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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

Claire Butters [a,b,1], Ntombi Benede [b,c,1], Thandeka Moyo-Gwete [d,e], Simone I. Richardson [d,e], Ursula Rohlwink [f,g,h], Muki Shey [b,i,j], Frances Ayres [d,e], Nelia P. Manamela [d,e], Zanele Makhado [d,e], Sashkia R. Balla [d,e], Mashudu Madzivhandila [d,e], Amkele Ngomti [b,c], Richard Baguma [b,c], Heidi Facey-Thomas [a], Timothy F. Spracklen [a,k], Jonathan Day [a], Hamza van der Ross [a], Catherine Riou [b,c,j], Wendy A. Burgers [b,c,j], Christiaan Scott [a,l], Liesl Zühlke [a,b,k,m], Penny L. Moore [d,e,n], Roanne S. Keeton [b,c,1], Kate Webb [a,h,*,1]

[a] Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital, University of Cape Town, Klipfontein Road, Rondebosch, 7700 Cape Town, South Africa
[b] Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Anzio Road, Observatory, 7935 Cape Town, South Africa
[c] Division of Medical Virology, Department of Pathology, University of Cape Town, Anzio Road, Observatory, 7935 Cape Town, South Africa
[d] SA MRC Antibody Immunity Research Unit, School of Pathology, University of the Witwatersrand, Modderfontein Road, Sandringham, 2192 Johannesburg, South Africa
[e] National Institute for Communicable Diseases of the National Health Laboratory Services, Modderfontein Road, Sandringham, 2192 Johannesburg, South Africa
[f] Division of Neurosurgery, Department of Surgery, Red Cross War Memorial Children's Hospital, University of Cape Town, Klipfontein Road, Rondebosch, 7700 Cape Town, South Africa
[g] Neuroscience Institute, University of Cape Town, Anzio Road, Observatory, 7935 Cape Town, South Africa
[h] Crick African Network, The Francis Crick Institute, Midland Road, London NW1 1AT, United Kingdom
[i] Department of Medicine, University of Cape Town, Anzio Road, Observatory, 7935 Cape Town, South Africa
[j] Wellcome Centre for Infectious Diseases Research in Africa, University of Cape Town, Anzio Road, Observatory, 7935 Cape Town, South Africa
[k] Cape Heart Institute, University of Cape Town, Anzio Road, Observatory, 7935 Cape Town, South Africa
[l] Clinical Research Centre, University of Cape Town, Groote Schuur Hospital, Observatory, 7935 Cape Town, South Africa
[m] South African Medical Research Council, Francie Van Zijl Drive, Parow Valley, 7501 Cape Town, South Africa
[n] Centre for the AIDS Programme of Research in South Africa, Umbilo Road, 4001 Durban, South Africa

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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 Vβ, 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.

* Corresponding author at: Department of Paediatrics and Child Health, Red Cross War Memorial Children's Hospital, University of Cape Town, Klipfontein Road, Rondebosch, 7700 Cape Town, South Africa.

E-mail addresses: claire.butters@uct.ac.za (C. Butters), bndnto005@myuct.ac.za (N. Benede), thandekam@nicd.ac.za (T. Moyo-Gwete), simoner@nicd.ac.za (S.I. Richardson), ukrohlwink@uct.ac.za (U. Rohlwink), muki.shey@uct.ac.za (M. Shey), francesa@nicd.ac.za (F. Ayres), neliam@nicd.ac.za (N.P. Manamela), sashkiab@nicd.ac.za (S.R. Balla), amkele.ngomti@uct.ac.za (A. Ngomti), Heidi.facey-thomas@uct.ac.za (H. Facey-Thomas), timothy.spracklen@uct.ac.za (T.F. Spracklen), hamza.vanderross@uct.ac.za (H. van der Ross), cr.riou@uct.ac.za (C. Riou), wendy.burgers@uct.ac.za (W.A. Burgers), chris.scott@uct.ac.za (C. Scott), liesl.zuhlke@uct.ac.za (L. Zühlke), pennym@nidc.ac.za (P.L. Moore), Roanne.keeton@uct.ac.za (R.S. Keeton), kate.webb@uct.ac.za (K. Webb).

[1] Equal contributions.

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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)-1β, 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 Vβ 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-1β, 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 μg/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× 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 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.

