022; Brodin, 2022; K. W. Ng et al., 2020; Sette & Crotty, 2020). Indeed, in Chapter 3 of this thesis, we aimed to investigate the impact of pre-existing immunity to endemic HCoVs on SARS-CoV-2 T cell immunity by quantifying SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses in a cross-sectional cohort that consisting of 41 seropositive and 30 seronegative children. We found 60% of SARS-CoV-2 seronegative children had detectable SARS-CoV-2-specific CD4⁺ T cells and the polyfunctional profile of SARS-CoV-2-responding CD4⁺ T cells

was different between seropositive and seronegative children, with a skewing towards a monofunctional T cell profile, mainly interferon gamma (IFN- γ) producing cells, in seronegative children. Moreover, we also found the frequency of SARS-CoV-2-responding CD4⁺ T cells correlated with HCoV-HKU1 antibody responses in seronegative children. Our findings are further supported by several studies that have shown that cross-reactive T cell responses from endemic HCoVs were detectable in SARS-CoV-2 unexposed donors (Bacher et al., 2020; Braun et al., 2020; Jay et al., 2023; Kundu et al., 2022; Le Bert et al., 2020; Loyal et al., 2021; Nelde et al., 2021; Saletti et al., 2020; C. C. S. Tan et al., 2021; Woldemeskel et al., 2020) including children and adolescents (Dowell et al., 2022a; Humbert et al., 2023; K. W. Ng et al., 2020; Niessl et al., 2021b; Selva et al., 2021; Woudenberg et al., 2021). It has thus been suggested that pre-existing immunity may translate into protective effects from SARS-CoV-2 infection. In accordance with our results, Dowell et al. showed that SARS-CoV-2 seronegative children with high SARS-CoV-2-specific responses had increased antibody levels to at least one of 4 endemic HCoVs, indicative of recent HCoV infection (Dowell et al., 2022a).

Cross-reactive SARS-CoV-2 T cell responses in seronegative children are possible due to T cell recognition of the conserved epitope regions which share sequence similarities between HCoV-HKU1 and SARS-CoV-2 spike (S) proteins. Despite SARS-CoV-2 peptide similarity, that can potentially be recognized by HCoV memory T cells, lack of 100% sequence homology may result in reduced peptide binding affinity and a different functional T cell profile (Jarjour et al., 2021; Mateus et al., 2020; Murray et al., 2023; Nelde et al., 2021; Spear et al., 2019; M. P. Tan et al., 2015). This suggests that the monofunctional profile of SARS-CoV-2-specific CD4⁺ T cells in seronegative children may be mediated by pre-existing immunity from primary infection with endemic HCoVs with partial sequence homology to SARS-CoV-2. It is noteworthy to report that we did not find an association between beta HCoV-OC43 IgG responses and SARS-CoV-2-specific CD4⁺ T cells, which may be related to differences in the seasonal prevalence of circulating HCoVs. Indeed, several epidemiological studies have documented that viral infections caused by HCoVs are different depending on the geographical region. Globally, HCoV infection peaks during winter season and is most prevalent in children (Audi et al., 2020; Friedman et al., 2018; S. Park et al.,

2020; Shah et al., 2022), whereas in SA, HCoVs circulate throughout the year, with peak infections of beta HCoVs occurring in the colder months of May to August (Baillie et al., 2021). Overall these results suggests SARS-CoV-2 T cells detected in SARS-CoV-2 seronegative children could be a result of cross-reactive immunity to recent endemic HCoV infection which may contribute towards lower risk of severe COVID-19 in children and by conferring partial protection against SARS-CoV-2.

In Chapter 4 of this thesis, we sought to confirm these findings in a separate cohort and also characterize the phenotypic profile of SARS-CoV-2-responding T cells, which was not addressed in Chapter 3. In this second cohort, we also found that seropositive and seronegative children have a comparable magnitude of SARS-CoV-2-specific T cell responses, although we found some discordance in the percentage of CD4 responders between the cohorts. In Chapter 3 and Chapter 4 of this thesis, we found that 60% and 28% of seronegative children, respectively, had detectable SARS-CoV-2-specific T cell responses. Secondly, the overall quality (polyfunctionality) of SARS-CoV-2-specific CD4⁺ T cells was different between seropositive and seronegative children for both cohorts, although we found no differences in the proportion of CD4⁺ T cells producing different combinations of cytokines in the second cohort. These differences could be attributed to the dissimilarities in the demographic characteristics of the two cohorts, including age and municipality of residence. Although the median age in both studies was 8 years, the first cross-sectional cohort had a wide age range of 3 months - 15 years compared to the second cohort that had a smaller age range of 5-8 years. Typically, children younger than 5 years old have been reported to have the higher risk of respiratory viral infection and hospitalizations compared to children older than 5 years and adults (Izurieta et al., 2000; Meury et al., 2004; Sakkou et al., 2011; H. Zhou et al., 2012). Therefore, we speculate that children in the cross-sectional cohort in Chapter 3, in which 32% (23/71) were under 5, may have had more recent infections with endemic HCoVs compared to the cohort in Chapter 4, thereby accounting for the increased proportion of cross-reactive SARS-CoV-2 responders in this more age variable cohort. In addition to age related differences in HCoV infections, the different regional municipality locations from which the cohorts are recruited may also contribute to differences in the proportion of SARS-CoV-

2 responders in the seronegative children in the two cohorts. The children from the DCHS cohort were enrolled with the mothers (1000 mother-child pairs) as part of population-based birth cohort established in 2014 in Paarl, Western Cape, SA, which follows the mother-child pairs annually since birth. The study assesses the epidemiology, risk factors, and long-term impact of early childhood lower respiratory diseases (Zar et al., 2015), potentially resulting in better primary healthcare than typical for similar socioeconomical groups. It is important to highlight that we did not measure the endemic beta-HCoVs IgG responses in the second cohort presented in Chapter 4 to contextualize the association with SARS-CoV-2-specific T cells.

Furthermore, we reported in Chapter 4, that the memory differentiation of SARS-CoV-2-specific CD4⁺ T cells were predominantly early differentiated (ED:CD45RA-CD27⁺) memory cells in both SARS-CoV-2 seropositive and seronegative children. In contrast, in the CD8 compartment, seropositive children exhibited higher proportion of ED memory CD8⁺ responding cells, while seronegative children were skewed more towards the effector (CD45RA⁺CD27⁻) CD8⁺ memory subset. This distinct profile of CD8⁺ T cell memory profiles in seronegative children could be a result of repeated exposures or infection with endemic HCoVs. Human immunodeficiency virus (HIV) and Cytomegalovirus (CMV) studies have shown that ongoing exposure during chronic or repeated infections drives the CD8 memory T cells towards a more differentiated effector phenotype (Appay et al., 2002; Day et al., 2006; McLane et al., 2019). These differences in memory phenotype, together with disparities in polyfunctionality and the correlation between T cell responses and HKU1 antibodies, supports the theory that the SARS-CoV-2 responding T cells in seronegative children are possibly due to recent or repeated exposure to HCoVs.

The second major focus of this thesis was to analyse adaptive immunity in children compared to adults as a potential driving factor behind lower disease severity in children. In the first part of our study reported in Chapter 3, we demonstrated that seropositive children have significantly lower SARS-CoV-2-specific T cell responses compared to COVID-19 convalescent unvaccinated adult HCW. These findings were consistent with several previous studies (C. A. Cohen et al., 2021; Goenka et al., 2021; Jacobsen et al., 2022; Kaaijk et al., 2022; Nattrass et al., 2022; Pierce et al.,

2020; Rowntree et al., 2022) but discordant with others (Dowell et al., 2022a; Khoo et al., 2023; Tosif et al., 2020). Due to the discordance in the literature, we questioned whether this was a true biological difference or a product of poorly matched opportunistic cohorts. Cohort related causes for differences in the magnitude of responses between children and adults and discordance in the literature could be due to multiple reasons, three of which are, 1) differences in the timing of SARS-CoV-2 infection, 2) number of infections prior to sampling and 3) differences in disease severity. Indeed, the RC children's cohort and HCW cohort reported on in Chapter 3 may have been mismatched. Although both children and adults had similar disease severity, the timing between infection and sampling or the number of exposures could not be confirmed. The adults had recent SARS-CoV-2 infections as evidenced by the PCR-confirmed data, whereas children had no PCR-confirmed infection leaving the possibility open that they may have had a more distant SARS-CoV-2 infection. Additionally, public health measures in SA, including school closures, were still in place around the peak of second and third waves during the time-period at which the baseline (BL) sample was taken. Thus adults, particularly HCWs, during that period would likely have been exposed or infected more than once prior to the initial visit of our study. In fact, a study by Cohen C. et al. (2022) reported that children and adolescents younger than 19 years were reported to have less successive SARS-CoV-2 infections during the first infection wave (42.7% of individuals infected) and second waves (49.3% of infections), which was 1.5-fold higher after the third wave (65.7% of infections) when the restrictions were relaxed (C. Cohen et al., 2022). SARS-CoV-2 seroprevalence in children was lower with approximate of 18% and 28% in rural and urban communities respectively, in comparison to adults ages 19-59 years (37% and 59% in rural and urban community, respectively), post the second wave in March 2021, in SA (Kleynhans et al., 2021; Sun et al., 2022). The number of SARS-CoV-2 exposures has been widely reported to impact the virus-specific immune phenotypic characteristics including memory differentiation and polyfunctional profiles (Cai et al., 2023; Kared et al., 2022; Keeton et al., 2023; Koutsakos et al., 2022, 2023; Minervina et al., 2022; Nicolas et al., 2023; Painter et al., 2023). This may imply the differences in magnitude of responses might be due to the number of exposures to SARS-CoV-2 in adults during the lockdown while children were still at household settings.

To control for cohort confounders, we quantified SARS-CoV-2-specific T cells responses in a cohort of child-mother pairs in Chapter 4, where we included child-mother pairs living in the same household to limit the possibility of discrepancies in the timing and number of exposures to SARS-CoV-2 infection in the child and adult cohort. In support of this, it is worth reiterating that during the time of sampling during the first visit, SA was still under a strict lockdown for public health measures, therefore children had limited in-person school activities, and were presumably infected or exposed at the same time and frequency by their mothers. In this cohort, despite similar disease severity and theorized similar timing and number of infections between children and mothers, we still reported that children had lower magnitude of SARS-CoV-2-specific T cell responses compared to adults, in this case their mothers. Thus, increasing the probability that the differences between the children and mothers were a true biological difference.

Strikingly, we found that a small proportion (36%) of children living in the same household as their seropositive mothers were seronegative, despite the possibility of similar SARS-CoV-2 exposure. Three reasons may account for this resistance to infection, namely, 1) possibly early rapid viral clearance by innate immunity, 2) early abortive SARS-CoV-2 infection, and/or 3) cross-reactivity to endemic HCoVs. The lack of SARS-CoV-2 antibody responses in SARS-CoV-2 exposed seronegative children could be related to stronger rapid innate and local immune responses resulting in early clearance of SARS-CoV-2 infection prior to the induction of adaptive immunity, which has been reported by several studies (de Candia et al., 2021; Loske et al., 2022; Muus et al., 2021; Neeland et al., 2021a; Neeland et al., 2021b; Pierce et al., 2021; Tosif et al., 2020; Vono et al., 2021; Yoshida et al., 2021). Lack of seroconversion in these children may also be as a result of early abortive SARS-CoV-2 infection as has been reported in SARS-CoV-2 exposed seronegative (repeatedly negative) HCW (Swadling et al., 2021). Swadling and colleagues reported that seronegative HCW had increased SARS-CoV-2-specific T cells targeting replication-transcription complex (RTC) consisting of non-structural proteins, along with robust early innate signature *IF127*, an IFN-inducible marker of early infection. Additionally, SARS-CoV-2 RTC-specific CD4+ and CD8+ T cell epitopes with higher sequence conservation with endemic HCoVs were found in seronegative HCW, suggesting that clonally expanded memory T cells were specific for RTC from

prior HCoVs infection (Swadling et al., 2021). In addition to this being documented in adults, a study by Rowntree et al. showed that SARS-CoV-2 exposed seronegative children had naïve SARS-CoV-2 tetramer-specific T cells and diverse T-cell receptor-(TCR) $\alpha\beta$ repertoires capable of selectively recruiting and recognizing multiple epitope-specific T cells, possibly RTC-specific T cells (Rowntree et al., 2022). Thus, SARS-CoV-2 exposed seronegative children without detectable SARS-CoV-2-SNM specific T cells may have had early abortive SARS-CoV-2 infection before the priming of the adaptive immune responses and those with cross-reactive T cell responses may be a result of prior infection with endemic HCoVs as discussed previously.

In the children that did seroconvert, a potential factor contributing to reduced SARS-CoV-2 T cell responses compared to adults could be age-related differences in adaptive immune maturation, such that children respond to new antigens with lower responses as a result of higher naïve T-cell populations, compared to adults with more-experienced immune system which has been exposed to different antigens (Briceño et al., 2016; B. V. Kumar et al., 2018; Van Epps et al., 2014; Yoshida et al., 2021). Indeed, we showed a significantly higher proportion of naïve T cells and lower proportion of ED memory T cells in convalescent children compared to their mothers. Previously, naïve B and T cells have been shown at high frequencies in SARS-CoV-2 infected children compared to infected adults. This was also accompanied by diverse TCR repertoires capable to recognizing multiple epitopes of the viral proteins leading to early adaptive immunity (Khoo et al., 2023; Yoshida et al., 2021).

Further analysis of SARS-CoV-2-specific memory phenotypic features revealed ED (CD45RA-CD27+) memory CD4+ T cells as the dominant memory subset in both children and mothers. In accordance with our findings, several studies have reported that SARS-CoV-2-specific memory subsets were comprised predominantly of ED (CD45RA-CD27+) (Moyo-Gwete et al., 2023), central memory (TCM) defined as CD45RA-CCR7+ or CD45RA-CD27+CCR7+ (Weiskopf et al., 2020) and effector memory 1 (EM1: CD45RA-CD27+CCR7-) cells (Goel et al., 2021b; Painter et al., 2021) depending on the memory markers used. Of note, in our study due to the exclusion of CCR7 in our flow cytometry panel, our ED (CD45RA-CD27+) memory subset would comprise both TCM

(CD45RA-CD27+CCR7+) and EM1 (CD45RA-CD27+CCR7-). Fewer studies have shown the memory differentiation profile of SARS-CoV-2-specific T cells in children compared to adults (C. A. Cohen et al., 2021; Jacobsen et al., 2022; Khoo et al., 2023; Rowntree et al., 2022). Although the ED memory subsets predominated in both children and adults, there were subtle differences with significantly higher proportion of naïve-like cells and significantly lower proportion of ED cells in children compared to adults. Similar to our findings, a study by Rowntree et al. showed that children displayed lower TCM-like cells (CD45RA-CD27+) and increased stem-cell memory (TSCM:CD45RA+CD27+CD95+) SARS-CoV-2-specific T cells compared to adults (Rowntree et al., 2022). The TSCM subsets are rare memory population with large resemblance of naïve phenotype although overexpressing CD95 antigen (death receptor), also possess stem cell-like self-renewal properties, multipotent capacity (Farber et al., 2014; Gattinoni et al., 2017; Mahnke et al., 2013). These findings are somewhat concordant with our findings, although we do not include the CD95 marker to differentiate our naïve cells from TSCM cells. Together this data suggests that SARS-CoV-2 infection in children, induces long-lived memory cells albeit lower than adults. These ED memory subsets are associated with high proliferation and self-renewal capacity and protective immunity from reinfections (Künzli & Masopust, 2023; Mahnke et al., 2013; Sallusto et al., 2004).

Polyfunctional T cells have been associated with superior functional capacity and have been shown to effectively control viral infections (Almeida et al., 2007; Betts et al., 2006; Ciuffreda et al., 2008; Collins et al., 2020; Kannanganat et al., 2007; Seder et al., 2008; Snyder et al., 2016). Despite children having a lower magnitude of SARS-CoV-2-specific T cells the quality of that response as defined by their polyfunctionality was not distinct between seropositive mothers with seropositive children and seronegative children. In accordance with our findings, Cohen C. A. et al. (2021) reported that the cytokine polyfunctionality of SARS-CoV-2 structural-specific T cells was comparable between convalescent children and adults after 2 months post infection (C. A. Cohen et al., 2021). Together this data suggests that SARS-CoV-2 infection in children results in reduced magnitude but similar quality of T cell responses which might be attributed by the age-associated naïve-phenotypes and robust innate immunity in this population compared to adults.

Overall, the reduced magnitude responses in children compared to adults raised two major concerns; 1) would these lower responses maintain durability, and 2) would cross-reactivity to variants of concern be as robust as previously reported in adults. Thus, we next assessed durability of SARS-CoV-2-specific T cell responses over a 16-month period. Here we demonstrated that children previously exposed to SARS-CoV-2 at BL, had an increased proportion of responders after 16 months, although magnitude within the responders was similar. Our findings are somewhat discordant with those reported by Jacobsen et al. whereby they found detectable but significantly reduced SARS-CoV-2-specific CD4⁺ T cell responses after 8 months, although they did report that these responses persisted over time (Jacobsen et al., 2022). In fact, several studies have reported a reduction in the magnitude of SARS-CoV-2-specific cellular responses 10 to 12 months post-infection in children younger than 19 years (Dowell et al., 2022a; Jacobsen et al., 2022; Kaaijk et al., 2022; Nattrass et al., 2022; Sieber et al., 2022). The reduction of responses reported by these studies might be, for instance, waning immunity as previously reported by Dan et al. (Dan et al., 2021) and Cohen K. W. et al. (K. W. Cohen et al., 2021) or migration from peripheral to secondary lymphoid tissues (Grau-Expósito et al., 2021; Niessl et al., 2021a; Poon et al., 2021; Sette et al., 2023). Our assessment of durability was confounded by the likelihood of repeated exposure to SARS-CoV-2 during the three waves of SARS-CoV-2 dominated by Delta, Omicron BA.1/2 and BA.4/5 variants in SA, between sampling time. This could account for the increased proportion of responders we detected over 16 months. The possibility of new exposures to SARS-CoV-2, was supported by the gaining of SARS-CoV-2-specific CD4⁺ T cell responses in children that had been seronegative at BL at the 16-month time-point. In contrast to our SA study, in the study by Jacobsen et al. only one infection wave occurred in Germany between sampling (Jacobsen et al., 2022; Mitze & Rode, 2022; Stich et al., 2021), whereas three waves of infection occurred between (Tegally et al., 2022; W. Yang & Shaman, 2022).