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% CO_2. Subsequently, 1×10^4 HEK293T cells engineered to overexpress ACE-2, kindly provided by Dr. Michael Farzan (Scripps Research, La Jolla, CA USA), were added and incubated at 37 °C, 5% CO_2 for 72 h, upon which the luminescence was measured. Both neutralisation ID_{50} values for the D614G SARS-CoV-2 variant and the neutralisation ID_{50} 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 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 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 FcγRIIIa receptor present on Jurak cells [50]. Briefly, HEK293T cells were transfected with 5 μg SARS-CoV-2 D614G spike variant plasmid and were incubated at 37 °C for 2 days. Subsequently, 1×10^5 spike 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 h at 37 °C. Jurkat-Lucia NFAT-CD16 cells (2×10^5 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 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 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 μg/ml and used at a concentration of 1 μg/ml. Nucleocapsid (N) and membrane (M) peptide pools were prepared by resuspending in dimethyl sulfoxide (DMSO, Sigma) at a concentration of 100 μg/ml and used at a final concentration of 1 μg/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 μL) 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 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 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 (SFCI12T4D11, 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 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.

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

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

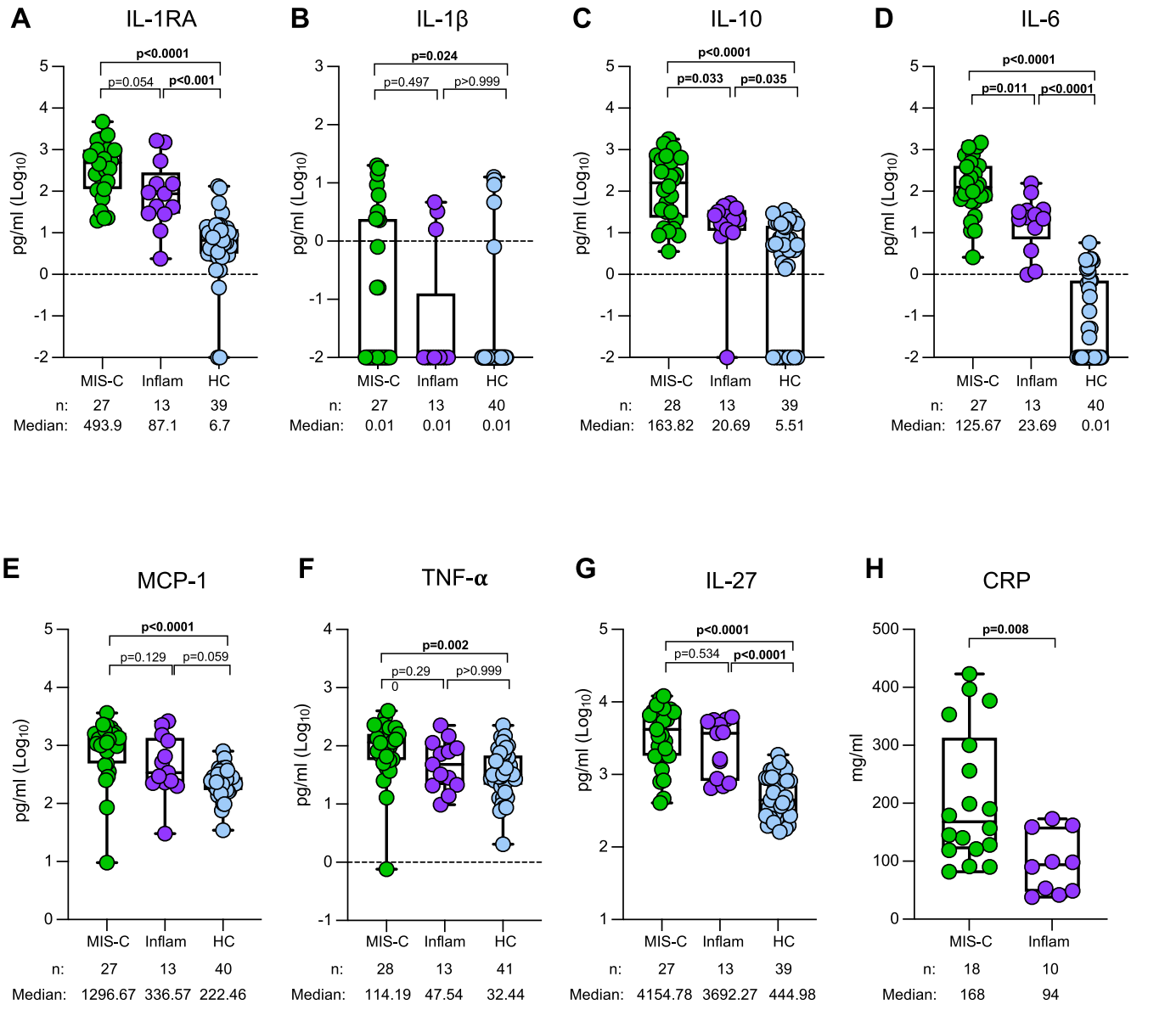

Fig. 1. Serum cytokine levels in pre-treatment samples from MIS-C patients, inflammatory controls, and healthy children.
Box and whisker plots demonstrate the median Log_{10} concentration, range, and interquartile range for (A) IL-1RA, (B) IL-1β, (C) IL-10, (D) IL-6, (E) MCP-1, (F) TNF-α, (G) IL-27 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-1β, 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

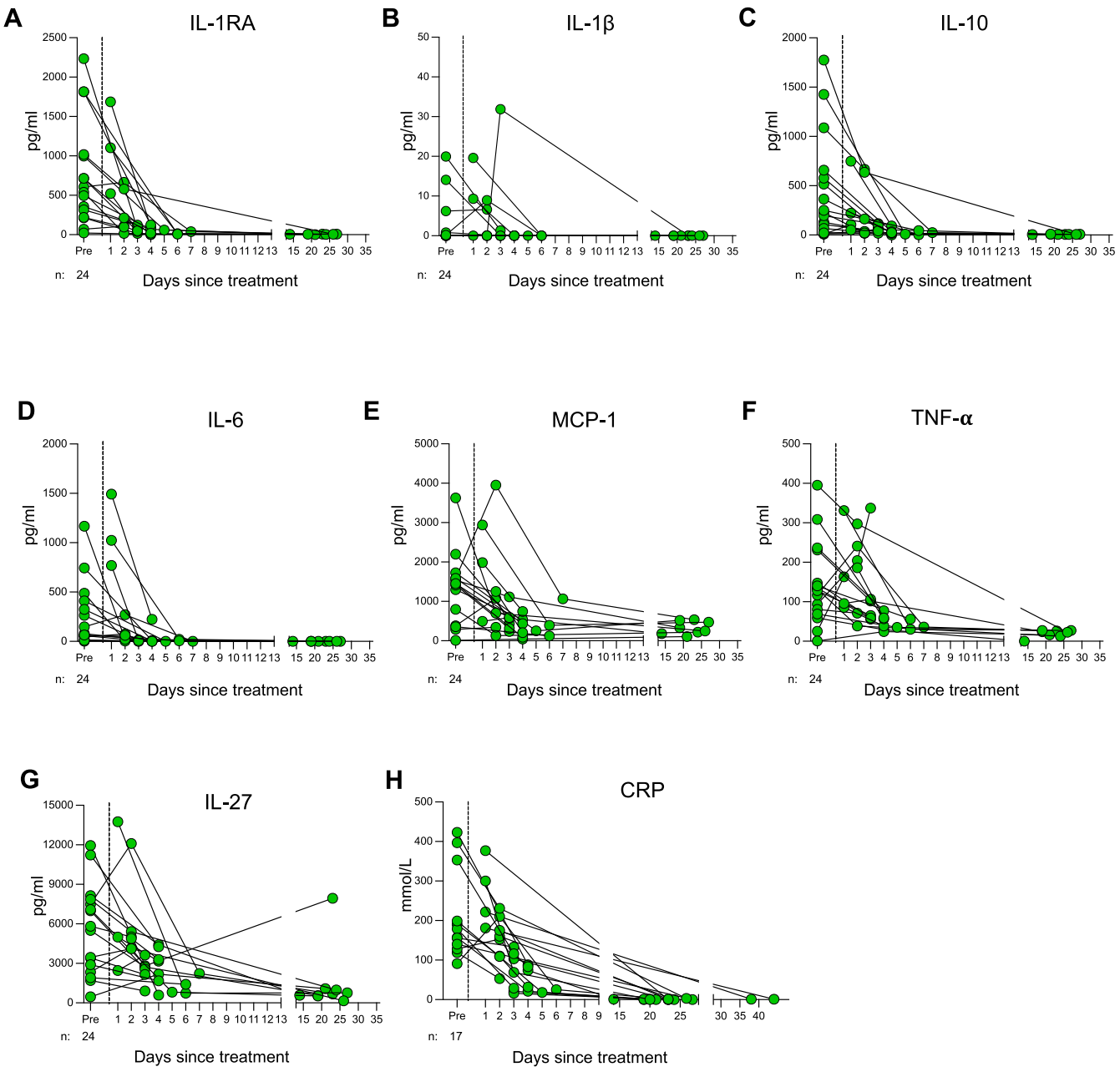

Fig. 2. Effect of treatment on the inflammatory cytokine profile in MIS-C cases.
Longitudinal profile of serum inflammatory marker concentrations in children with MIS-C pre- and post-treatment. (A) IL-1RA, (B) IL-1β, (C) IL-10, (D) IL-6, (E) MCP-1, (F) TNF-α, (G) IL-27 and (H) CRP.