The polyfunctional profile of SARS-CoV-2-specific CD4⁺ T cells in BL seropositive children was distinct between the initial visit and 16-month sampling, with a reduced proportion of cells co-producing IL-2+TNF- α + counterbalanced by an increase of virus-specific CD4⁺ T cells producing IFN- γ + and IFN- γ +IL-2+ cytokines at the second compared to the first visit. Strikingly, a study by

Keeton et al. (2023) that specifically analysed the evolution of SARS-CoV-2 T cell responses to repeated exposure to SARS-CoV-2 infection and vaccination in adults showed concordant results, with a reduced proportion of cells co-producing IL-2+TNF- α + and an increase in IFN- γ +IL-2+ cytokines in response to increasing numbers of exposures (Keeton et al., 2023). PCR-confirmed SARS-CoV-2 infection, serial nucleocapsid (N) ELISAs and vaccination dates in the Keeton et al. study corroborate that the evolution of the polyfunctional profile detected in both their study and by inference, the current study, was a result of multiple exposures (Keeton et al., 2023). This could be attributed to the production of IL-2 in autocrine manner to promote expansion of memory T cell development, survival and function (Keeton et al., 2023; McKinstry et al., 2014; Nattrass et al., 2022; Seder et al., 2008; M. A. Williams et al., 2006). We thus further determined the memory differentiation profile of SARS-CoV-2-responding T cells in children with responses at BL and V2. We found a dominance of ED memory subsets at both time-points, although there was an increase in ED and a decrease in naïve-like proportion at 16 months compared to BL. We and others have previously shown that SARS-CoV-2-S re-exposure by vaccination boosting induced an increase in ED memory cells (Moyo-Gwete et al., 2023) or EM1, a subset of ED cells (Goel et al., 2021b). The dominance of ED memory subsets in children, suggests long-lived memory T cells that may provide immunological memory for rapid recall responses upon re-infections (Mahnke et al., 2013; Siggins et al., 2021). Most notably, a study by Goel et al. demonstrated that an increased proportion of EM1 cells, a subset of ED memory T cells, at the peak of the CD4 T cell response was associated with improved durability (Goel et al., 2021b). Although we were not able to directly measure durability due to the possible repeat exposures, our results highlight that SARS-CoV-2-specific T cells present after 16 months are characterized by long-lived ED memory T cells that may confer protection upon re-infection, even in the absence of COVID-19 vaccination in children (Mahnke et al., 2013; Painter et al., 2021; Sallusto et al., 2004).

The emergence of Omicron BA.1/BA.2 variants by the end of 2021, resulted in increased infectivity, and great humoral immune escape, which caused a challenge on the long-term immunity induced by prior infection or first-generation vaccination (Carabelli et al., 2023; Petrone

et al., 2023). Numerous studies have demonstrated that T cell immunity induced by previous SARS-CoV-2 infection and/or vaccination is cross-reactive with SARS-CoV-2 variants in adults (Collier et al., 2022; Gao et al., 2022; Geers et al., 2021; GeurtsvanKessel et al., 2022; Goel et al., 2021b; Keeton et al., 2022, 2023; Müller et al., 2024; Naranbhai et al., 2022; Nesamari et al., 2024; Reynolds et al., 2021; Riou et al., 2021; Tarke et al., 2022a; Tarke et al., 2021b). In fact, recent studies by Nesamari et al. and Müller et al. showed that CD4+ and CD8+ T cell responses were cross-reactive to Omicron BA.2.86 subvariant a highly mutated variant with 30 additional mutations in S protein compared to Omicron BA.2, in individuals that were both vaccinated and also had had prior SARS-CoV-2 Omicron infection (Müller et al., 2024; Nesamari et al., 2024).

In this study, we measured cross-reactivity to Delta and Omicron BA.1 S cross-sectionally at the 16-month time-point. In the design of our study, children who were seropositive at BL had their first exposure to either Ancestral or Beta variant, while those that were seronegative at BL but had been exposed before the 16-month sampling and had been first exposed to either Delta or Omicron BA.1. By comparing children who had been SARS-CoV-2 seropositive at the initial visit to those that were seronegative, allowed us to better evaluate cross-reactivity. During childhood, immune memory cells develop over time as result of diverse antigen encounter and accumulate throughout life before reaching plateau at adulthood (Farber et al., 2014; B. V. Kumar et al., 2018). The initial antigen exposure to influenza infection during childhood determines the recalled immune responses later in life upon re-exposure to influenza strains or vaccinations, this was described as the original antigenic sin by Thomas Francis in 1960 (Francis, 1960) and the recalled responses may contribute to protection through immunological imprinting of conserved epitopes shared from the first-ever exposure to new viral strains (Aguilar-Bretones et al., 2023; Chemaitelly et al., 2022; Cobey & Hensley, 2017; Gostic et al., 2016; Monto et al., 2017; Vatti et al., 2017; A. Zhang et al., 2019). It has been speculated that antigenic sin or immune imprinting by prior exposure to ancestral or Wuhan strain or first-generation COVID-19 vaccines may result in reduced *de novo* responses against SARS-CoV-2 VOC infections (Aguilar-Bretones et al., 2023; Chemaitelly et al., 2022; Rijkers & van Overveld, 2021; Roncati & Palmieri, 2020; Torresi & Edeling, 2024; Wheatley et al., 2021; Z. Zhou et al., 2023). Antigenic sin or immune imprinting through the

first exposure of SARS-CoV-2 infection in children might provide viral-specific long-lived immunity against SARS-CoV-2 variants (Chou et al., 2022; Pierce et al., 2022).

We demonstrated that overall, T cell responses can cross-recognize SARS-CoV-2 Delta and BA.1 variants irrespective of the variant of their primary exposure. The majority of children with possible primary infection with either ancestral or Beta variant (77%) that had an ancestral response had preservation of CD4+ T cell responses to Delta and Omicron BA.1, suggesting that T cells induced from prior infection were directed against epitopes conserved within Delta and BA.1. Whilst a small proportion of children (47%) likely infected with Delta/Omicron variants as primary infection had no detectable CD4+ responses against ancestral S subsequently gained responses (66%) to Delta and BA.1 variants, likely reflecting *de novo* generation of CD4 responses to mutated S epitopes in variants. The increased responses could potentially confirm that some of these children were exposed to and/or infected with Delta and/or BA.1 variants over 3 infection waves. We also demonstrated that a small fraction (16%) of children likely with primary infection with ancestral or Beta variants had stable CD8 responses against all three tested variant S and 10% had no ancestral response, 2 gained responses to Delta and BA.1 variants, this indicates there is less robust preservation of CD8+ T cells. Importantly, we also observed that 21% (4/19) of children that were possibly first exposed to Delta or Omicron, had increased or new CD8 responses to BA.1 S compared to Ancestral S, implying that potentially these children were exposed to or infected with BA.1 variant. Furthermore, the quality of SARS-CoV-2-S specific T cell responses against Ancestral, Delta and B.A.1 variants was assessed by polyfunctional cytokine co-expression in children with ancestral/ Beta as primary infection and those likely infected with Delta/Omicron as primary infection, showed no changes in the overall profiles, suggesting that cross-reactive T cell responses does not impact the prominent cytokine production. Our findings are concordant with previous cross-reactivity studies focused on children and adolescents (Lan et al., 2023; Paniskaki et al., 2023; Paul et al., 2022; Saggau et al., 2022; Seidel et al., 2023; Sieber et al., 2022). Similar to our findings, Sieber et al. reported that SARS-CoV-2 seropositive children had comparable CD4+ and CD8+ T cell responses against SARS-CoV-2 Wildtype and Omicron BA.1 (B.1.1.529) variants, suggesting that T cell recognition to the Omicron BA.1 variant (Sieber et al.,

2022). Our findings highlight the preservation of T cell responses across Delta and BA.1 variants in children. This may be a result of T cell targeting of immunodominant epitopes which are conserved between variants and/or viral mutations creating new immunogenic epitopes in Delta and/or BA.1 variants which compensate for lost ancestral epitopes.

A rare COVID-19 complication in children is the development of MIS-C, a post-infectious hyperinflammatory syndrome and it has been suggested that T cells play a role in mediating MIS-C immune dysfunction (Brodin, 2022, 2023; Riphagen et al., 2020). It was therefore important to determine the role of adaptive immunity in MIS-C patients in comparison to other pediatric inflammatory conditions. In Chapter 5 of this thesis, we quantified SARS-CoV-2-specific T cell responses in children diagnosed with MIS-C (based on WHO criteria) and compared them to SARS-CoV-2 seropositive children with clinically similar paediatric febrile diseases (non-MIS-C) and healthy SARS-CoV-2 seropositive children. We found that children with MIS-C mounted SARS-CoV-2-specific T cells similarly to non-MIS-C children and seropositive healthy children. Our findings are supported by several studies which have reported that children with MIS-C mount comparable (Lam et al., 2022; Pierce et al., 2020) SARS-CoV-2-specific T cells when compared to healthy children but discordant with other studies reporting higher responses (Conway et al., 2022; Rybkina et al., 2023) or lower responses (Moreews et al., 2021; Singh et al., 2022).

Additionally, we also showed that the quality of SARS-CoV-2-responding CD4⁺ T cells in children with MIS-C was skewed toward a monofunctional profile of IL-2 or TNF- α single-producing cells, which was similar to that of non-MIS-C but distinct from that of healthy seropositive children. Our findings are in contrast to a study by Rybkina and colleagues that reported children recovered from MIS-C had increased SARS-CoV-2-specific CD4⁺ T cells producing IL-2 cytokine and CD8⁺ T cells producing TNF- α and IFN- γ compared to convalescent COVID-19 children and suggested that MIS-C recovery is associated robust functional memory Th1 responses for future subsequent immune protection. The differences of the functional profile between studies could be the study cohorts. In our study, we included children presenting with MIS-C, prior to receiving treatment

while in the Rybkina et al. study, the children that recovered from MIS-C were outpatient from 0.5-19 months post infection (Rybkina et al., 2023).

We hypothesised that the monofunctional profile in children with MIS-C and non-MIS-C children in our findings, is either a result of T cell exhaustion due to persistent systemic inflammation in both MIS-C and non-MIS-C groups or SARS-CoV-2 S superantigen-mediated responses (Cheng et al., 2020; Rivas et al., 2021, 2022). To support the latter, we compared the expression of TCR V β 21.3 receptor on SARS-CoV-2-specific T cells and we found that the frequency of TCR V β 21.3+ SARS-CoV-2-specific CD4+ T cells was significantly increased in children with MIS-C compared to healthy seropositive children. While our results are in concordance with several previous studies that have shown the expansion of TCR V β 21.3+ or *TRBV11-2+* polyclonal T cells in MIS-C patients (Hoste et al., 2021; Moreews et al., 2021; Porritt et al., 2021a; Porritt et al., 2021b; Ramaswamy et al., 2021; Sacco et al., 2022), in our study, we also found that children with non-MIS-C have comparable frequencies of TCR V β 21.3+ SARS-CoV-2-specific CD4+ T cells to children with MIS-C. In contrast, a study by Moreews et al. using flow cytometry identified the distribution of V β subunits in T cells and transcriptomic analyses of V β expression and showed that only patients with MIS-C (75%) had expansion of V β 21.3+ T cells in comparison to other clinical groups consisting of TSS, KD and COVID-19 patients (Moreews et al., 2021). The disparities between the studies could be the experimental approaches, in our study we analyzed TCR V β 21.3+ on cytokine-producing cells following stimulation with SARS-CoV-2 peptides, whereas Moreews et al. used two approaches simultaneously to show that the expansion of V β 21.3+ T cells is distinctive to MIS-C patients and not the other paediatric inflammatory conditions including COVID-19 (Moreews et al., 2021). The comparable TCR V β 21.3+ expression on SARS-CoV-2-responding CD4+ T cells in MIS-C and non-MIS-C groups could be a result of partial non-specific TCR binding of V β 21.3+ cells following SARS-CoV-2 infection, which could result of systemic inflammation similar to TSS (Choi et al., 1990; Krakauer, 2019; Rivas et al., 2021; F. R. Shepherd et al., 2023) in both groups therefore leading to virus-specific T cell exhaustion and the skewed functional T cell profile. Our results emphasize that the expression of TCR V β 21.3 receptor upon stimulation with SARS-CoV-2-specific peptides may not be ideal as a differentiating feature for

MIS-C in our setting but should rather be used with additional approaches such as genetic quantification of TCR V β 21.3 gene (*TRBV11-2*).

COVID-19 pediatric vaccination strategies currently vary for different countries, with the US recommending vaccination (COVID-19 Pfizer-BioNTech; BNT162b2 mRNA vaccine) of healthy children aged 12-17 years by May 2021, 5-11 years in November 2021 and 6 months - 4 years children in June 2022 (American Academy of Pediatrics, 2023; Maldonado et al., 2022). In SA, the National Department of Health, and the South African Health Products Regulatory Authority (SAHPRA) approved the use of COVID-19 BNT162b2 mRNA vaccines for children aged 12 years and older in September 2021, and by February 2023, vaccination was approved for children aged 5-11 years at risk of severe disease with significant comorbidities (Department: Health Republic of South Africa, 2023; Kruger et al., 2021). Paediatric COVID-19 vaccination remains lower compared to adult populations, with only 39% of children aged 5-11 years in the US having received at least 1 and/or 2 doses of COVID-19 mRNA vaccines, while in SA approximately less than 1% (4374/39 294 790) of children under the age of 11 years have received at least one dose of COVID-19 vaccine (American Academy of Pediatrics, 2023; Department: Health Republic of South Africa, 2024). One of the factors contributing to the different vaccine roll-out between first world countries like the USA and African countries, is vaccine access/availability. In resource-poor setting vaccinations were prioritized to populations at higher risk of severe disease (Hunter et al., 2022; Watson et al., 2022). Since children typically present with less severe disease than adults, they were not prioritized for vaccine campaigns early in the pandemic (Watson et al., 2022). However severe sequelae such as MIS-C highlighted the need for vaccination in this cohort. Indeed, SARS-CoV-2 vaccination among children has been associated with decreased MIS-C incidence cases in adolescents (Jain et al., 2022; Levy et al., 2022; Nygaard et al., 2022). Although the incidence of MIS-C has greatly reduced, the factors driving the progression to MIS-C have yet to be understood. In addition, although comorbidities are lower in children, there are still a subset of high-risk children with comorbidities. Therefore, pediatric COVID-19 vaccinations must continue to be prioritized by policymakers and practitioners committed to global child health (Pierce et al., 2022).

Overall, the findings of this thesis provide important relevant data for understanding the adaptive immunity to SARS-CoV-2 in children compared to adults and provide insight into the cross-reactive immunity that may provide protection against re-infections in children.

Limitations of this thesis

The studies described in this thesis had several limitations. Due to the lack of PCR-confirmed SARS-CoV-2 infection in children, infection/exposure was based upon serological detection of SARS-CoV-2-specific IgG responses and therefore the exact timing between infection and sampling could not be determined. Serology assays recommended by WHO for serosurveillance in epidemiological studies include antibody ELISA, neutralization assays, and rapid tests (Clapham et al., 2020). The use of serology assays to define previous infection in the absence of PCR-confirmed or other nucleic acid amplification tests, such as in our cohort, can present a limitation. IgG antibodies against SARS-CoV-2 wane over time; therefore, this could underestimate the true number of individuals that have been infected with SARS-CoV-2 and those recently infected may be missed since antibody responses may not yet been mounted (X. Chen et al., 2021; Clapham et al., 2020; Murhekar & Clapham, 2021). The sample size of participants in MIS-C and non-MIS-C groups in chapter 5 was very low. We assessed SARS-CoV-2 T cell response durability over 16-month period since the initial visit, however, 3 epidemiological waves occurred and were dominated by SARS-CoV-2 Delta, Omicron BA.1/2 and BA.4/5 variants during this period; therefore, children may have been reinfected between the two sampling days. Our study mainly focused on type 1 CD4 T helper (Th1) cytokine analysis, while other Th CD4 subtypes such as Th2, common in early infancy and childhood, Th17 and circulating T follicular helper (cTfh) cells were not included which have been associated with distinct severity (Niessl et al., 2021a; Sette & Crotty, 2021; Shahbaz et al., 2021). We also measured T cell responses in blood samples which may differ from responses in mucosal tissue, including tissue-resident memory T cells (Trm) which may provide optimal protection at the site (Bertoletti et al., 2021b; Niessl et al., 2021a; Sette et al., 2023; Weiskopf et al., 2020). Finally, our analysis was limited to the measurement of T cell responses to structural proteins (S, N and M) which excluded T cell targets to non-structural

proteins (nsp7, 12 & 13) or ORF1/3 which have been associated with early abortive SARS-CoV-2 infection in seronegative HCW (Jay et al., 2023; Swadling et al., 2021).

Future direction for ongoing research

The serology data to elucidate and expand more on the number of SARS-CoV-2 variant infections is being generated by our collaborators. The COVID-19 pandemic is no longer a global public health emergency. Nevertheless, circulating variants may cause uncertainties and SARS-CoV-2 may continue to circulate and evolve into endemic seasonal waves of infection (Lenharo, 2023; Rahimi et al., 2023; WHO, 2023b). This would potentially lead to incidence cases of viral co-infections in children, who are vulnerable to other respiratory viral acute infections. While children were largely spared from severe COVID-19 illness, a recent study reported that co-infection with respiratory syncytial virus (RSV) was associated with a higher risk of severe respiratory illness and that these co-infections were highest in infants less than 1 year of age (23.9%) and children aged 1-4 years (21.3%) (Choudhary et al., 2022). Following the COVID-19 pandemic, a resurgence of RSV infections in infants and young children was reported, and RSV-associated hospitalization rates were markedly increased (CDC, 2023; NICD, 2023a). T cell immunity provides protection against severe disease caused by either RSV or SARS-CoV-2 infection, both viruses can also induce a strong Th1 immune response characterized by polyfunctional T cells (Altmann & Boyton, 2020; Bueno et al., 2008; Cannon et al., 1988; Lambert et al., 2014; Moss, 2022; Openshaw & Tregoning, 2005). Nevertheless, co-infection with RSV and SARS-CoV-2 may lead to excessive inflammation and immunopathology, as has been reported for other viral infections (N. Kumar et al., 2018; Tregoning & Schwarze, 2010). In addition to immunopathology, and it is plausible that increased systemic inflammation could drive excessive proliferation, differentiation, and subsequent exhaustion of antigen-specific T cells. In order to better understand the immunological mechanisms contributing to the increased severity of RSV disease observed after the COVID-19 pandemic, future work will examine systemic and local immune responses to RSV in the context of recent or concurrent SARS-CoV-2 infection in children.