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-1β, 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

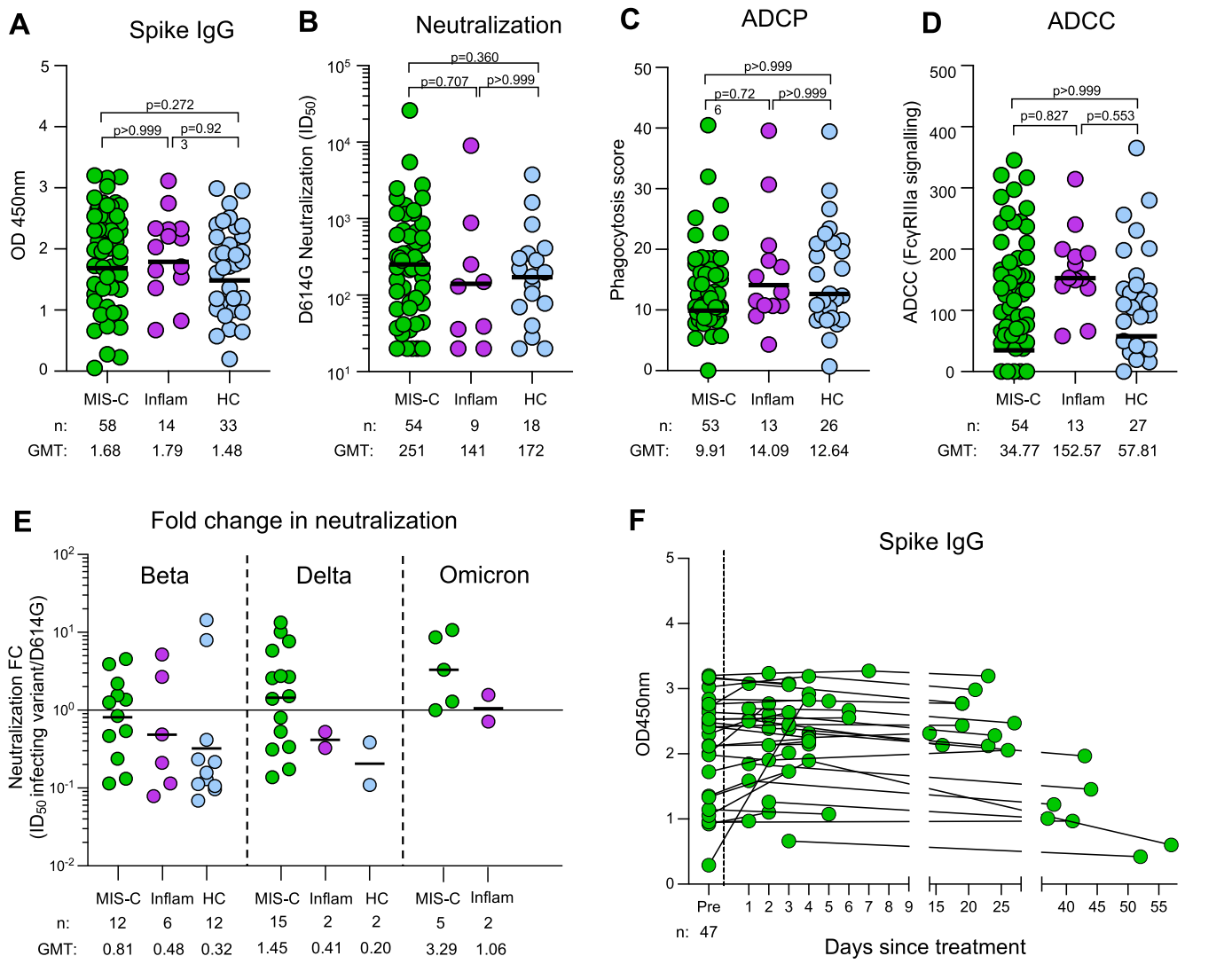

Fig. 3. Comparison of SARS-CoV-2 spike IgG binding antibody titre, neutralisation activity and antibody-dependent processes between groups.
Serum samples were analysed to measure (A) SARS-CoV-2 D614G spike IgG binding antibodies quantified by OD450nm values, (B) Neutralisation activity of the SARS-CoV-2 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 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-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-C[24], 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

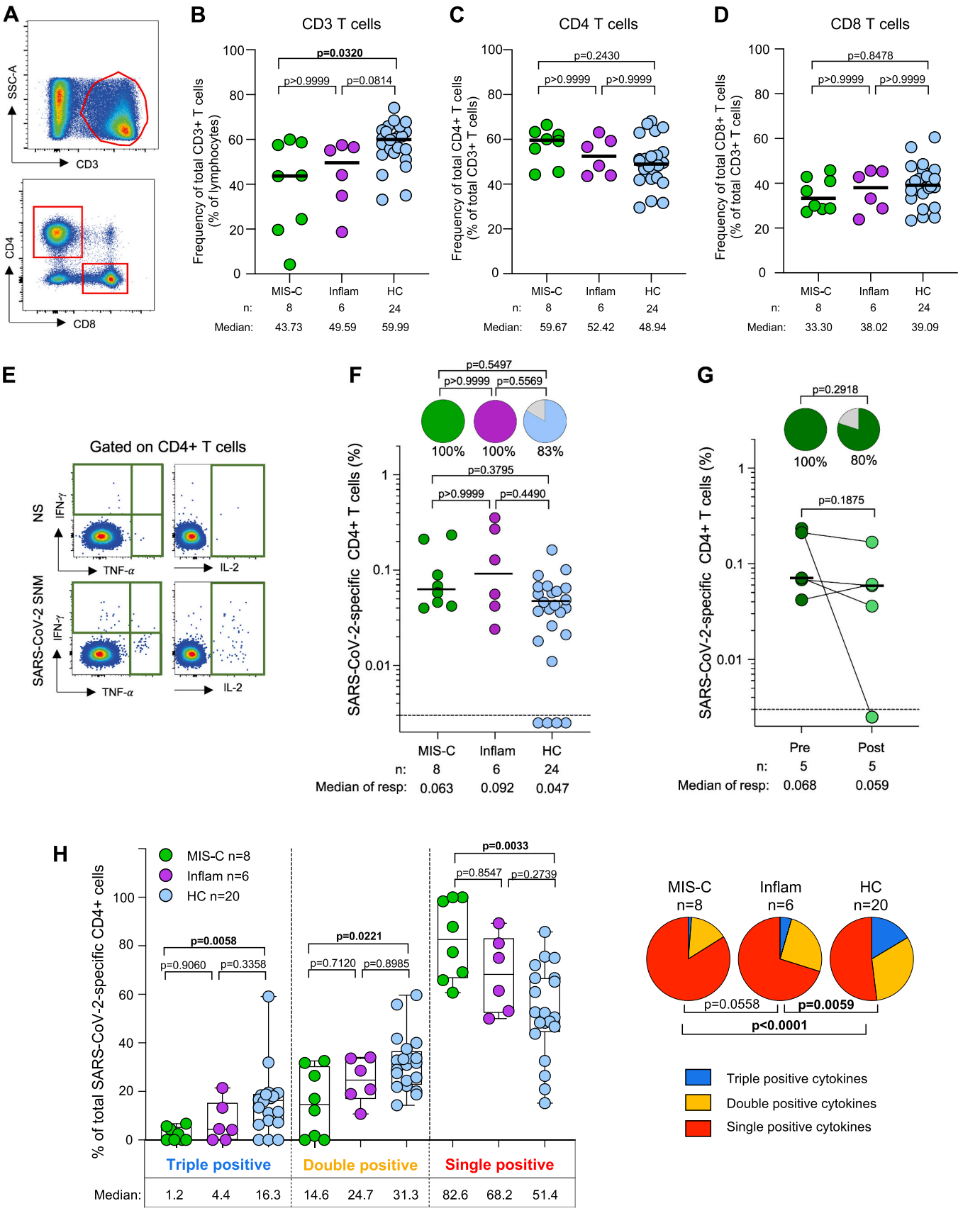

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Fig. 4. T cell frequency, SARS-CoV-2-specific T cell responses and functional profile in children presenting MIS-C, SARS-CoV-2 seropositive inflammatory controls and healthy controls.
A) Representative examples of CD4 and CD8 T cell distribution. The 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 Vβ 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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