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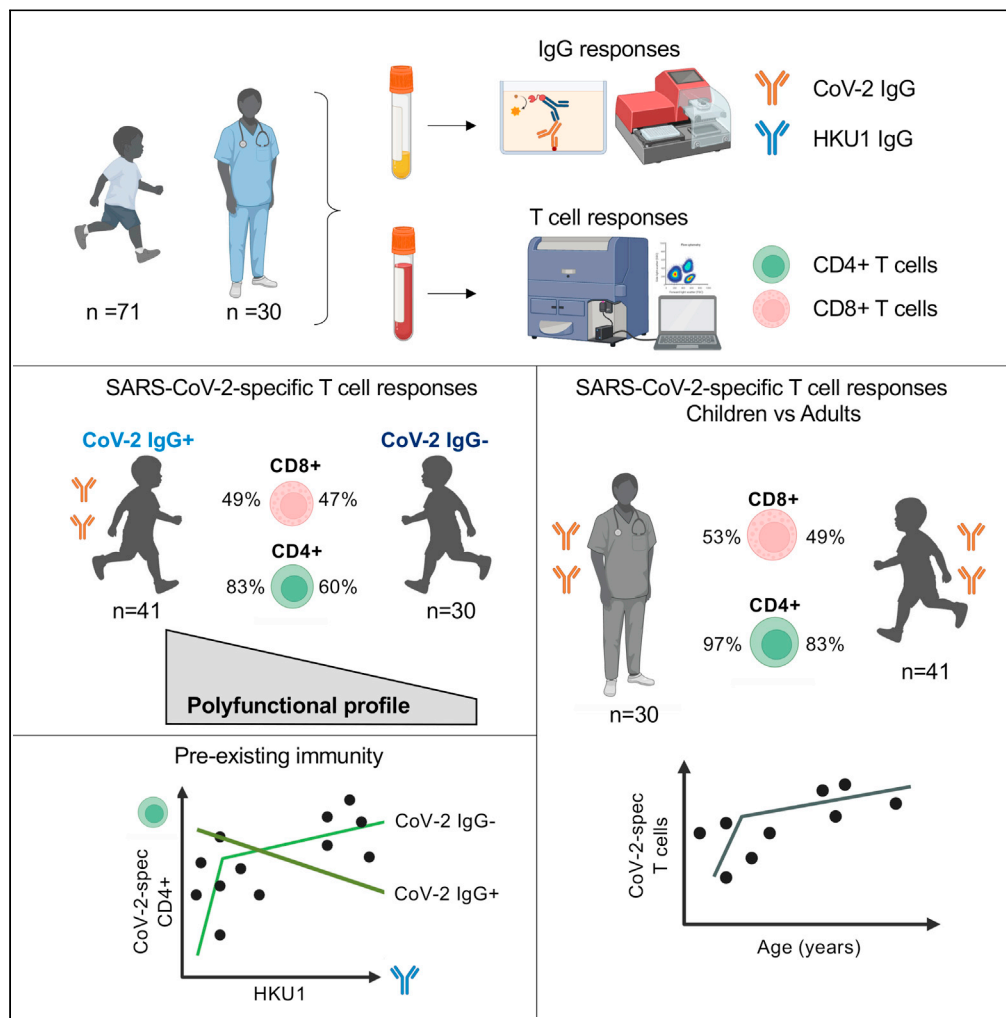
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APPENDICES
PUBLICATIONS

Article

Distinct T cell polyfunctional profile in SARS-CoV-2 seronegative children associated with endemic human coronavirus cross-reactivity



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Highlights

SARS-CoV-2-specific T cell responses are detected in 60% of seronegative children

Similar magnitude T cell response between seropositive and seronegative children

Seronegative children exhibit a lower proportion of polyfunctional T cells

CoV-2-specific T cell and endemic HKU1 IgG responses correlate in seronegative children

Article

Distinct T cell polyfunctional profile in SARS-CoV-2 seronegative children associated with endemic human coronavirus cross-reactivity

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SUMMARY

SARS-CoV-2 infection in children typically results in asymptomatic or mild disease. There is a paucity of studies on SARS-CoV-2 antiviral immunity in African children. We investigated SARS-CoV-2-specific T cell responses in 71 unvaccinated asymptomatic South African children who were seropositive or seronegative for SARS-CoV-2. SARS-CoV-2-specific CD4⁺ T cell responses were detectable in 83% of seropositive and 60% of seronegative children. Although the magnitude of the CD4⁺ T cell response did not differ significantly between the two groups, their functional profiles were distinct, with SARS-CoV-2 seropositive children exhibiting a higher proportion of polyfunctional T cells compared to their seronegative counterparts. The frequency of SARS-CoV-2-specific CD4⁺ T cells in seronegative children was associated with the endemic human coronavirus (HCoV) HKU1 IgG response. Overall, the presence of SARS-CoV-2-responding T cells in seronegative children may result from cross-reactivity to endemic coronaviruses and could contribute to the relative protection from disease observed in SARS-CoV-2-infected children.

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection in children usually causes asymptomatic or mild illness, contrasting with the high rate of severe disease reported in older adults.^{1,2} As a result, global reports of coronavirus disease 2019 (COVID-19) cases among children and adolescents are underreported. The United Nations International Children's Emergency Fund (UNICEF) estimates that 21% of all reported confirmed cases occur in individuals younger than 20 years.³

In the USA, it has been documented that COVID-19-associated hospitalization rates among children less than 18 years were lower compared to those in older individuals.⁴ Indeed, the COVID-19-Associated Hospitalization Surveillance Network (COVID-NET) reported that children younger than 18 years accounted for only 4.2% of COVID-19-associated hospitalization and 0.2% of COVID-19-associated in-hospital deaths.⁵ In South Africa, during the first 2 years of the pandemic, 12.5% of confirmed cases and 0.7% of COVID-19 associated in-hospital deaths were in individuals younger than 19 years.⁶

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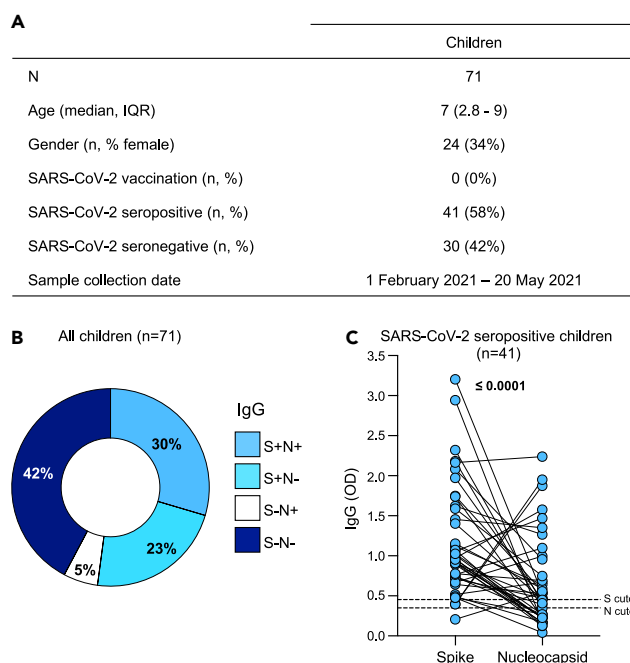


Figure 1. SARS-CoV-2-specific antibody responses in children

(A) The demographic characteristics of 71 unvaccinated children included in this study. Age, gender, SARS-CoV-2 vaccination status, SARS-CoV-2 serology and collection date are shown.

(B) Proportion of children exhibiting antibody responses to SARS-CoV-2 spike (S) and nucleocapsid (N) proteins.

(C) The magnitude of SARS-CoV-2 S and N IgG antibodies (OD_{490nm}) measured by ELISA in seropositive children ($n = 41$). The dotted lines indicate the cut-off for positivity which was calculated as the mean optical density of COVID-19 prepandemic control samples. Statistical analysis was performed using the Wilcoxon signed rank test; p values < 0.05 were considered statistically significant and are bolded.

See also [Figure S1](#).

In contrast to the low COVID-19 severity in the majority of younger individuals, it is known that children are more susceptible than adults to other acute viral respiratory tract infections including respiratory syncytial virus (RSV), rhinovirus (RV), influenza virus and common circulating endemic human coronaviruses (HCoV).⁷ Several age-associated factors have been proposed to play a role in the reduction of severity to SARS-CoV-2 infection in children,^{2,8} including limited comorbidities,^{9–11} differences in the expression of SARS-CoV-2 viral entry factors,^{12–14} robust innate immune responses,^{15–18} humoral and cellular immunity^{19–21} and pre-existing immunity against common cold circulating endemic HCOVs.^{22–26} Endemic HCOVs account for 15 to 30% of respiratory infections reported annually in children.^{27,28} These HCOVs belong to the alpha-coronavirus subfamily (HCoV-229E and HCoV-NL63) and the beta-coronavirus subfamily (HCoV-OC43 and HCoV-HKU1), are generally considered to have seasonal infection peaks during the winter season and are responsible for high rates of infection among children.^{29,30} In contrast, the influenza surveillance data from India showed that HCoV-HKU1 peaked during spring to summer over the 2015–2016 period.³¹

It is well established that protective immune responses to SARS-CoV-2 encompass both an antibody and T cell component.³² Several studies have reported robust and durable antibody and T cell responses against SARS-CoV-2, which are maintained up to 6–12 months following infection.^{21,33–36} However, data on T cell responses in children compared to adults are conflicting, with studies reporting lower SARS-CoV-2-specific T cell responses in children,^{23,37,38} no differences³⁹ or greater T cell responses in children.²² Many of these studies make use of ELISPOT assays which preclude analysis of T cell phenotypes or functional cytokine profiles.

In this study, we prospectively characterized specific T cell responses in SARS-CoV-2 seropositive and seronegative children during the COVID-19 pandemic and determined the functional profiles of SARS-CoV-2-specific T cells, in the context of their pre-existing immunity against endemic beta-HCOVs. This study provides insights into cross-reactive immunity to SARS-CoV-2 in children.

RESULTS

Study cohort

To investigate SARS-CoV-2-specific immune responses, we measured immunoglobulin (Ig) G and T cell responses in 71 healthy children recruited in Cape Town, Western Cape, South Africa. The participants are described in [Figure 1A](#). The median age of the children was 7 years (interquartile range (IQR) 2.8–9 years) and 34% (24/71) were female. The children included in this study had not received any SARS-CoV-2 vaccine prior to recruitment and no PCR-confirmed infection data were available, although 58% showed SARS-CoV-2 seropositivity (defined

as being positive for either anti-spike or anti-nucleocapsid IgG). Samples were collected between 1 February 2021 and 20 May 2021, after two infection waves in South Africa, that were dominated by the ancestral D614G strain followed by the Beta variant of concern.⁴⁰

SARS-CoV-2-specific antibody responses in children

To characterize the children serologically, we measured IgG responses against SARS-CoV-2 spike and nucleocapsid proteins using an indirect Enzyme-linked immunosorbent assay (ELISA) (Figure 1B). A large proportion (58%, 41/71) of unvaccinated children had detectable SARS-CoV-2-specific IgG against spike and/or nucleocapsid proteins and were classified as seropositive. Of the seropositive children, 51% (21/41) had spike- and nucleocapsid-specific IgG, 39% (16/41) had only spike-specific IgG and 10% (4/41) had only nucleocapsid-specific IgG only (Figure 1B). The remaining 42% (30/71) had undetectable SARS-CoV-2-spike and nucleocapsid-specific IgG and were classified as seronegative (Figure 1B). IgG responses to spike and nucleocapsid are shown for the 41 seropositive children in Figure 1C. Moreover, we compared SARS-CoV-2-spike- and nucleocapsid-specific IgG levels among SARS-CoV-2 seropositive ($n = 41$) children stratified by age. Children under the age of 5 years ($n = 13$) had significantly higher SARS-CoV-2-spike-specific IgG responses ($p = 0.0007$) compared to children older than 5 years old ($n = 28$) (Figure S1A). However, no significant differences ($p = 0.972$) were observed for SARS-CoV-2-nucleocapsid-specific IgG responses (Figure S1B).

SARS-CoV-2-specific T cell responses in children

The magnitude of SARS-CoV-2-specific T cell responses in children was quantified using a whole blood assay and intracellular cytokine staining followed by flow cytometry. SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses were measured as the total cytokine production of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) or interleukin-2 (IL-2) in response to a combined peptide pool covering SARS-CoV-2 spike (S), nucleocapsid (N) and membrane (M) proteins (Figure 2A).

We assessed the proportion of responders and magnitude of SARS-CoV-2-specific T cell responses according to the serostatus of the children (Figures 2B and 2C). As expected, 83% (34/41) of seropositive children had detectable SARS-CoV-2-specific CD4⁺ T cells. Interestingly, 60% (18/30) of seronegative children also had detectable SARS-CoV-2-specific CD4⁺ T cells, although this was a significantly lower proportion than observed in the seropositive group ($p = 0.031$). The magnitude of the SARS-CoV-2-specific CD4⁺ T cell response in seronegative responders was comparable to that observed in seropositive children (median of responders: 0.04% and 0.057% respectively; $p = 0.437$, Figure 2B). Conversely, we did not observe significant differences in the proportion of SARS-CoV-2 CD8⁺ T cell responders (~50%) or the frequency of SARS-CoV-2-specific CD8⁺ T cells between seropositive and seronegative children (median of responders: 0.019% and 0.025% respectively; $p = 0.214$, Figure 2C).

Next, we evaluated the polyfunctional profile of SARS-CoV-2-responding CD4⁺ and CD8⁺ T cells based on the co-production of IFN- γ , IL-2 or TNF- α (Figures 2D and 2E). The overall functional profile of SARS-CoV-2-specific CD4⁺ T cells in seropositive children was distinct from that of seronegative children ($p = 0.007$, Figure 2D). SARS-CoV-2-specific CD4⁺ T cells in seropositive children were more polyfunctional, exhibiting a higher proportion of triple functional IFN- γ +IL-2+TNF- α -producing CD4⁺ T cells ($p = 0.0044$), and dual expressing cells (IL-2+TNF- α +, $p = 0.0048$; IFN- γ +IL-2+, $p \leq 0.0001$) compared to the seronegative children. In contrast, seronegative children were characterized by an increased proportion of IFN- γ monofunctional CD4⁺ T cells ($p = 0.0048$). Considering the CD8 compartment, we detected no significant differences in the overall functional profile of SARS-CoV-2-responding CD8⁺ T cells between the two groups ($p = 0.098$), although seropositive children did have a larger proportion of CD8⁺ T cells producing both IFN- γ and IL-2 ($p = 0.027$) and single IFN- γ ($p = 0.011$) (Figure 2E).

Taken together, these data show that a significant proportion of seronegative children have detectable SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells. Notably, these CD4⁺ T cells exhibit a distinct polyfunctional cytokine profile compared to those found in SARS-CoV-2-exposed children.

Pre-existing immunity to endemic common circulating human coronaviruses

The presence of SARS-CoV-2-responding CD4⁺ T cells primarily exhibiting a monofunctional profile observed in seronegative children led us to hypothesize that the T cell response might be due to cross-reactivity resulting from prior infection with common circulating endemic HCoVs. Therefore, we measured endemic beta HCoV-HKU1 and HCoV-OC43 spike IgG in SARS-CoV-2 seropositive and seronegative children. The magnitude of both HCoV-HKU1 (Figure 3A) and HCoV-OC43 (Figure S2A) spike IgG was comparable in the seropositive and seronegative groups (median OD: 0.746 vs. 0.420; $p = 0.153$ for HCoV-HKU1 and 1.769 vs. 1.281; $p = 0.07$ for HCoV-OC43, respectively).

We found a moderate correlation between the frequency of SARS-CoV-2-specific CD4⁺ T cells and HCoV-HKU1-spike IgG in seronegative children ($p = 0.036$, $r = 0.392$, Figure 3B), while no correlation was observed in seropositive children ($p = 0.936$, $r = -0.013$, Figure 3C). For HCoV-OC43, no correlation was detected in either the seronegative ($p = 0.324$, $r = -0.187$, Figure S2B) or the seropositive group ($p = 0.171$, $r = -0.218$, Figure S2C). Overall, these results may suggest the SARS-CoV-2-reactive CD4⁺ T cell responses in a proportion seronegative children could be due in part to cross-reactive T cell immunity resulting from prior infection with common cold HCoV-HKU1.

SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive children compared to COVID-19 convalescent adults

Blood and plasma samples were collected from 30 COVID-19 convalescent healthcare workers (HCWs) participating in a longitudinal study at Groote Schuur Hospital (Cape Town, Western Cape, South Africa) (Figure 4A). All convalescent adults had a prior mild/asymptomatic PCR-confirmed SARS-CoV-2 infection (median 224 days prior to sampling) and had not received a COVID-19 vaccine at the time of sampling. Samples were collected between 22 January 2021 and 23 February 2021.

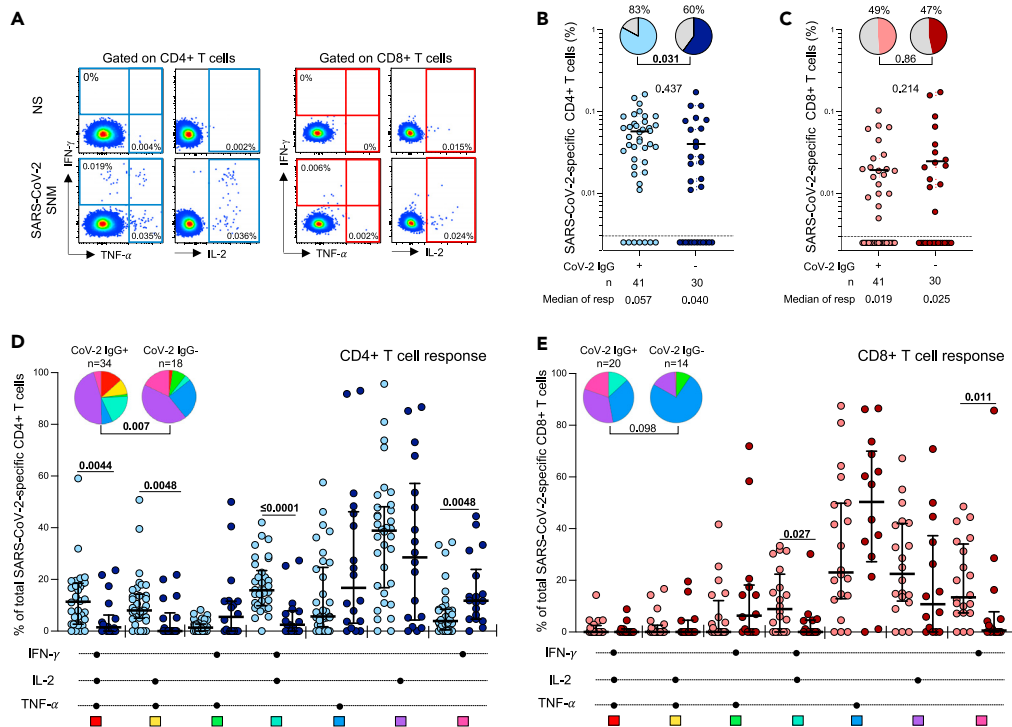


Figure 2. SARS-CoV-2-specific T cell responses in children

(A) Representative flow cytometry plots of SARS-CoV-2-specific interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and interleukin-2 (IL-2) cytokine production from CD4⁺ (left) and CD8⁺ (right) T cells in response to SARS-CoV-2 peptide stimulation. NS: no stimulation, SNM: combined peptide pool of SARS-CoV-2-spike, nucleocapsid and membrane proteins.

(B) Frequency of SARS-CoV-2-specific CD4⁺ T cells producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). Children were grouped according to SARS-CoV-2 serostatus (light blue: n = 41 SARS-CoV-2 seropositive; dark blue: n = 30 SARS-CoV-2 seronegative). Bars represent the median of the responders, and median values are indicated. The pie charts represent the proportion of responders with detectable T cell response to SARS-CoV-2 SNM peptides.

(C) Frequency of SARS-CoV-2-specific CD8⁺ T cells producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). Children were grouped according to SARS-CoV-2 serostatus (light red: n = 41 SARS-CoV-2 seropositive children; dark red: n = 30 SARS-CoV-2 seronegative children). Statistical comparisons in (B) and (C) were performed using the Mann-Whitney test between seropositive and seronegative children and the Chi-square test to compare the percentage of responders; p values < 0.05 were considered statistically significant and are bolded.

(D) Polyfunctional profile of SARS-CoV-2-specific CD4⁺ and (E) CD8⁺ T cells in seropositive and seronegative unvaccinated children. The x axis illustrates each combination which is indicated with a black circle for the presence of IFN- γ , IL-2 and TNF- α . The medians and interquartile range are shown. Each response pattern (any possible combination of IFN- γ , IL-2 and TNF- α production) is color coded and summarized in the pie charts, with each pie slice representing the median contribution of each combination to the total SARS-CoV-2 responses. The permutation test was used to compare the statistical differences between the pie charts and the Mann Whitney Sum Test to compare response patterns between seropositive and seronegative children; p values < 0.05 were considered statistically significant and are bolded.

A trend toward a higher proportion of SARS-CoV-2 CD4 responders was observed in convalescent adults (97%) compared to seropositive children (83%) (p = 0.07, Figure 4B). Moreover, in SARS-CoV-2 responders, convalescent adults did have a significantly higher frequency of SARS-CoV-2-specific CD4⁺ T cells compared to seropositive children (median of responders: 0.092% vs. 0.057%; p = 0.0004, respectively). Unlike CD4⁺ T cell responses, there were no significant differences in either the proportion of CD8 responders (53% vs. 49%, p = 0.705) or the frequency of SARS-CoV-2-specific CD8⁺ T cells (median of responders: 0.038% vs. 0.019%; p = 0.096) between convalescent HCWs and seropositive children (Figure 4C). Furthermore, we observed a moderate correlation between age and the frequency of SARS-CoV-2-specific CD4⁺ T cells (p = 0.0006, r = 0.396, Figure 4D). No correlation was observed with SARS-CoV-2-specific CD8⁺ T cells and age (p = 0.116, r = 0.188, Figure 4D). Overall, these results show that convalescent adults mount higher SARS-CoV-2-specific CD4⁺ T cell responses compared to children. This disparity may, in part be attributed to the incomplete maturation of the adaptive immune system in children.^{41,42}

DISCUSSION

T cells have been associated with protection from severe COVID-19.⁴³ It is therefore important to understand the nature of T cell responses targeting SARS-CoV-2 in children, who have been largely spared from severe COVID-19. In this study, comparing SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive and seronegative children, a sizable proportion of seronegative children had SARS-CoV-2-reactive

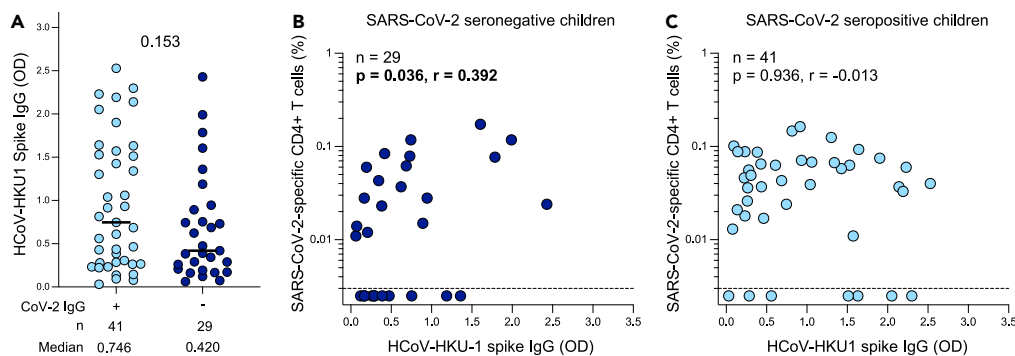


Figure 3. SARS-CoV-2 cross-reactivity to endemic beta-HCoV in children

(A) The magnitude of HCoV-HKU-1 spike IgG levels were measured by ELISA in SARS-CoV-2 seropositive (light blue; $n = 41$) and seronegative (dark blue; $n = 29$) children. Plasma sample was insufficient for one seronegative child therefore OD for HCoV-HKU1 was not measured for this participant. The bars represent the median values. A statistical comparison was performed using the Mann-Whitney test between seropositive and seronegative children; a p value < 0.05 was considered statistically significant.

(B) Correlation between the frequency of SARS-CoV-2-specific CD4⁺ T cells and HCoV-HKU-1-spike IgG levels in SARS-CoV-2 seronegative children ($n = 29$). One participant had insufficient sample available to be included in this assay.

(C) Correlation between the frequency of SARS-CoV-2-specific CD4⁺ T cells and HCoV-HKU-1-spike IgG levels in SARS-CoV-2 seropositive children ($n = 41$). Statistical comparisons for (B) and (C) were performed using a two-tailed non-parametric Spearman rank tests; p values < 0.05 were considered statistically significant and are bolded and correlation coefficients values are shown.

See also Figure S2.

CD4⁺ and CD8⁺ T cells, but their CD4⁺ T cells exhibited a distinct functional cytokine profile compared to seropositive children. Importantly, in seronegative children, the frequency of SARS-CoV-2-reactive CD4⁺ T cells positively associated with HCoV-HKU1 spike-specific IgG antibodies. Additionally, we showed that convalescent adults had a higher magnitude of CD4⁺ T cell responses against SARS-CoV-2 compared to seropositive children, which associated positively with age.

Our data are in accordance with several studies showing that children develop robust humoral and cellular immunity to SARS-CoV-2 infection^{20,22,39,44,45} and BNT162b2 vaccine.⁴⁶ In addition, SARS-CoV-2-specific T cell responses have been detected in 40–60% of SARS-CoV-2 unexposed individuals including children and adults^{22,23,47–52} suggesting possible cross reactivity to HCoVs. One study demonstrated that the proportion of T cell responders was higher in children than adults.²² In contrast, a study by Tsang et al. found that SARS-CoV-2 uninfected children failed to mount detectable T cell responses.⁴⁴ A further study showed that only a small proportion of seronegative children mounted a SARS-CoV-2-specific CD4⁺ T cell response (13%) compared to 60% in seropositive siblings, despite similar exposure in shared households.⁴⁵ These two studies differ from our findings, where we showed that 60% of seronegative children had detectable SARS-CoV-2-specific T cell responses, consistent with Dowell et al.²² The discrepancies between the studies could be due to a difference in the seasonal prevalence of circulating HCoVs in each study setting,^{29,30,53} different T cell assays used to analyze T cell responses, and/or the cohort demographics.

Although the source of SARS-CoV-2 reactive T cells in seronegative children remains unclear, mounting evidence argues for cross-reactivity to endemic HCoVs. HCoVs (including beta HCoVs-HKU1 and OC43, and alpha HCoVs-NL63 and 229E) have partial sequence homology with SARS-CoV-2.^{52,54} While cross reactive humoral responses have been shown not to underly this protection,⁵⁵ studies have reported pre-existing T cell responses to endemic HCoVs with cross-reactivity to SARS-CoV-2 in unexposed adults^{50,56–61} and children.^{22,24,62,63} These cellular responses were targeted mainly toward the S2 subunit which is highly conserved among coronaviruses.^{22,25} In our study, we showed a positive correlation between the frequency of SARS-CoV-2 CD4⁺ T cells and the magnitude of HKU1 spike-specific IgG in SARS-CoV-2 seronegative children. Our findings are supported by similar findings showing that 58% (7/12) seronegative children had binding antibodies against alpha and/or beta HCoV spike proteins, which they concluded were likely due to recent HCoV infection.²²

Pre-existing immunity from endemic HCoV infection or exposure has been shown to be associated with a protective effect against COVID-19 disease in adults.^{56,59–61,64} Sagar et al. showed that individuals with a documented recent or ongoing HCoV infection and SARS-CoV-2 infection had fewer COVID-19-associated complications when hospitalized, compared to individuals without HCoV infection.⁶⁴ Previously, the clinical relevance of pre-existing immunity and cross-reactive cellular responses from endemic HCoVs in providing protection from SARS-CoV-2 infection in children was unclear. Recently however, Dowell et al. demonstrated that SARS-CoV-2-reactive T cell responses in SARS-CoV-2 seronegative children were associated with relative protection against Omicron infection, suggesting a protective role in SARS-CoV-2 unexposed children.⁶⁵

Polyfunctional T cells have been reported to be associated with protection against several viral diseases.^{66–69} These include protection against cytomegalovirus (CMV) infection after lung transplantation,⁶⁷ improved viral control of hepatitis C virus (HCV)⁶⁸ and detection during human immunodeficiency virus (HIV) infection in long term non-progressors.⁶⁹ We found that seropositive children had a more polyfunctional profile of SARS-CoV-2-specific CD4⁺ T cells while CD4 responses in seronegative children exhibited a predominantly monofunctional profile.

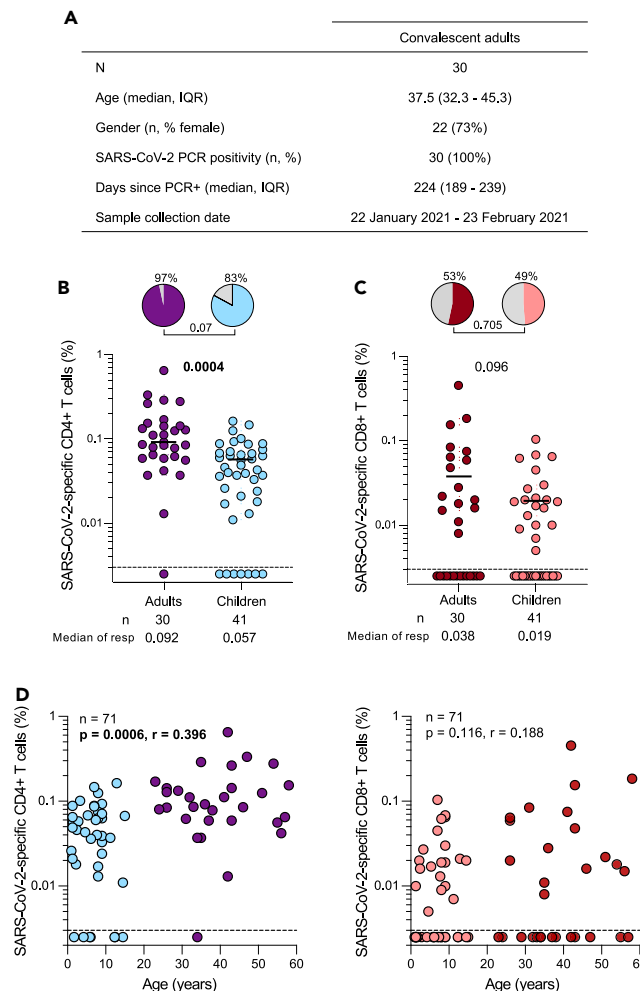


Figure 4. SARS-CoV-2-specific T cell responses in children compared to convalescent adults

(A) The demographic characteristics of 30 unvaccinated convalescent adults included in this study. Age, sex, SARS-CoV-2 PCR-positivity, days since PCR test and collection date are shown.

(B) Frequency of SARS-CoV-2-specific CD4⁺ T cells producing any of the measured cytokines (IFN- γ , TNF- α , or IL-2) in SARS-CoV-2 convalescent HCW (purple; n = 30) and seropositive children (light blue; n = 41). The pie charts represent the proportion of responders with a detectable T cell response to SARS-CoV-2 SNM combined peptide pools. Bars represent median of the responders.

(C) Frequency of SARS-CoV-2-specific CD8⁺ T cells producing any of the measured cytokines (IFN- γ , TNF- α , or IL-2) in SARS-CoV-2 convalescent HCW (dark red; n = 30) and seropositive children (light red; n = 41). The bars represent the median of the responders and median values are indicated. Statistical comparisons were performed using the Mann-Whitney test between seropositive children and adults and the Chi-square test was used to compare the percentage of responders for (B) and (C); p values < 0.05 were considered statistically significant and are bolded.

(D) Correlations between SARS-CoV-2-specific CD4⁺ or CD8⁺ T cells and age in convalescent HCW (purple and dark red; n = 30) and seropositive (light blue and light red; n = 41) children. Statistical comparisons were performed using a two-tailed non-parametric Spearman rank tests; p values < 0.05 were considered statistically significant and are bolded and correlation coefficients are shown.

The SARS-CoV-2-specific polyfunctional Th1 CD4 T cell response (characterized by the co-expression of IFN- γ , TNF- α and/or IL-2), as seen in seropositive children, may be necessary for effective viral control and has been documented in COVID-19 convalescent adults.^{70,71}

The distinct CD4 and CD8 polyfunctional profiles reported in our findings could be a result of differing degrees of memory T cell differentiation and/or lower SARS-CoV-2 peptide binding affinity with HCoV-specific T cells resulting in low T cell activation. The affinity between MHC molecules presenting peptides (pMHC) and the T cell receptor (TCR) plays a role in antigen recognition. It has been reported high TCR-pMHC complex affinity is associated with increased overall T cell functional efficacy *in vitro* and *in vivo* and therefore an increased polyfunctional profile compared to low affinity TCRs.^{72–74} TCR cross-recognition of pMHCs that are not structurally identical results in lower binding affinity which decreases T cell polyfunctionality.^{73,75} It is therefore plausible that the distinct polyfunctional profile of SARS-CoV-2 CD4⁺ T cell responses in seronegative children observed in our study may be mediated by the cross-recognition of pre-existing T cell immunity to HCoV-HKU1, where

partial sequence homology between HCoV-HKU1 and SARS-CoV-2 results in low peptide binding affinity and a different polyfunctional T cell profile.^{52,58,72,75,76} It is noteworthy to report that we did not find any association with the other beta HCoV-OC43 spike-specific IgG and SARS-CoV-2-specific T cells in seronegative children, which could be related to differences in seasonal prevalence of types of HCoV infections. Indeed, multiple studies have shown differences in the prevalence of HCoV infections which change yearly and depend on regional environment.^{31,77–80} In particular, a study by Friedman et al. showed a high prevalence of HCoV-OC43 was the most prevalent HCoV in winter but HCoV-HKU1 was the most prevalent in spring to summer during the 2015–2016 time period.³¹

Numerous studies have now compared the magnitude of the SARS-CoV-2 specific T cell response in children to those detectable in adults.^{22,23,37–39,44} Initially it was thought that children may have a higher magnitude of T cell responses given their relative resistance to severe disease and the link between T cell responses and protection from severe disease.^{8,81} However, children were shown to have lower T cell responses than adults in most studies, including the current study.^{23,37,38} Several hypotheses could possibly explain the differences in the magnitude of SARS-CoV-2-specific CD4⁺ T cell responses between children and adults in our study. These include 1) differences in disease severity between the groups, 2) the immaturity of children's immune system and 3) the timing of infection in relation to sampling. Adults have a mature immune system with more differentiated memory T cell subsets endowed with increased cytokine capacity, whereas children have an immature immune system, with many more naive T cells which have reduced cytokine producing capacity, are enriched for monofunctional responses, and have increased antigen dependence.⁸² However, as the exact time of SARS-CoV-2 infection in the children group could not be defined, we cannot fully exclude that the higher magnitude of SARS-CoV-2-specific CD4⁺ T cells observed in the adult group could be related to a more recent infection compared to the children group. Despite lower circulating SARS-CoV-2-specific T cells, children and adults may also have different responses in the respiratory tract. There is evidence in adults demonstrating the presence of SARS-CoV-2-specific T cell responses in the nasal mucosa after infection and vaccination.^{83,84} However, comparative studies have yet to be performed in children. Additionally, several studies have proposed that one potential contributing factor to age-related COVID-19 clinical outcome is the robust innate immune responses observed in children, which may contribute to early control of viral replication.^{15–17,85} Recent studies have shown that, compared to adults, SARS-CoV-2 seronegative children exhibited an increased number of innate immune cells with pre-activated signatures leading to the early production of interferon-mediated antiviral effects in the upper respiratory tract.^{18,19}

In conclusion, our study shows that a robust SARS-CoV-2-specific T cell response is observed in children, including those with no evidence of prior SARS-CoV-2 infection. We demonstrate that the magnitude of SARS-CoV-2-reactive CD4⁺ T cells in seronegative children correlates with HCoV-HKU1 exposure. This, together with a distinct functional profile of SARS-CoV-2-specific responding CD4⁺ T cells observed between seropositive and seronegative children may provide further evidence for pre-existing T cell responses cross-reactive to SARS-CoV-2.

Limitations of the study

This study relied on serology to determine SARS-CoV-2 exposure in the pediatric cohort. With no PCR confirmation of SARS-CoV-2 infection, the exact time of infection of the children could not be determined. This caveat in our study may have an impact in the differences of SARS-CoV-2-specific T cell responses seen in adults compared to seropositive children. Additionally, our study investigated T cell responses against SARS-CoV-2 spike, nucleocapsid and membrane proteins, and not the non-structural viral proteins that can also serve as targets for the T cell response, thus not capturing the full extent of T cell reactivity in infected participants. While our study focused solely on Th1-associated cytokines, it remains to define whether other Th CD4 subtypes such Th2 or Th17 could as also play a role in COVID-19 protection. Furthermore, we did not address durability of the T cell responses in children due to the cross-sectional design of the study. Moreover, it would be of interest to assess the HCoV-HKU1-specific memory T cell responses and define the relationship with SARS-CoV-2 specific immune responses and their potential protective effect against SARS-CoV-2. Further experiments assessing HCoV-IgG responses as well as the neutralizing antibody activity against HCoVs, SARS-CoV-2 and its variants of concern are warranted to gain a better understanding of the relative protection from disease observed in SARS-CoV-2-infected children compared to adults.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.108728>.

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AUTHOR CONTRIBUTIONS

R.S.K., K.W., and W.A.B. conceived the study and designed the experiments. N.S.B.B., A.N. and R.B. performed T cell assays. M.B.T., A.W., and V.S. performed SARS-CoV-2 ELISAs. S.V.G. and S.R.B. performed HCoV ELISAs. N.S.B.B., T.M.G., P.L.M., C.R., W.A.B., and R.S.K. analyzed the data. L.Z. and K.W. established and led the Immunology Biorepository and MIS-C sub-study, and C.B., H.F.T., C.S., J.D. and T.F.S. recruited participants and managed samples from Red Cross War Memorial Children's Hospital. H.J.Z. established and led the DCHS cohort including a COVID sub-study. M.B. and L.W. managed the DCHS cohort and repository. R.M., M.J., and D.G. ran serology to characterize the DCHS cohort. N.A.B.N. established and led the adult HCW cohort and L.H., M.M., S.S., and M.A. recruited participants and managed the cohort. N.S.B.B., C.R., W.A.B., and R.S.K. wrote the article. All authors reviewed and edited the article.

DECLARATION OF INTERESTS

The authors have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified NA/LE mouse anti-human CD28 (clone 28.2)	BD Pharmingen	Cat# 555725; RRID:AB_2130052
Purified NA/LE mouse anti-human CD49d (clone L25)	BD Pharmingen	Cat# 555501; RRID:AB_396068
CD4 ECD (clone SFC112T4D11)	Beckman Coulter	Cat# 6604727; RRID:AB_394488
CD8 BV510 (clone RPA-8)	Biolegend	Cat# 301048; RRID:AB_2561942
CD3 BV650 (clone OKT3)	Biolegend	Cat# 317324; RRID:AB_2563352
IFN- γ AlexaFluor® 700 (clone B27)	BD Biosciences	Cat# 557995; RRID:AB_396977
TNF- α BV786 (clone Mab11)	Biolegend	Cat# 502948; RRID:AB_2565858
IL-2 APC (clone MQ1-17H12)	Biolegend	Cat# 500310; RRID:AB_315097
Anti-human IgG (Fab-specific)-horseradish peroxidase	Sigma Aldrich	Cat# A0170; RRID:AB_257868
Biological samples		
Unvaccinated children blood samples	Red Cross War Memorial Children's Hospital	https://www.childrenshospitaltrust.org.za/the-hospital/
Unvaccinated children blood samples	Mbekweni and TC Newman clinics	https://www.westerncape.gov.za/facility/mbekweni-community-day-centre-cdc https://www.westerncape.gov.za/facility/tc-newman-community-day-centre-cdc
Convalescent health care worker blood samples	Groote Schuur Hospital	https://www.gsh.co.za
Chemicals, peptides, and recombinant proteins		
Brefeldin A	Sigma Aldrich	Cat# B7651-5MG
PepTivator® SARS-CoV-2 Prot_S	Miltenyi Biotech	Cat # 130-126-701
Peptide Array, SARS-Related Coronavirus 2 Nucleocapsid (N) Protein	BEI resources	Cat# 52404
Peptide Array, SARS-Related Coronavirus 2 Membrane (M) Protein	BEI resources	Cat# 52403
PtX™ SARS-CoV-2 Spike Protein (S1, Rabbit FC)	Cape Bio Pharms (Pty) Ltd	Cat# CB_0002.2
Recombinant SARS-CoV-2 Spike RBD His-tag (HEK293 Expressed)	Bio-Techne	Cat#10500-CV
BTA SARS-CoV-2 Nucleocapsid (1mg)	BioTech Africa	Cat# BA25-C
Recombinant HCoV-HKU1 spike (HEK293F Expressed)	In-house	N/A
Recombinant HCoV-OC43 spike (HEK293F Expressed)	In-house	N/A
O-phenylenediamine dihydrochloride (OPD), Sigmafast TM	Sigma Aldrich	P9187-50SET
3',5,5'-Tetramethylbenzidine (TMB) substrate	Thermofisher Scientific	Cat# 34029
Casein, Hammarsten Bovine	Sigma Aldrich	E0789-500
Phosphate buffered saline with 0.05% tween, pH 7.4	Sigma Aldrich	P3563-10PAK
Critical commercial assays		
BD FACS™ Lysing Solution	BD Biosciences	Cat # 349202
BD Perm/Wash Buffer	BD Biosciences	Cat# 554723
CellFIX buffer	BD Biosciences	Cat# 340181

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human Embryonic Kidney (HEK) 293F	Dr Nicole Doria-Rose, VRC, USA	N/A
Recombinant DNA		
HCoV-HKU1 and -OC43 spike plasmids	Dr Gaurav Kwatra and Prof Shabir A. Madhi, Wits VIDA, SA Prof Barney Graham, VRC, USA	N/A
Software and algorithms		
FACSDiva 9	BD Biosciences	https://www.bdbiosciences.com
FlowJo 10	FlowJo, LLC	https://www.flowjo.com
Graphpad Prism 9	Graphpad	https://graphpad.com
BioRender	BioRender	https://biorender.com

RESOURCE AVAILABILITY**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Roanne S. Keeton (roanne.keeton@uct.ac.za).

Materials availability

Requests for materials should be directed to and will be fulfilled by the [lead contact](#), Roanne S. Keeton (roanne.keeton@uct.ac.za).

Data and code availability

- All data generated in this study is included in the accompanying tables, figures, and supplemental materials.
- No code was generated in this study.
- Any additional information required to reanalyse the data reported in this paper is available from the [lead contact](#) Roanne S. Keeton (roanne.keeton@uct.ac.za) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Study participants**

Pediatric participants (n = 71) were recruited from two cohorts in the Western Cape Province, South Africa. The first cohort enrolled 50 children from the Red Cross War Memorial Children's Hospital (Cape Town, Western Cape, South Africa). In this cohort 50/71 children were hospitalized for non-COVID-19-related elective procedures and were confirmed to be clinically well, with no chronic or intercurrent infectious or inflammatory disease. This study is approved by University of Cape Town Human Research Ethics Committee as a Pediatric Immunology Biorepository (UCT HREC 112/2012) and MIS-C substudy (UCT HREC 599/2020). A further 21 children were enrolled from the Drakenstein Child Health Study (DCHS) (Cape Winelands Western Cape, South Africa), a birth cohort study.⁸⁶ The participants from this study were recruited between 1 February 2021 and 20 May 2021, after the first and the second infection waves with Ancestral strain and Beta variant in South Africa.⁸⁷ The study was approved by the University of Cape Town Human Research Ethics Committee and renewed annually (HREC 401/2009). Parents or legal guardians provided written informed consent for all pediatric participants.

COVID-19 convalescent unvaccinated adults from a longitudinal study of healthcare workers (HCW) enrolled from Groote Schuur Hospital (Cape Town, Western Cape, South Africa) were included in the study (n = 30). HCW in this cohort were recruited between July 2020 and January 2021 and were selected for inclusion based on a prior PCR-confirmed SARS-CoV-2 infection at least 3 months earlier. All participants were asymptomatic or had mild symptoms and did not require hospitalization for COVID-19 and were symptom-free at the time of sampling. Written informed consent was obtained from all participants and the study was approved by the University of Cape Town Human Research Ethics Committee (HREC 190/2020 and 209/2020).

METHODS DETAILS**SARS-CoV-2 and HCoV antigens**

For serology assays, recombinant SARS-CoV-2 spike S1 (Cape Bio Pharms), and SARS-CoV-2 nucleocapsid (BioTech Africa) proteins were used for this study.

HKU1 and OC43 spike proteins were expressed in Human Embryonic Kidney (HEK) 293F suspension cells by transfecting the cells with the spike plasmid. After 6 days, proteins were purified using a nickel resin followed by size-exclusion chromatography. Relevant fractions were collected and frozen at -80°C until use.

SARS-CoV-2 peptides used for T cell assays included a commercially available peptide pool (15mers peptides with 11 amino acid overlap) covering the immunodominant regions of SARS-CoV-2 spike protein (PepTivator SARS-CoV-2 Prot_S, Miltenyi Biotech) based on the Wuhan-1 strain. The Spike peptide pool was prepared by resuspending in distilled water and used at a final concentration of $1\text{ }\mu\text{g/mL}$. SARS-CoV-2 (Wuhan-1) nucleocapsid and membrane peptides (17mers with 10 amino acid overlap spanning the full proteins) were obtained from BEI Resources and were prepared by resuspending in dimethyl sulfoxide (DMSO, Sigma) and used at a concentration of $1\text{ }\mu\text{g/mL}$.

Enzyme-linked immunosorbent assay (ELISA)

SARS-CoV-2-specific enzyme-linked immunosorbent assay (ELISA) was performed to characterize the serostatus of participants. Two $\mu\text{g/mL}$ of spike or nucleocapsid protein was used to coat 96-well high-binding plates and incubated overnight at 4°C . The plates were incubated in a blocking buffer consisting of 1% casein, 0.05% Tween 20, $1\times$ Phosphate-Buffered Saline (PBS) for SARS-CoV-2 or $1\times$ PBS, 5% skimmed milk powder, 0.05% Tween 20 for HCoV-229E for 1 h. Plasma samples were diluted to 1:50 for SARS-CoV-2 or 1:100 for HCoV-229E in the respective blocking buffer and added to the plates for 2 h. For the SARS-CoV-2 ELISA, secondary antibody was diluted to 1:5000 in dilution buffer and added to the plates for 1 h followed by SigmaFast O-phenylenediamine dihydrochloride (OPD) substrate for 12 min. For the HCoV-229E ELISAs, secondary antibody was diluted to 1:3000 in blocking buffer and added to the plates followed by TMB substrate (ThermoFisher Scientific). Upon stopping the reaction with 1–3 M sulfuric acid, absorbance was measured at a wavelength of 490 nm for SARS-CoV-2 or 450 nm for HCoV-229E. A cut-off for positivity was set at two standard deviations (SD) above the mean optical density (OD) of prepandemic samples ($n = 40$) for SARS-CoV-2 ELISAs.

Whole blood-based T cell assay

Blood was collected in sodium heparin tubes and processed within 4–6 h of collection. The whole blood assay sample processing used for this study was adapted from a whole blood intracellular cytokine detection assay designed to detect SARS-CoV-2 specific T cells in adults.^{88,89} For this study, $500\text{ }\mu\text{L}$ of blood was stimulated for 24 h at 37°C with a combined pool of SARS-CoV-2 peptides including S, N and M, all at $1\text{ }\mu\text{g/mL}$ in the presence of costimulatory antibodies against CD28 (clone 28.2) and CD49d (clone L25) ($1\text{ }\mu\text{g/mL}$ each; BD Biosciences) and Brefeldin A ($10\text{ }\mu\text{g/mL}$, Sigma-Aldrich). Unstimulated blood was incubated with costimulatory antibodies, Brefeldin A and an equimolar amount of DMSO as a background control. After 24 h, blood was treated with EDTA (2 mM) for 15 min followed by red blood cell lysis and white cell fixation using FACS lysing solution (BD Biosciences) for 10 min. Cells were then cryopreserved in freezing media (90% fetal bovine serum (FBS) and 10% DMSO) and stored at -80°C until batched analysis.

Cell staining and flow cytometry

Cell staining was performed on cryopreserved fixed cells that were thawed and washed with 1% FACS washing buffer (1% FBS in PBS). A viability dye was not included during the staining process, as it has been reported that the omission of a viability dye during the staining process does not impact the detection and quantification of cytokines when using the whole blood-based T cell assay.⁸⁸ Cells were stained with the following surface antibody markers: CD4 ECD (SFC112T4D11, Beckman Coulter), CD8 BV510 (RPA-8, Biolegend) and incubated at room temperature for 20 min. Cells were permeabilized and stained with intracellular antibody markers CD3 BV650 (OKT3), IFN- γ AlexaFluor 700 (B27), TNF- α BV786 (Mab11) and IL-2 APC (MQ1-17H12) (all from Biolegend). Finally, cells were washed and fixed with Cellfix (BD Biosciences). Samples were acquired on a multiparameter BD Fortessa flow cytometer using Diva software version 9 and analyzed using FlowJo v10. Results are expressed as the frequency of CD4⁺ or CD8⁺ T cells expressing IFN- γ , TNF- α or IL-2. Cytokine responses presented are background subtracted values (from the frequency of cytokine produced by unstimulated cells).

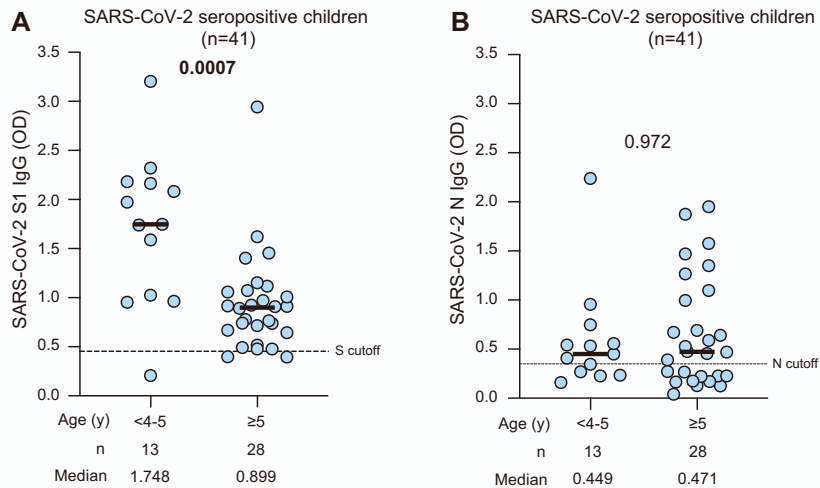
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed in Prism (v9.4.1; GraphPad Software Inc, San Diego, CA, USA). Non-parametric tests were used for all comparisons. The Mann-Whitney and Wilcoxon signed rank tests were used for unmatched and paired samples, respectively. Chi-square tests were used for comparisons between the proportion of responders represented as pie charts. All correlations reported are non-parametric Spearman's correlations. Analysis of cytokine co-expressing populations was performed using SPICE version 5.1. *p* values less than 0.05 were considered statistically significant. Details of analyses performed for each experiment are described in the figure legends.

Supplemental information

Distinct T cell polyfunctional profile in SARS-CoV-2 seronegative children associated with endemic human coronavirus cross-reactivity

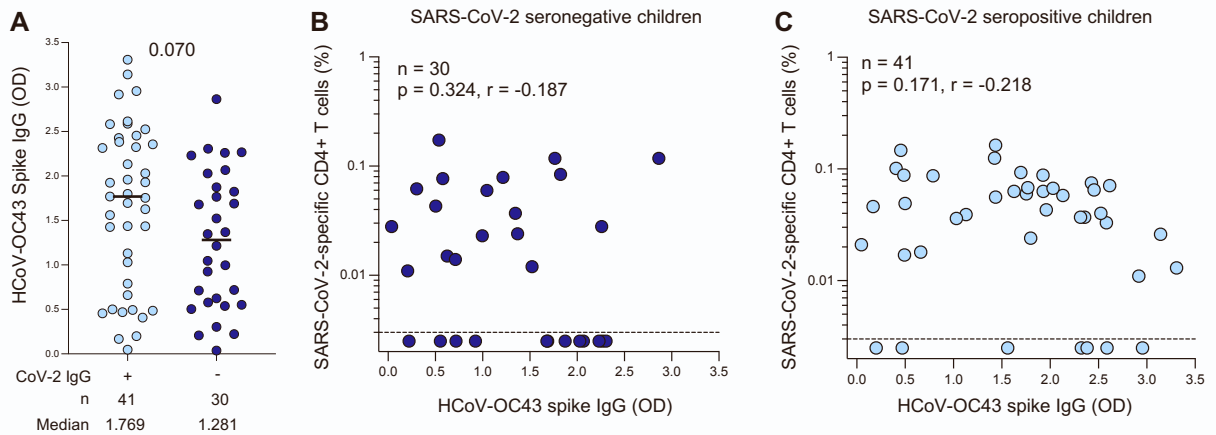
Ntombi Benede, Marius B. Tincho, Avril Walters, Vennesa Subbiah, Amkele Ngomti, Richard Baguma, Claire Butters, Lina Hahnle, Mathilda Mennen, Sango Skelem, Marguerite Adriaanse, Heidi Facey-Thomas, Christiaan Scott, Jonathan Day, Timothy F. Spracklen, Strauss van Graan, Sashkia R. Balla, Thandeka Moyo-Gwete, Penny L. Moore, Rae MacGinty, Maresa Botha, Lesley Workman, Marina Johnson, David Goldblatt, Heather J. Zar, Ntobeko A.B. Ntusi, Liesl Zühlke, Kate Webb, Catherine Riou, Wendy A. Burgers, and Roanne S. Keeton



Supplementary Figure 1: SARS-CoV-2-specific antibody responses in children stratified by age, related to Figure 1.

(A) The magnitude of SARS-CoV-2 spike-specific IgG measured by ELISA in SARS-CoV-2 seropositive children younger than 5 years (n=13) and 5 years or older (n=28).

(B) The magnitude of SARS-CoV-2-nucleocapsid-specific IgG in in SARS-CoV-2 seropositive children younger than 5 years (n=13) and 5 years or older (n=28). Statistical comparisons in (A) and (B) were performed using the Mann-Whitney test between groups; p values <0.05 were considered statistically significant and are bolded.



Supplementary Figure 2: SARS-CoV-2 cross-reactivity to endemic HCoV-OC43 in unvaccinated children, related to Figure 3.

(A) The magnitude of HCoV-OC43 spike IgG levels were measured by ELISA in SARS-CoV-2 seropositive (light blue; n=41) and seronegative (dark blue; n=30) children. The bars represent the median values. Statistical comparisons were performed using the Mann-Whitney test between seropositive and seronegative children; p values <0.05 were considered statistically significant.

(B) Correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-OC43-spike IgG levels in SARS-CoV-2 seronegative.

(C) Correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-OC43-spike IgG levels in SARS-CoV-2 seropositive children (n=41). Statistical comparisons in (B) and (C) were performed using a two-tailed non-parametric Spearman rank tests; p values <0.05 were considered statistically significant and are bolded and correlation coefficients values are shown.

CONTRIBUTION FOR PUBLICATION

Contribution details for the manuscript “**Distinct T cell polyfunctional profile in SARS-CoV-2 seronegative children associated with endemic human coronavirus cross-reactivity**” published in *iScience* (doi: 10.1016/j.isci.2023.108728).

- Kate Webb, Wendy A. Burgers and Roanne S. Keeton contributed to the conception and design of the study.
- **Ntombi Benede**, Amkele Ngomti and Richard Baguma received and processed samples using whole blood assays and stored the samples.
- **Ntombi Benede** performed the antibody panel building and optimization for flow cytometry.
- **Ntombi Benede** generated the data, through sample staining followed by acquisition on the flow cytometer.
- **Ntombi Benede**, Marius B Tincho, Avril Walters, Vennesa Subbiah performed the SARS-CoV-2 serology assays.
- Strauss van Graan and Sashkia R. Balla performed the HCoV serology assays.
- **Ntombi Benede** analyzed the SARS-CoV-2 and HCoV serology and flow cytometry data.
- **Ntombi Benede**, Catherine Riou, Wendy A. Burgers and Roanne S. Keeton contributed to the data interpretation.
- **Ntombi Benede** and Roanne S. Keeton drafted the manuscript.
- Catherine Riou, Wendy A. Burgers and Roanne S. Keeton edited and proofread the manuscript.
- Liesl Zühlke and Kate Webb established and led the Immunology Biorepository and MIS-C sub-study from Red Cross War Memorial Children’s Hospital.
- Claire Butters, Heidi Facey-Thomas, Christiaan Scott, Jonathan Day, and Timothy F. Spracklen, recruited pediatrics participants and managed the MIS-C sub-study cohort.
- Heather J. Zar established and led the Drakenstein Child Health Study (DCHS) cohort including a COVID sub-study.
- Maresa Botha and Lesley Workman managed the DCHS cohort and repository.
- Rae MacGinty, Marina Johnson and David Goldblatt ran and manage the serology of the DCHS cohort.
- Ntobeko A.B. Ntusi established and led the adult healthcare workers cohort.
- Lina Hahnle, Mathilda Mennen, Sango Skelem and Marguerite Adriaanse recruited the adult healthcare worker participants and managed the cohort.
- Thandeka Moyo-Gwete, Penny L. Moore, Wendy A. Burgers and Roanne S. Keeton contributed to the execution and oversight of experimental work.
- All authors read, revised, and approved the final version of the manuscript.



Comparing the immune abnormalities in MIS-C to healthy children and those with inflammatory disease reveals distinct inflammatory cytokine production and a monofunctional T cell response

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ARTICLE INFO

Keywords:

MIS-C

Inflammatory cytokine profile

ABSTRACT

Multisystem inflammatory syndrome in children (MIS-C) is a severe, hyperinflammatory disease that occurs after exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The underlying immune pathology of MIS-C is incompletely understood, with limited data comparing MIS-C to clinically similar paediatric febrile diseases at presentation. SARS-CoV-2-specific T cell responses have not been compared in these groups to assess

Abbreviations: MIS-C, multisystem inflammatory syndrome in children; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; IL, interleukin; CRP, C reactive protein; CD, cluster of differentiation; Ig, immunoglobulin; COVID-19, coronavirus disease 2019; IVIG, intravenous immunoglobulins; IFN, interferon; KD, Kawasaki Disease; TCR V β , T cell receptor β chain variable region; WHO, World Health Organisation; RNA, ribonucleic acid; RA, receptor antagonist; MCP, monocyte chemoattractant protein; NICD, National Institute for Communicable Diseases; ADCP, antibody dependent cellular phagocytosis; ADCC, antibody dependent cellular cytotoxicity; ANOVA, analysis of variance; TNF, tumour necrosis factor; Fc, fragment crystallisable; ICU, intensive care unit; S, SARS-CoV-2 spike protein; N, SARS-CoV-2 nucleocapsid protein; M, SARS-CoV-2 membrane protein.

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Antibody effector function
SARS-CoV-2-specific T cells

whether there is a T cell profile unique to MIS-C. In this study, we measured inflammatory cytokine concentration and SARS-CoV-2-specific humoral immunity and T cell responses in children with fever and suspected MIS-C at presentation ($n = 83$) where MIS-C was ultimately confirmed ($n = 58$) or another diagnosis was made ($n = 25$) and healthy children ($n = 91$). Children with confirmed MIS-C exhibited distinctly elevated serum IL-10, IL-6, and CRP at presentation. No differences were detected in SARS-CoV-2 spike IgG serum concentration, neutralisation capacity, antibody dependant cellular phagocytosis, antibody dependant cellular cytotoxicity or SARS-CoV-2-specific T cell frequency between the groups. Healthy SARS-CoV-2 seropositive children had a higher proportion of polyfunctional SARS-CoV-2-specific CD4+ T cells compared to children with MIS-C and those with other inflammatory or infectious diagnoses, who both presented a largely monofunctional SARS-CoV-2-specific CD4+ T cell profile. Treatment with steroids and/or intravenous immunoglobulins resulted in rapid reduction of inflammatory cytokines but did not affect the SARS-CoV-2-specific IgG or CD4+ T cell responses in MIS-C. In these data, MIS-C had a unique cytokine profile but not a unique SARS-CoV-2 specific humoral or T cell cytokine response.

1. Introduction

Multisystem inflammatory syndrome in children (MIS-C) is a novel and severe hyperinflammatory disease associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. MIS-C generally occurs 2–4 weeks after primary infection [2,3], with an estimated incidence of 31.6 cases per 100,000 paediatric SARS-CoV-2 cases in the USA [4], and 22 cases per 100,000 paediatric SARS-CoV-2 infections in Cape Town, South Africa [5]. As children are relatively spared from acute severe SARS-CoV-2, MIS-C represents the most severe manifestation of SARS-CoV-2 infection in children; with approximately 40% of children with MIS-C requiring intensive care unit admission [4] and a 2% mortality rate, which is markedly higher than primary coronavirus disease 2019 (COVID-19) in children [6,7].

MIS-C is widely reported throughout the world, and affects black children disproportionately [8,9]. Despite this, MIS-C has been poorly reported in Africa, with scattered clinical case series [10]. In Cape Town, South Africa, we have documented and described the clinical phenotype of a large cohort of children with MIS-C throughout the COVID-19 pandemic at the two major paediatric referral hospitals in the city [5,11,12]. These data show that MIS-C does affect children in Africa and the dearth of reported cases likely reflects poor recognition of the disease in children from less resourced areas. Children with MIS-C present acutely unwell with fever, rash, multi-organ disease and shock [5,9,13]. This is not an uncommon clinical presentation in children, especially in South Africa and other African countries where sepsis and gastrointestinal shock are common [14]. The unique immune phenotype in children with MIS-C as compared to clinically similar diseases at presentation in this setting is unknown. MIS-C is treated with intravenous immunoglobulins (IVIG) and/or steroids, with recent evidence showing that steroid therapy alone may be sufficient [15,16]. These treatments may be contra-indicated in sepsis, infections and other diseases that present similarly. Immunological markers may therefore be useful in identifying MIS-C at presentation and thereby initiating therapy sooner.

The underlying immune pathology and cause of MIS-C is incompletely understood. There is a mature immunoglobulin (Ig) response in MIS-C as compared to acute SARS-CoV-2 infection, confirming the post-infectious nature of the disease [17], with no unique production of SARS-CoV-2 specific antibodies [17–27], however the function of these antibodies has been incompletely assessed. Multiple cytokines, including interleukin (IL)-1 β , IL-6, IFN- γ , IL-10 and clinical markers of inflammation such as C-reactive protein (CRP) are markedly elevated compared to children with acute SARS-CoV-2 infection, previous SARS-CoV-2 infection or Kawasaki Disease (KD) [5,9,17,18,28–40]. These comparator groups are less representative of the clinical setting, where clinicians must distinguish MIS-C from other common paediatric febrile diseases at presentation. Fewer data compare the cytokine production at presentation, prior to therapy, between MIS-C and similar inflammatory or infectious childhood diseases [33,38,41].

Children with MIS-C have low T cell frequencies, but increased T cell activation and expansion of non-specific TCR V β 21.3+ cells

[17,28,32,41–43]. This expansion bears the hallmark of a superantigen response [44]; indeed, a superantigen-like motif has been identified in Spike using *in silico* modelling [45] but it remains to be confirmed experimentally, and it is unclear whether T cells respond differently to SARS-CoV-2 in patients with MIS-C with conflicting published data available. Hsieh et al. 2022 report that SARS-CoV-2-specific T cell responses were similar in children with MIS-C compared to children with previous SARS-CoV-2 infection [46], while Singh et al. 2022 report that SARS-CoV-2-specific T cell responses in children with MIS-C were reduced compared to COVID-19 convalescent children although comparable to healthy children [19]. Both studies included children who had received steroids which may have affected T cell function, and neither quantified the virus-specific T cell response in MIS-C. In addition, SARS-CoV-2-specific T cell responses in MIS-C have not been compared to other inflammatory conditions to assess whether the T cell response is unique in MIS-C or rather reflects non-specific inflammation.

In this study we aimed to discover the unique immune phenotype of MIS-C in treatment-naïve South African children with MIS-C, SARS-CoV-2 exposed healthy children and children with diseases that are clinically similar to MIS-C at presentation within a similar time. We investigated the cytokine production, SARS-CoV-2 antibody responses and function, and SARS-CoV-2-specific T cell responses in this cohort and measured the change in immune variables with time and treatment in children with MIS-C.

2. Methods

2.1. Patient cohort and sample collection

From 26 June 2020 to 9 February 2022, children presenting to the emergency room with suspected MIS-C and at least 3 days of fever at the Red Cross War Memorial Children's Hospital in Cape Town, South Africa were recruited. Clinical data and samples were collected at presentation and throughout hospitalisation. Patients were ultimately diagnosed by attending clinicians as having confirmed MIS-C (WHO criteria [47]) or another final diagnosis and these children were categorised as 'inflammatory controls'. Children with Kawasaki disease (classified as meeting AHA 2017 criteria [48] and SARS-CoV-2 antibody negative) were excluded from this study. During the same period, healthy children without chronic or inflammatory diseases who were hospitalised for elective surgeries were recruited and classified as healthy controls. The detailed clinical features and whole blood RNA expression of this cohort has been published [5,49].

This protocol was approved by the relevant ethics committees at both the University of Cape Town (UCT HREC 112/2012; 599/2020) and Red Cross War Memorial Children's Hospital (RXH RCC240/WC 202008.113). Consent, and assent where appropriate, were provided prior to recruitment as per ethical requirements.

2.2. Serum cytokines and chemokines

Patient serum was used to measure inflammatory cytokine concentrations in a subset of children with MIS-C, inflammatory and healthy controls. A customised Milliplex kit (Millipore, St Charles, Missouri) was used to quantify the serum cytokines IL-1 receptor antagonist (RA), IL-1 β , IL-10, IL-6, IL-27, monocyte chemoattractant protein 1 (MCP-1) and tumour necrosis factor alpha (TNF- α) on the Bio-Plex platform (Bio-Rad Laboratories, Hercules, California), according to manufacturer instructions. Briefly filter plates were coated with wash buffer and assay buffer thereafter internal standards, controls and serum samples were added to respective wells. Pre-mixed beads (antibody immobilized) of the makers of interest were added to all wells and incubated overnight at 4 °C. Plates were washed and detection antibody was added and incubated for 1 h. Thereafter Streptavidin-Phycoerythrin was added and incubated for 30 min in the dark. Following this, Sheath Fluid was added to each well and sheath and results were read on the Luminex® instrument plate reader.

2.3. Serum CRP

Patient CRP tests were performed for clinical indications at the local clinical laboratory.

2.4. Antibody enzyme-linked immunosorbent assay

Antibody titres were measured in serum from children with MIS-C, seropositive inflammatory controls, and seropositive healthy controls. Briefly, two μ g/ml of SARS-CoV-2 Spike protein was used to coat 96-well, high-binding plates and incubated overnight at 4 °C. The plates were incubated in a blocking buffer consisting of 5% skimmed milk powder, 0.05% Tween 20, 1 $\times$ PBS. Plasma samples were diluted to a 1:100 dilution and added to plates followed by the addition of secondary antibody diluted to 1:3000 in blocking buffer. TMB substrate (ThermoFisher Scientific) was then added and upon stopping the reaction with 1 M H₂SO₄, absorbance was measured at a 450 nm wavelength. In all instances, monoclonal antibody CR3022 was used as a positive control and Palivizumab was used as a negative control.

2.5. Neutralisation assays

Methods for neutralisation assay were performed as previously described [50]. Briefly, SARS-CoV-2 pseudotyped lentiviruses were prepared by co-transfecting the HEK 293 T cell line with either the SARS-CoV-2614G spike (D614G), Beta (L18F, D80A, D215G, 242-244del, K417N, E484K, N501Y, D614G, and A701V), Delta (T19R, 156-157del, R158G, L452R, T478K, D614G, P681R, and D950N) and Omicron (BA.1) (A67V, D69-70, T95I, G142D, D143-145, D211, L212I, 214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, and L981F) plasmids, in conjunction with a firefly luciferase encoding lentivirus backbone plasmid and a murine leukaemia virus backbone plasmid. The parental plasmids were kindly provided by Dr. Elise Landais and Dr. Devin Sok (International AIDS Vaccine Initiative, New York, NY USA). Heat-inactivated plasma samples from MIS-C patients were incubated with the SARS-CoV-2 pseudotyped virus for 1 h at 37 °C, 5% CO₂. Subsequently, 1 $\times$ 10⁴ HEK293T cells engineered to over-express ACE-2, kindly provided by Dr. Michael Farzan (Scripps Research, La Jolla, CA USA), were added and incubated at 37 °C, 5% CO₂ for 72 h, upon which the luminescence was measured. Both neutralisation ID₅₀ values for the D614G SARS-CoV-2 variant and the neutralisation ID₅₀ values for the SARS-CoV-2 variant that the patient was likely exposed to (autologous) was assessed. This autologous variant was assigned using the NICD definition of 30 cases per 100,000 population in the province [51]. Additionally, the cross reactivity of anti-SARS-CoV-2

S antibodies was assessed.

2.6. Antibody-dependent cellular phagocytosis (ADCP)

The ADCP assay measures the level at which antibody-opsonised target beads are engulfed by THP-1 cells. ADCP assays were performed as previously described [52]. Briefly, fluorescent neutravidin beads were coated with biotinylated SARS-CoV-2 spike protein and incubated for 2 h with either monoclonal antibodies at a starting concentration of 50 μ g/ml titrated fivefold or participant plasma samples at a dilution of 1:100. CR3022 and a plasma pool of SARS-CoV-2 positive individuals were used as a positive control while Palivizumab and a plasma pool of negative individuals were used as a negative control. The beads were then incubated with THP-1 cells per well overnight, fixed with 4% paraformaldehyde and interrogated on the FACSaria II. Phagocytosis score was calculated as the percentage of THP-1 cells that engulfed fluorescent beads multiplied by the geometric mean fluorescence intensity of the population in the FITC channel with background (no antibody control) subtraction.

2.7. Antibody-dependant cellular cytotoxicity (ADCC)

The ADCC assay measures the ability of antibodies to cross link spike-expressing target cells and the Fc γ RIIIa receptor present on Jurak cells [50]. Briefly, HEK293T cells were transfected with 5 μ g SARS-CoV-2 D614G spike variant plasmid and were incubated at 37 °C for 2 days. Subsequently, 1 $\times$ 10⁵ spike transfected cells per well were incubated with heat inactivated plasma (1:100 final dilution) or monoclonal antibodies (final concentration of 100 μ g/ml) in RPMI 1640 media supplemented with 10% FBS 1% Pen/Strep (Gibco, Gaithersburg, MD) for 1 h at 37 °C. Jurkat-Lucia NFAT-CD16 cells (2 $\times$ 10⁵ cells/well; Invivogen) were added and were incubated at 37 °C for 24 h. Twenty microlitres supernatant was removed and transferred to a white 96-well plate and luciferase activity was measured following the addition of 50 μ l reconstituted QUANTI-Luc secreted luciferase reagent with a one second integration time. Relative light units (RLU) of a no antibody control were subtracted as background. Palivizumab was used as a negative control, while CR3022 was used as a positive control. To induce the transgene 1 $\times$ cell stimulation cocktail (ThermoFisher Scientific, Oslo, Norway) and 2 μ g/ml ionomycin in R10 was added as a positive control to confirm sufficient expression of the Fc receptor. RLUs for spikes were normalised to each other and between runs using CR3022.

2.8. SARS-CoV-2 antigens

Commercially available peptide pools (15mer sequences with 11 amino acid overlap) covering the immunodominant regions of the Wuhan-1 SARS-CoV-2 spike, (PepTivator, Miltenyi Biotech), nucleocapsid and membrane (17mer with 10 amino acid overlap; BEI resources) proteins were used. The spike (S) peptide pool was prepared by resuspending in distilled water at a concentration of 50 μ g/ml and used at a concentration of 1 μ g/ml. Nucleocapsid (N) and membrane (M) peptide pools were prepared by resuspending in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 100 μ g/ml and used at a final concentration of 1 μ g/ml.

2.9. Whole blood-based T cell detection assay

Whole blood was collected in sodium heparin tubes and processed within 4–8 h of collection. Blood (500 μ L) was stimulated for 24 h, at 37 °C with SARS-CoV-2 S, N and M peptide pools in the presence of Brefeldin-A (10 μ g/ml, Sigma-Aldrich) and co-stimulatory antibodies against CD28 (clone 28.2) and CD49d (clone L25) (1 μ g/ml each; BD Biosciences). Unstimulated blood was incubated with an equimolar amount of dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a background control. After 24 h, blood was treated with 2 mM EDTA (Sigma-Aldrich)

and followed by red blood cell lysis and cell fixation using BD FACS lysing solution (BD Biosciences). Cells were cryopreserved in freezing media (90% foetal bovine serum (FBS) and 10% DMSO) and stored at -80°C until batch analysis.

Fixed, cryopreserved cells were subsequently thawed, washed in FACS washing buffer (1% FBS and 99% PBS) and transferred to 96-well V-bottom plate prior to surface staining with CD4 ECD (SFC12T4D11, Beckman Coulter) and CD8 BV510 (RPA-8, Biolegend). Cells were permeabilized using BD Perm/wash buffer (BD Bioscience) and stained with CD3 BV650 (OKT3, Biolegend) IFN- γ AlexaFluor® 700 (B27, Biolegend), TNF- α BV786 (Mab11, Biolegend) and IL-2 APC (MQ1-17H12, Biolegend). Stained cells were washed and fixed with BD CellFIX buffer (BD Bioscience). Samples were acquired on a BD LSR-II flow cytometer and analysed using FlowJo (v10). Cells were gated on singlets, lymphocytes and CD3 cells. Results are expressed as the frequency of CD4+ or CD8+ T cells expressing IFN- γ , IL-2 or TNF- α . Cytokine responses are presented as background subtracted values (from the frequency of cytokine produced in unstimulated cells).

2.10. Quantification and statistical analysis

Statistical analyses of serum inflammatory markers, antibody titres and antibody-dependent processes were performed in SPSS Statistics (v28, IBM Corp., Armonk, NY) while statistical analyses of T cell responses were performed in Prism (v9.4.1; GraphPad Software Inc). One-way analysis of variants (ANOVA) was used for comparing three or more cohorts. Inflammatory marker concentrations were log transformed and therefore a parametric ANOVA was used, and non-parametric tests were used for all other comparisons. The Mann-Whitney and Kruskal-Wallis test with Dunn's multiple comparisons post-test were used for unmatched samples. Fisher's exact tests were used for comparisons between the proportion of responders represented as pie charts, and Chi-square test was used to compare the statistical differences between the median contributions of the polyfunctional profiles. P values <0.05 were considered statistically significant. Details of analyses performed for each experiment are described in the figure legends.

3. Results

3.1. Study cohort description

A total of 83 children with suspected MIS-C were enrolled: 58 had ultimately confirmed MIS-C and 25 had an alternate diagnosis (inflammatory controls). Ninety-one healthy children (healthy controls) were recruited (Table 1). The clinical phenotype of these patients has been previously described in larger cohort studies [5,11,12,49], but the basic clinical details and treatments are provided in Supplementary Table 1. The inflammatory controls had a range of diagnoses which are further outlined in Supplementary Table 2. Briefly, the median age among the children recruited in the study was similar; children with MIS-C had a median age of 5.07 years, and the median age of both the inflammatory

Table 1
Study cohort demographic features and assay sample sizes.

	MIS-C	Inflammatory controls	Healthy
n	58	25	91
Age, median (IQR)	5.07 (2.45–8.75)	5.32 (2.39–8.73)	5.32 (2.38–9.33)
Female, n (%)	30 (51.7%)	12 (48.0%)	27 (29.7%)
Black African, n (%)	31 (53.4%)	10 (40.0%)	37 (40.7%)
SARS-CoV-2 antibody positive, n (%)	57 (98.3%)	14 (56.0%)	34 (37.3%)
Sample analysed, n			
Serum cytokine	28	13	41
Antibody response	58	14	33
T cell response	8	6	24

controls and healthy controls was 5.32 years. There was no sex bias in the MIS-C and inflammatory control cohort, with 51% and 48% of children being female respectively, while 29% of healthy controls were female. The racial and ethnic distribution of the cohort consisted mostly of black and mixed-race children (Table 1). Samples were collected prior to any MIS-C treatment (methylprednisolone or IVIG) in 74% (43/58) of children with MIS-C (Supplementary Table 1). Based on the SARS-CoV-2-spike specific serology, 98% (57/58) of children with MIS-C were SARS-CoV-2 seropositive, whilst 56% (14/25) of inflammatory controls and 37% (34/91) of the healthy controls had detectable SARS-CoV-2-spike specific antibodies (Table 1). No patients had been vaccinated at the time of sampling and none of the seropositive healthy children or inflammatory controls had a history of confirmed SARS-CoV-2 infection.

3.2. Cytokines and CRP are upregulated in MIS-C compared to other inflammatory conditions

Of the consented patients, 28 MIS-C, 13 inflammatory controls and 41 healthy controls had pre-treatment serum inflammatory cytokine concentrations measured (Table 1). Compared to inflammatory controls, children with MIS-C had significantly increased concentrations of IL-10 ($p = 0.033$, Fig. 1C), IL-6 ($p = 0.01$, Fig. 1D) and CRP ($p = 0.008$, Fig. 1H). IL-1RA ($p = 0.054$, Fig. 1A) and MCP-1 ($p = 0.059$, Fig. 1E) were elevated in MIS-C, although this did not reach statistical significance. Compared to healthy controls, children with MIS-C had increased concentrations of all cytokines measured (Fig. 1A–G).

Following treatment with IVIG and/or methylprednisolone, there was a rapid decrease in the serum concentration of all measured cytokines and CRP in children with MIS-C (Fig. 2).

3.3. Anti-SARS-CoV-2 IgG binding antibody titres, neutralising activity and Fc effector function do not differ between groups but are affected by epidemiological wave

There was no difference in the wild-type D614G variant spike IgG titres in patients with MIS-C compared to SARS-CoV-2-seropositive inflammatory controls or SARS-CoV-2-seropositive healthy controls (Fig. 3A). There was no difference in the 'autologous' neutralising ability (as measured towards the SARS-CoV-2 variant that the patient was likely exposed to in a specific infection wave), ADCP or ADCC between groups (Fig. 3B–D). Children with MIS-C did not have altered neutralising cross-reactivity of SARS-CoV-2 antibodies across the SARS-CoV-2 waves compared to SARS-CoV-2-seropositive inflammatory controls or SARS-CoV-2-seropositive healthy controls (Fig. 3E). Spike IgG responses were unaffected by treatment in children with MIS-C (Fig. 3F).

Children with MIS-C who presented in the first SARS-CoV-2 infection wave driven by the D614G variant had significantly higher spike-specific IgG antibody titres compared to those who presented in the waves driven by the Beta ($p = 0.032$), Delta ($p = 0.013$) and Omicron (BA.1) ($p < 0.0001$) variants (Supplementary Fig. 1A). Overall, there were no significant differences in the autologous neutralisation activity in MIS-C patients who were infected with any SARS-CoV-2 variant (Supplementary Fig. 1B).

3.4. SARS-CoV-2-specific T cell profiling reveals a largely monofunctional CD4+ T cell profile in MIS-C patients and inflammatory controls compared to healthy controls

T cell responses were measured pre-treatment in children with MIS-C ($n = 8$), seropositive inflammatory controls ($n = 6$) and seropositive healthy controls ($n = 24$) (Fig. 4A and E). Children with MIS-C had lower frequencies of CD3+ T cells than SARS-CoV-2-seropositive healthy controls ($p = 0.0320$) but similar to SARS-CoV-2 seropositive inflammatory controls ($p > 0.9999$, Fig. 4B). No difference in CD3+ T cell frequency was observed between SARS-CoV-2-seropositive healthy controls and inflammatory controls ($p = 0.0814$, Fig. 4B). There was no

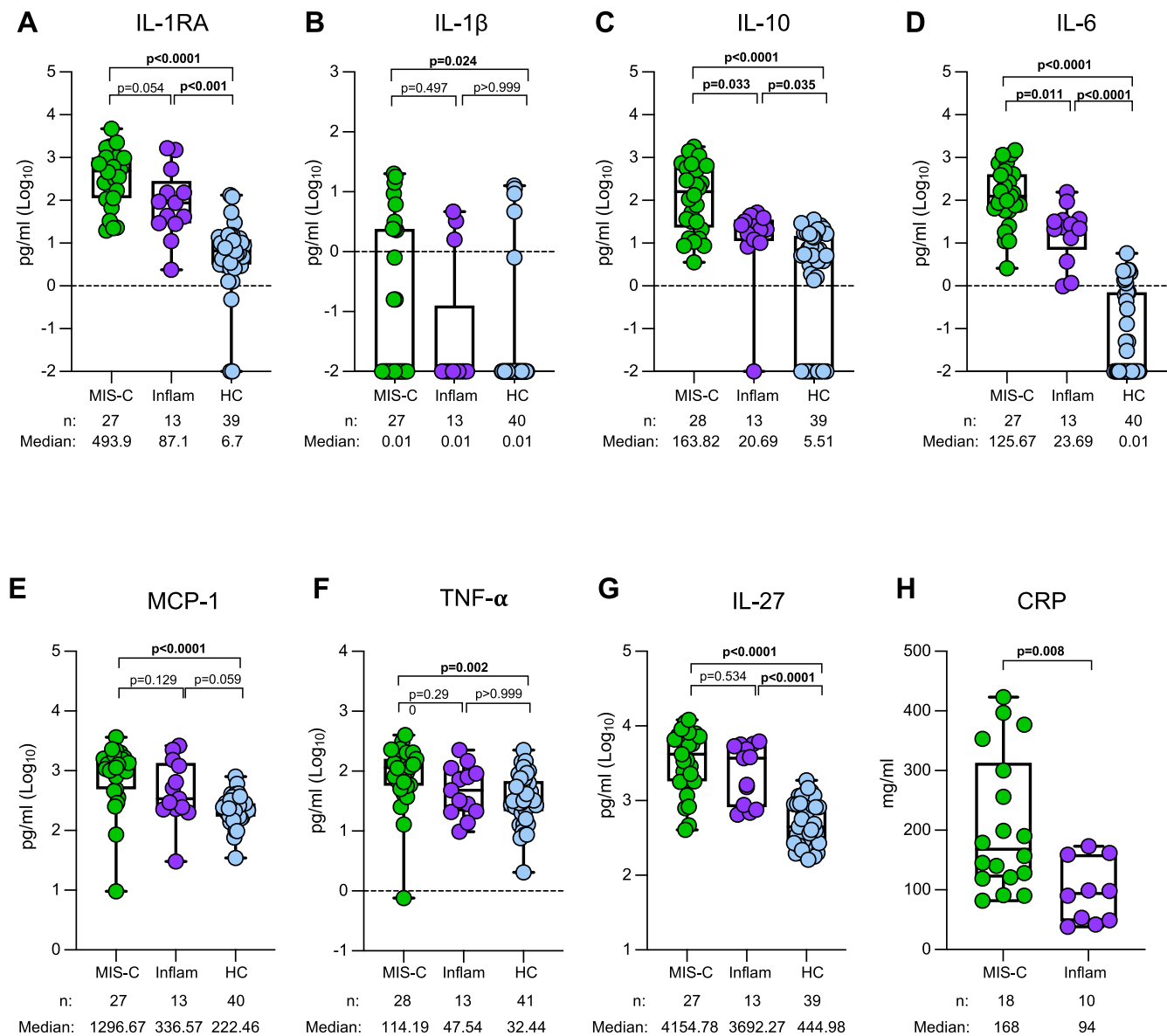


Fig. 1. Serum cytokine levels in pre-treatment samples from MIS-C patients, inflammatory controls, and healthy children.

Box and whisker plots demonstrate the median Log_{10} concentration, range, and interquartile range for (A) IL-1RA, (B) IL-1 β , (C) IL-10, (D) IL-6, (E) MCP-1, (F) TNF- α , (G) IL-27 and (H) CRP in each group (Green - MIS-C: multisystem inflammatory syndrome in children, purple - Inflam: inflammatory controls, blue - HC: healthy controls). Detection limits were 1.29 pg/ml for IL-1RA, 0.52 pg/ml for IL-1 β , 0.91 pg/ml for IL-10, 0.14 pg/ml for IL-6, 3.05 pg/ml for MCP-1, 5.39 pg/ml for TNF- α and 50.78 pg/ml for IL-27. The median concentrations are shown below the graphs. Statistical comparisons were assessed using a one-way analysis of variants (ANOVA) with Bonferroni correction for multiple testing. *p* values <0.05 were considered statistically significant and are bolded.

difference in the frequency of CD4 $^{+}$ and CD8 $^{+}$ T cells between all the groups (Fig. 4C and D).

There was no difference in the proportion of SARS-CoV-2-specific CD4 $^{+}$ T cell responders between all groups, with all (8/8) children with MIS-C (pre-treatment), all inflammatory controls (6/6) and 83% (20/24) of the healthy controls having detectable SARS-CoV-2-specific CD4 $^{+}$ T cell responses against SARS-CoV-2 S, N, and M combined peptide pools. Similarly, no differences were observed in the proportion of responders (*p* = 0.5569) and the magnitude of responses between inflammatory and healthy controls (*p* = 0.4490, Fig. 4F).

Overall, 25% (2/8) of children with MIS-C, 50% (3/6) of inflammatory controls and 42% (10/24) of healthy controls had detectable SARS-CoV-2-specific CD8 $^{+}$ T cell responses, with no differences in the proportion of responders or magnitude of CD8 $^{+}$ T cell responses between each of the groups (Supplementary Fig. 2A).

Furthermore, no differences were observed between the proportion

of responders (*p* = 0.2918) or the magnitude of SARS-CoV-2-specific CD4 $^{+}$ T cell responses (*p* = 0.1875) pre- and post-treatment in children with MIS-C (Fig. 4G).

The functional profile of the responding T cells was measured by their capacity to produce different combinations of IFN- γ , TNF- α or IL-2 after stimulation with SARS-CoV-2 S, N and M peptide pools (Fig. 4H). The overall CD4 $^{+}$ T cell functional profile in children with MIS-C and inflammatory controls was similar (*p* = 0.0558), whereas in children with MIS-C this was found to be different compared to healthy controls (*p* < 0.0001, Fig. 4H). Indeed, in healthy controls a higher proportion of SARS-CoV-2-specific CD4 $^{+}$ T cells were polyfunctional, characterized by the production all three measured cytokines (*p* = 0.0058), and dual-functional cells producing any two of the measured cytokines (*p* = 0.0221) compared to children with MIS-C, who presented a largely monofunctional SARS-CoV-2-specific CD4 $^{+}$ T cell profile compared to healthy controls (*p* = 0.0033, Fig. 4H). The predominant cytokines

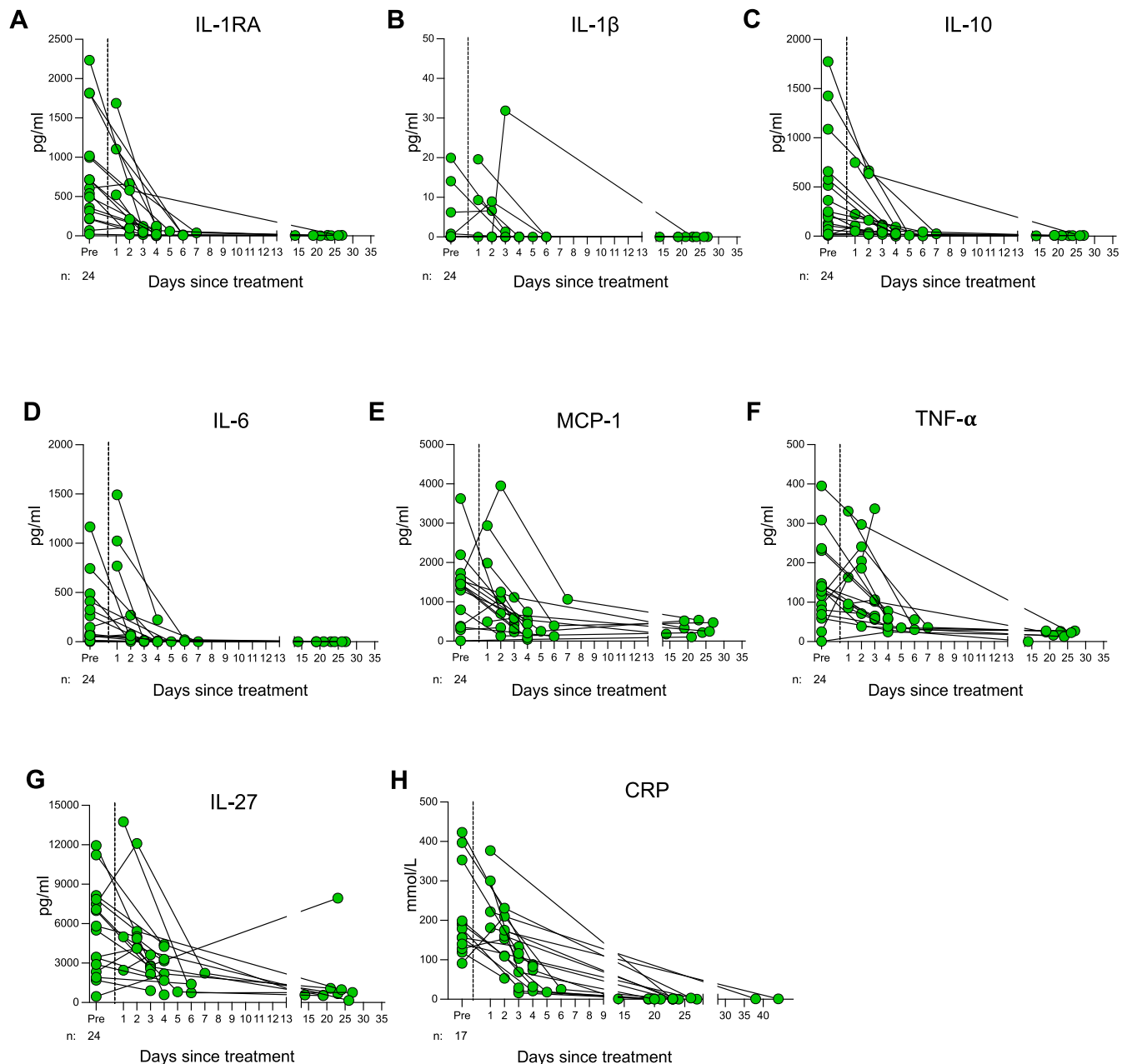


Fig. 2. Effect of treatment on the inflammatory cytokine profile in MIS-C cases. Longitudinal profile of serum inflammatory marker concentrations in children with MIS-C pre- and post-treatment. (A) IL-1RA, (B) IL-1 β , (C) IL-10, (D) IL-6, (E) MCP-1, (F) TNF- α , (G) IL-27 and (H) CRP.

produced by monofunctional T cells in MIS-C and inflammatory controls were TNF- α (50.57% and 28.39%, respectively; $p = 0.439$) or IL-2 (25.25% and 29.17%, respectively) compared to 12.7% and 21.52%, respectively, for TNF- α and IL-2 in healthy controls. The proportion of TNF- α ⁺ monofunctional T cells among MIS-C patients was highly variable (IQR: 5.19%–92.42%).

4. Discussion

In this study we observed that serum IL6, IL10 and CRP were distinctly raised in MIS-C, suggesting a unique MIS-C cytokine response. This inflammatory cytokine response resolved quickly with treatment in MIS-C, mirroring the clinical phenotype [5]. Intriguingly for a disease related to SARS-CoV-2 infection, the SARS-CoV-2 specific antibody and T cell responses were not unique to MIS-C.

It has been documented that patients with MIS-C have an overproduction of IL-6, IL-10, IL-1 β , IL-27, IFN- γ , MCP-1, IL-1RA, TNF- α and CRP [17,18,28–36,40], which is mirrored in our data when comparing the serum levels of these inflammatory cytokines to healthy controls. The normalization of cytokines with disease improvement that we have observed matches other data [32,37]. A unique strength of this study is that it agnostically recruited patients with fever and suspected MIS-C at presentation and pre-therapy. These groups represent the true conundrum that clinicians face in identifying cases with true MIS-C vs other clinically similar, severe diseases in the acute setting, especially in a region where shock, sepsis and infectious diseases are common. In this setting, we have shown that IL-6, IL-10, and CRP, are elevated in MIS-C compared to children with similar clinical diseases and may be useful diagnostic markers for further investigation. A previous study reported that that IL-6 and CRP concentrations were increased in ICU-admitted

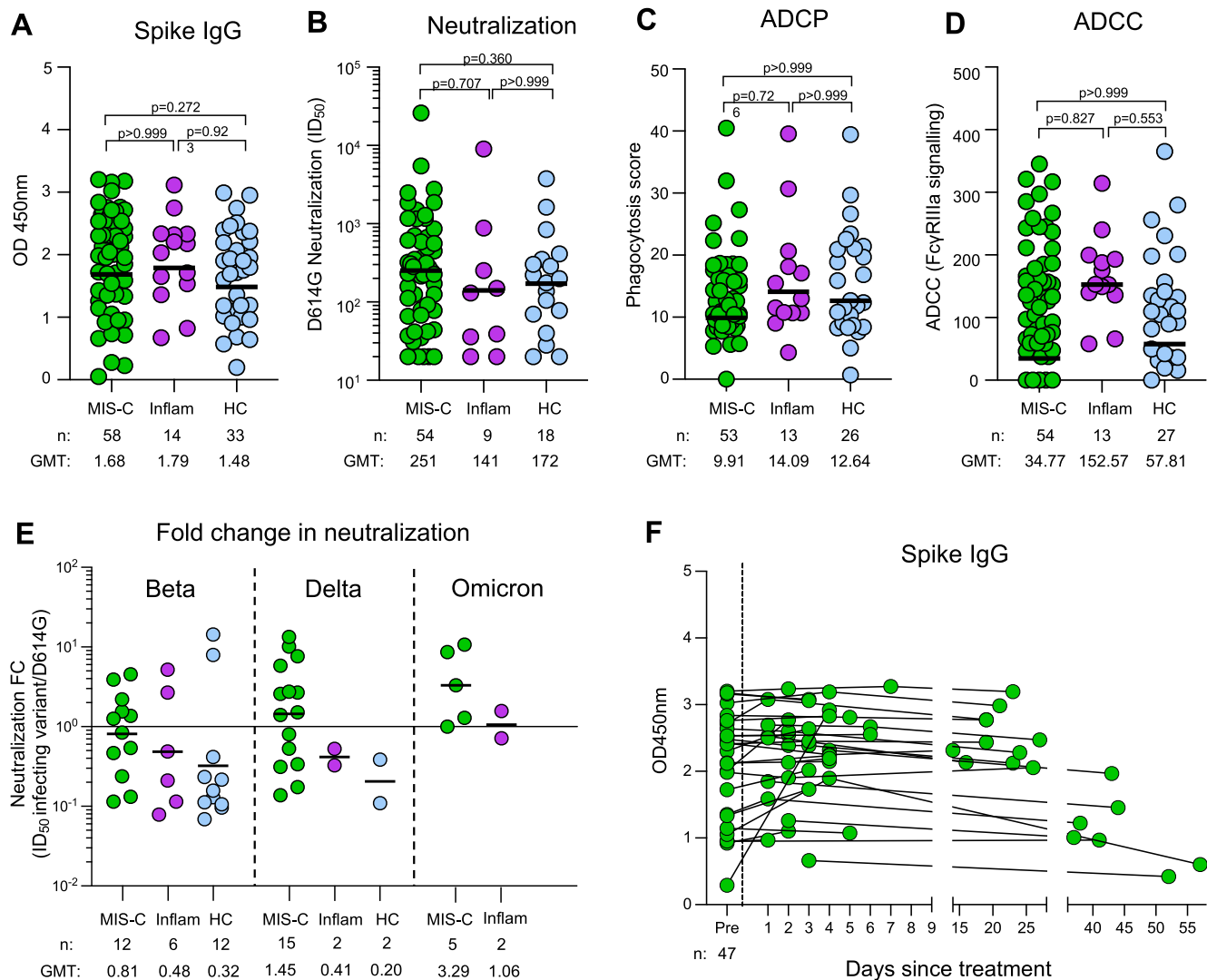


Fig. 3. Comparison of SARS-CoV-2 spike IgG binding antibody titre, neutralisation activity and antibody-dependent processes between groups.

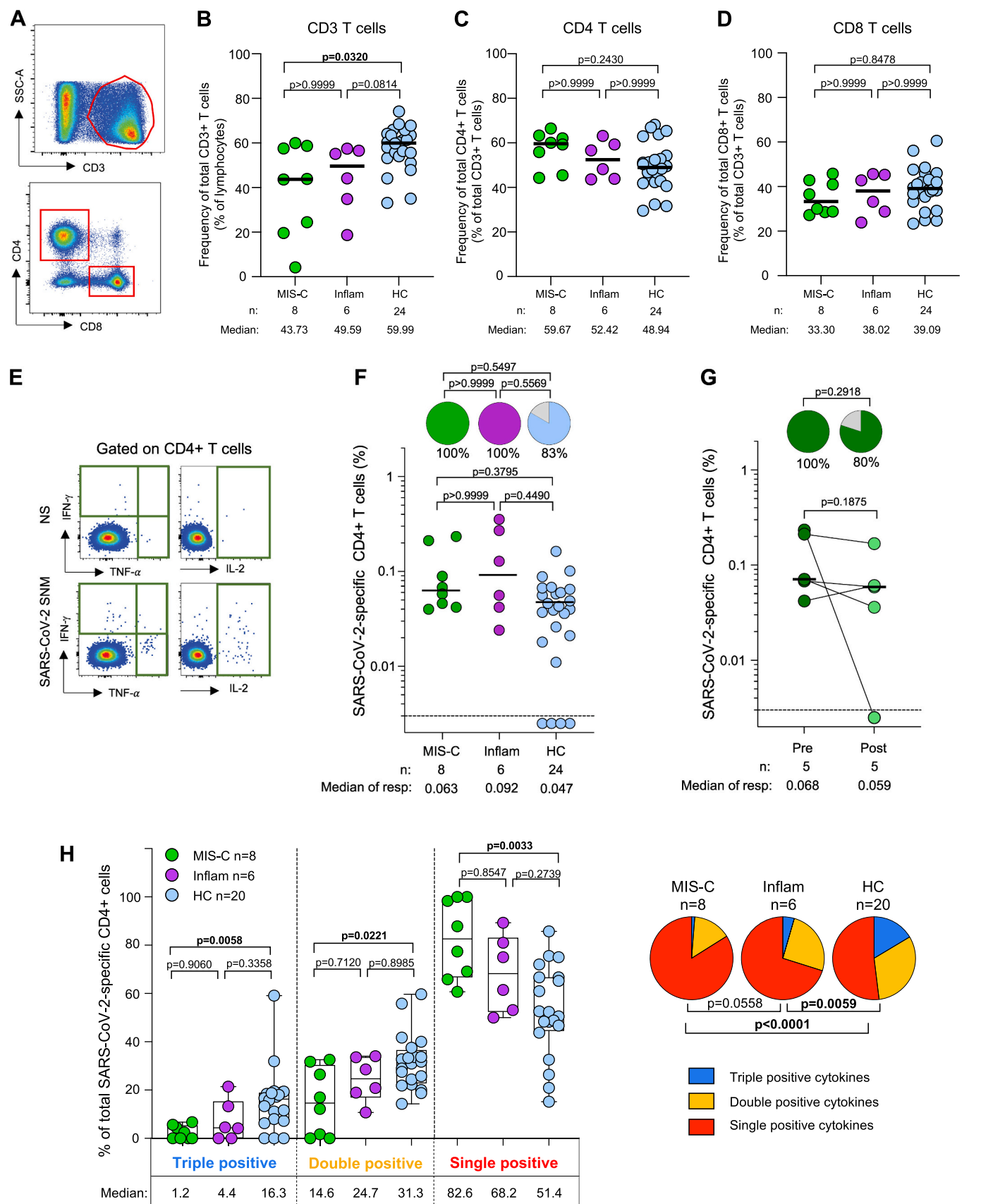
Serum samples were analysed to measure (A) SARS-CoV-2 D614G spike IgG binding antibodies quantified by OD450nm values, (B) Neutralisation activity of the SARS-CoV-2 autologous pseudovirus indicated by 50% inhibitory dilution (ID_{50}) values, (C) Antibody-dependent cellular phagocytosis (ADCP) activity, (D) Antibody-dependent cellular cytotoxicity (ADCC) activity measured in $Fc\gamma RIIIa$ signalling. (E) Fold change in D614G neutralising activity relative to SARS-CoV-2 Beta, Delta, and Omicron (BA.1) variants in children with MIS-C (green), inflammatory controls (purple) and healthy controls (blue). The cross-reactivity of anti-SARS-CoV-2-spike antibodies was assessed by dividing the neutralisation ID_{50} of the autologous SARS-CoV-2 variant to the neutralisation ID_{50} of D614G SARS-CoV-2 variant. Geometric mean titre (GMT) is shown below the graphs. (F) longitudinal profile of SARS-CoV-2 D614G spike IgG pre- and post-treatment in children with MIS-C. Statistical comparisons were assessed using a one-way analysis of variants (ANOVA) test with multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

children with sepsis compared to children with MIS-C [41]. We argue that ICU admitted children with sepsis are not an optimal comparator group, as only about 38% of children with MIS-C require ICU admission [5] and sepsis is probably the most severe of the mimics of MIS-C. This may skew these findings resulting in higher inflammatory cytokines in the ICU group. It is noteworthy that IL-27 has previously been shown to have increased concentrations in 8 children with MIS-C pre-treatment compared to 14 febrile controls who were recruited in a similar manner to this study [53]. This is of particular interest to us as we have recently shown that the gene expression of *IL-27* was the best discriminator between MIS-C and inflammatory controls in a panel of 84 immune genes [49]. The current data, however, did not support this, with no significant difference in IL-27 concentrations between MIS-C and inflammatory controls. These data further enforce that MIS-C is a hyperinflammatory disease that has its own distinct inflammatory signature.

Our data complement other studies that have reported that children with MIS-C have comparable titres of SARS-CoV-2 antibodies compared

to healthy children exposed to SARS-CoV-2 who did not develop MIS-C [18,23–26]. These antibodies have been shown to neutralize the SARS-CoV-2 virus to the same degree as COVID-19 convalescent children [18,24,25], similar to our reported findings. There is less published data on the SARS-CoV-2 antibody Fc effector function in children with MIS-C. In this study we investigated ADCP and ADCC function in children with MIS-C, inflammatory and healthy controls. Rostad et al. found that healthy exposed children had comparable ADCC function compared to patients with MIS-C²⁴, which was also found in the current study. Therefore, accumulating evidence demonstrates no difference in the levels or function of SARS-CoV-2 antibodies in MIS-C, suggesting that these antibodies are unlikely to be driving the unique pathology of MIS-C.

Multiple studies have analysed SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses in COVID-19 convalescent, mild and severely ill adults, confirming a role for T cells in ameliorating disease severity [54–56]. Similar to severe COVID-19 illness, lymphopenia has been



(caption on next page)

Fig. 4. T cell frequency, SARS-CoV-2-specific T cell responses and functional profile in children presenting MIS-C, SARS-CoV-2 seropositive inflammatory controls and healthy controls.

A) Representative examples of CD4 and CD8 T cell distribution. The top plot shows the gating of total CD3+ T cells and the bottom plot shows CD4+ and CD8+ T cells. Frequency of CD3+ T cells (B), CD8+ T cells (C) and CD4+ T cells (D) in each study group. The bars represent the median. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's correction. (E) Representative flow cytometry plots of interferon-gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α) and interleukin-2 (IL-2) cytokine production by CD4+ T cells in response to SARS-CoV-2 peptide pools in one child with MIS-C. NS: no stimulation, SNM: SARS-CoV-2-spike, nucleocapsid and membrane combined peptide pools. (F) The magnitude of SARS-CoV-2-specific CD4+ T cell responses producing any of the measured cytokines (IFN- γ , IL-2 or TNF- α). The pie charts represent the proportion of responders with a detectable SARS-CoV-2-specific CD4 T cell response. Bars represent the median of the responders. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's correction and Fisher's exact test was used to compare the percentage of responders. (G) The magnitude of SARS-CoV-2-specific CD4+ T cell responses in children with MIS-C ($n = 5$) pre- and post-treatment (steroids and/or intravenous immunoglobulins). The pies represent the proportion of responders with a detectable SARS-CoV-2-specific CD4 response. Bars represent the median of the responders. Statistical comparison was performed using the Wilcoxon matched pairs signed rank test and Fisher's exact test was used to compare the percentage of responders. (H) Polyfunctional profile of SARS-CoV-2-specific CD4+ T cells. The pie charts represent the proportions of triple (IFN- γ + IL-2 + TNF- α), double (IFN- γ + IL-2+ or IFN- γ + TNF- α + or IL-2 + TNF- α +) and single (IFN- γ + or IL-2+ or TNF- α +) cytokine producing cells. Each pie slice represents the median contribution of produced cytokines to the total SARS-CoV-2 responses. Bars represent the median and interquartile range. The Kruskal-Wallis test with Dunn's correction was used to compare the response patterns between the groups and Chi-square test was used to compare the statistical differences between the pie charts. p values <0.05 were considered statistically significant and are bolded.

reported in MIS-C [43,57]. In this study we found that the frequency of CD3+ T cells was lower in children with MIS-C compared to healthy controls, although the CD3+ T cell frequency was measured as the proportion of lymphocytes using flow cytometry. We can therefore not conclude that this reduction indicates lymphopenia and could be as a result of expansion of CD3- populations. To this end, a study by Vella et al. showed a decreased proportion of CD3+ T cells and increased proportion of B cells in MIS-C compared to COVID-19 in children, demonstrating lymphocyte dysregulation [43].

Numerous studies have analysed SARS-CoV-2-specific T cell responses in MIS-C, compared to acute or convalescent COVID-19 in children and adults [19,23,27,42,58,59]. The data are largely conflicting, with some reports showing increased antigen-specific T cell responses in children with MIS-C [23,59], some showing equivalent responses [27,58] and yet others demonstrating a reduced magnitude T cell response [19,42] compared to either acute or convalescent children or adults. Several factors could account for the differences between studies, such as: 1) the different types of T cell assays employed; 2) antigens used to quantify responses; and 3) cohort differences, including timing of sampling during acute or convalescent MIS-C, differing comparator groups, as well as the severity of disease in MIS-C or convalescent groups. It is important to note that we, and others [27,60–62] have previously shown that the SARS-CoV-2-specific T cell responses in adults are higher than in children, rendering comparisons to adults difficult to interpret. Our data showing a similar magnitude of responses in children with MIS-C and healthy children exposed to SARS-CoV-2 are concordant with that of Pierce et al. and Lam et al. [27,58] Our data also extend these studies to demonstrate that the magnitude of SARS-CoV-2-specific T cell responses in children with MIS-C is comparable to SARS-CoV-2 seropositive children with non-MIS-C inflammatory conditions, suggesting that generalized inflammation does not impair the capacity of T cells to produce a SARS-CoV-2-specific T cell response.

We observed that in children with MIS-C, SARS-CoV-2-specific responding cells were predominantly monofunctional, producing a single cytokine (either IL-2 or TNF- α), while SARS-CoV-2 seropositive healthy controls exhibited a polyfunctional CD4+ T cell profile. In contrast, the study by Rybkina et al. showed an increased frequency of IL-2 producing CD4+ T cells, as well as IFN- γ and TNF- α CD8+ T cells in convalescent MIS-C compared convalescent COVID-19 patients [59] from which they concluded that MIS-C T cells had increased functionality. However, it is important to note that polyfunctional analysis to determine the capacity of these T cells to co-produce 2 or more cytokines was not undertaken. In our study, a similar mono-functional profile was observed in SARS-CoV-2 seropositive inflammatory controls as was seen in MIS-C patients. Two possible explanations for the observed shift towards a monofunctional response are: 1) the superantigen-like properties of the spike protein that have been described; and 2) T cell

exhaustion due to sustained systemic inflammation. In support of the first, it is plausible that non-specific superantigen-TCR binding [63] does not fully engage the TCR, resulting in suboptimal T cell activation and a skewing towards monofunctional cells producing TNF- α or IL-2. Interestingly, there is a precedent for TNF- α or IL-2 dominance in the T cell responses to other known superantigens [64]. The highly variable proportions of monofunctional TNF-producing T cells could be explained by different frequencies of TCR V β 21.3+ T cells in individual patients, although this requires experimental confirmation. In contrast, it is possible that the monofunctional profile, shared by MIS-C patients and inflammatory controls, results from sustained systemic inflammation that could drive excessive proliferation, differentiation, and possible exhaustion of antigen-specific T cells, leading to a skewed T cell response enriched with monofunctional cells. Indeed, studies have reported increased expression of PD-1 on SARS-CoV-2-specific T cells in MIS-C, possibly indicative of T cell exhaustion [43,65]. In this scenario, the highly variable proportions of monofunctional TNF-producing T cells could be explained by different degrees of T cell exhaustion in individual patients.

5. Conclusions

Our data provide an immunological comparison between children with MIS-C, other inflammatory diseases, and healthy children. At presentation, serum IL6, IL10 and CRP were raised in children with MIS-C, and rapidly returned to normal following treatment. SARS-CoV-2-specific antibody titres, antibody-dependent functions, and the magnitude of the SARS-CoV-2-specific T cell responses, however, were not unique in MIS-C. The quality of the T cell response to SARS-CoV-2, however, was compromised in children with MIS-C displaying decreased T cell polyfunctionality which was similar to that observed in non-MIS-C inflammatory controls. These results suggest that the monofunctional T cell cytokine production may be due to non-specific inflammation and not unique to MIS-C.

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Author contributions

K-W acted as principal investigator and led and designed the clinical study and co-ordinated all samples, institutional requirements, teams, and collaborations. C-B, H-F-T, J.D, H. VDR, A.F and K-W consented patients into the study. C-B, D.A and T.F-S processed patient serum samples. C-B, J.D, H.VDR and K-W extracted patient data from medical records. C-B and U.R performed serum cytokine analyses with the assistance of M.S. P.L.M, S.I.R and T.M-G designed the antibody experiments. F.A, N.P.M, Z.M, S.R.B, M.M, and C-B performed antibody experiments. R.S-K and W.A.B designed the T cell experiments. N-B, A.N, and R.B performed T cell assays. N-B, C.R, W.A.B and R.S-K analysed the T cell data. C-B, N-B, R.S.K and K.W wrote the paper. All authors reviewed and edited the manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2023.109877>.

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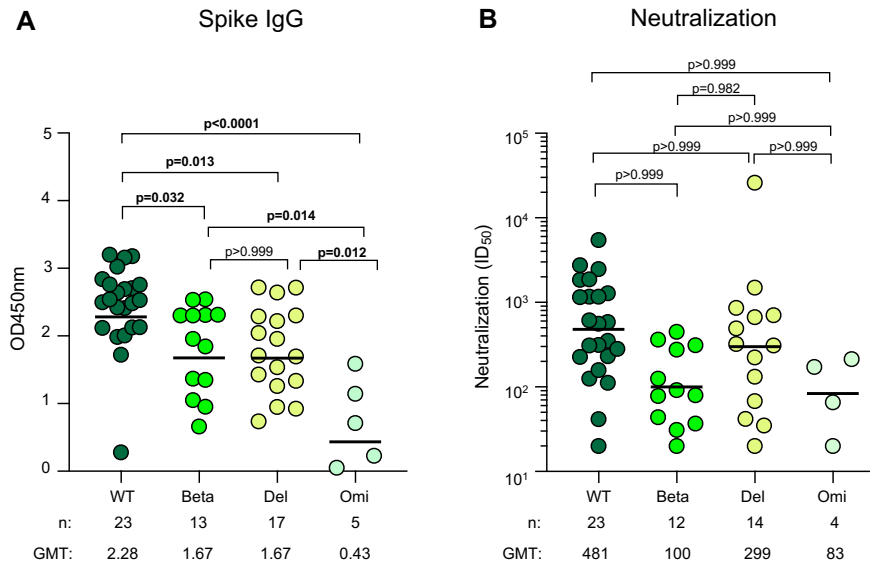
Supplementary Table 1

	MIS-C (n=58)	Inflammatory controls (n=25)
Age (years), median (IQR)	5.28 (2.81, 8.73)	5.31 (2.39, 8.73)
Female, n (%)	29 (50.0)	13 (52.0)
Days of fever (IQR)	6 (4, 7)	6 (3, 8)
Tachycardia (%)	57 (98.3)	19 (76.0)
Rash (%)	54 (93.1)	10 (40.0)
Conjunctivitis (%)	49 (84.5)	6 (24.0)
Received IVIG, n (%)	54 (93.1)	7 (28.0)
Received methylprednisolone, n (%)	33 (56.9)	3 (12.0)
Admitted to ICU, n (%)	17 (29.3)	4 (16.0)
Received Inotropes, n (%)	16 (27.6)	4 (16.0)
Ventilated, n (%)	2 (3.4)	4 (16.0)
Highest CRP (mmol/L), median (IQR)	193 (129, 300)	145 (44, 230)

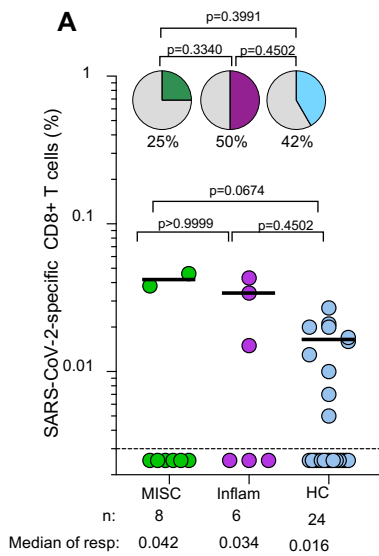
Supplementary Table 2

	Number of patients
Adenovirus	1
Tuberculosis	2
Typhoid	2
Pneumonia	1
Dysentery	4
Appendicitis	1
Abdominal sepsis	2
Systemic JIA with MAS	1
Gastroenteritis	2
Kukuchi Disease	1
Salmonella	1
Pyrexia of unknown origin	2
Viral upper RTI	3
Viral lower RTI	1

Supplementary Figure 1



Supplementary Figure 2



CONTRIBUTION FOR PUBLICATION

Contribution details for the manuscript “**Comparing the immune abnormalities in MIS-C to healthy children and those with inflammatory disease reveals distinct inflammatory cytokine production and a monofunctional T cell response**” published in *Clinical Immunology* (doi: 10.1016/j.clim.2023.109877).

- Kate Webb contributed to the conception and design of the study.
- **Ntombi Benede**, Amkele Ngomti and Richard Baguma received and processed samples using whole blood assays and stored the samples.
- **Ntombi Benede** performed the antibody panel building and optimization for flow cytometry.
- **Ntombi Benede** generated the data, through sample staining followed by acquisition on the flow cytometer and analyzed the flow cytometry data.
- **Ntombi Benede**, Catherine Riou, Wendy A. Burgers and Roanne S. Keeton contributed to the flow cytometry data interpretation.
- **Ntombi Benede** wrote and drafted the manuscript specific on T cell data including introduction, results, methods, discussions and conclusion and Claire Butters wrote and drafted the manuscript specifically on the serum cytokine concentration and antibody responses.
- Catherine Riou, Wendy A. Burgers, Roanne S. Keeton and Kate Webb edited and proofread the manuscript.
- Liesl Zühlke and Kate Webb established and led the Immunology Biorepository and MIS-C sub-study.
- Claire Butters, Heidi Facey-Thomas, Jonathan Day, Timothy F. Spracklen, Hamza van der Ross, Christiaan Scott and Kate Webb recruited participants and managed samples from the Immunology Biorepository and MIS-C sub-study cohort.
- Claire Butters, Ursula Rohlwink and Muki Shey performed the serum cytokines experiments.
- Thandeka Moyo-Gwete, Simone I. Richardson and Penny L. Moore designed the antibody experimental study.
- Frances Ayres, Nelia P. Manamela, Zanele Makhado, Sashkia R. Balla and Mashudu Madzivhandila performed the antibody experiment work.
- Kate Webb, Wendy A. Burgers and Roanne S. Keeton contributed to the execution and oversight of the experimental work.
- All authors read, revised, and approved the final version of the manuscript.

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06 May 2024

Dear Colleagues:

Supervisor letter of support for Ms Ntombi Sindiswa Buhle Benede's PhD thesis plagiarism Turnitin report.

I would like to motivate to the UCT Doctoral Degree Board and examiners for the approval of Ms NSB Benede's (student number: BNDNTOO05) plagiarism report despite the high similarity score. On the 4th of March 2024, Ms Benede received approval from the UCT Doctoral Degree Board to incorporate two of her publications, as detailed below, into her PhD thesis. The integration of these articles has contributed to a higher similarity score of 44% in Ms Benede's final plagiarism report generated by Turnitin. However, a total of 33% of that similarity can be attributed to her own work. Specifically, 19% of the similarity is due to publication 1, and 14% due to publication 2. Thus, only 11% of the similarity can be traced to other sources. Moreover, I have personally verified that Source 2, identified solely as "internet source www.ncbi.nih.gov" in the Turnitin report, corresponds to the Pubmed internet source of publication 1. Additionally, source 3 and 4 are correctly identified as preprint versions from MedRxiv, which are associated with publication 1.

1. **Ntombi Benede**, Marius B Tincho, Avril Walters, Vennesa Subbiah, Amkele Ngomti, Richard Baguma, Claire Butters, ... Wendy A Burgers & Roanne S Keeton. (2023). Distinct T cell polyfunctional profile in SARS-CoV-2 seronegative children associated with endemic human coronavirus cross-reactivity. iScience, <https://doi.org/10.1016/j.isci.2023.108728>.
2. Claire Butters*, **Ntombi Benede***, Thandeka Moyo-Gwete, Simone I Richardson, Ursula Rohlwink, ... Roanne S Keeton & Kate Webb. (2024). Comparing the immune abnormalities in MIS-C to healthy children and those with inflammatory

disease reveals distinct inflammatory cytokine production and a monofunctional T cell response. 2024; Clinical Immunology, <https://doi.org/10.1016/j.clim.2023.109877>. (*Co-first author)

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Source 1 (14%) is the publication listed as no 2 above.

Source 2 (10%) is the PubMed internet source for the publication listed as no 1 above.

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Based on the above, I hereby confirm, that I have reviewed and discussed the high similarity score with Ms Benede and support the inclusion of this report in her final thesis submission.

Yours sincerely,

Signed by candidate

Roanne S. Keeton, Ph.D.
PhD Supervisor of Ms NSB Benede
Senior Research Officer,
Division of Medical Virology,
Department of Pathology,
Faculty of Health Sciences,
University of Cape Town.