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## SARS-CoV-2-specific T cell responses in patients with multisystem inflammatory syndrome in children

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### ABSTRACT

Multisystem inflammatory syndrome in children (MIS-C) is a severe complication of SARS-CoV-2 infections that occurs in the pediatric population. We sought to characterize T cell responses in MIS-C compared to COVID-19 and pediatric hyperinflammatory syndromes.

MIS-C was distinct from COVID-19 and hyperinflammatory syndromes due to an expansion of T cells expressing *TRBV11-2* that was not associated with HLA genotype. Children diagnosed with MIS-C, but who were negative for SARS-CoV-2 by PCR and serology, did not display V $\beta$  skewing. There was no difference in the proportion of T cells that became activated after stimulation with SARS-CoV-2 peptides in children with MIS-C compared to convalescent COVID-19. The frequency of SARS-CoV-2-specific TCRs and the antigens recognized by these TCRs were comparable in MIS-C and COVID-19.

Expansion of V $\beta$ 11-2<sup>+</sup> T cells was a specific biomarker of MIS-C patients with laboratory confirmed SARS-CoV-2 infections. Children with MIS-C had robust antigen-specific T cell responses to SARS-CoV-2.

### 1. Introduction

Multisystem inflammatory syndrome in children (MIS-C) emerged as a complication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in children during the first wave of the COVID-19 pandemic [1,2]. MIS-C is notable for several remarkable features, which distinguish this syndrome from COVID-19 in pediatric patients. While children with acute SARS-CoV-2 infections are often

asymptomatic or present with mild upper respiratory symptoms, patients with MIS-C have prominent fevers, mucocutaneous involvement, and gastrointestinal symptoms [3–5]. The characteristic rashes, conjunctival injection, and mucositis of MIS-C is reminiscent of Kawasaki disease (KD) and toxic shock syndrome (TSS) [1,2]. MIS-C is further defined by its severity and a significant proportion of patients present in shock and require admission to the intensive care unit (ICU) [3–6]. Curiously, MIS-C manifests about a month after the primary SARS-CoV-2

**Abbreviations:** Multisystem inflammatory syndrome in children, MIS-C; T cell Receptor Beta Variable gene, TRBV; human leukocyte antigens, HLA; severe acute respiratory syndrome coronavirus 2, SARS-CoV-2; Kawasaki disease, KD; toxic shock syndrome, TSS; intensive care unit, ICU; T cell receptor, TCR; systemic juvenile idiopathic arthritis, sJIA; macrophage activation syndrome, MAS; Centers for Disease Control, CDC; World Health Organization, WHO; coronary artery aneurysms, CAAs; polymerase chain reaction, PCR; electronic medical record, EMR; institutional review board, IRB; peripheral blood mononuclear cells, PBMCs; complementarity determining region 3, CDR3; multiplexed identification of antigen-specific T cell receptors assay, MIRA; spike protein of SARS-CoV-2, (S); nucleocapsid protein of SARS-CoV-2, (N); membrane protein of SARS-CoV-2, (M); body mass index, BMI; National Marrow Donor Program, NMDP; T Cell Receptor Beta Joining gene, TRBJ; single cell RNA sequencing, scRNA-seq; multisystem inflammatory syndrome in adults, MIS-A; intravenous immunoglobulin, IVIG.

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infection in children who mount an appropriate antibody response to the virus [3,4,7–10].

The delayed presentation of MIS-C in comparison to the primary viral infection suggests that the adaptive immune response plays a role in driving the pathology of this disorder. While the majority of children with MIS-C are seropositive for antibodies that neutralize SARS-CoV-2, less is known about T cell responses [2,5–10]. Circulating T cells are decreased in patients with MIS-C but display features of lymphocytes that are activated and proliferating, particularly in CD8<sup>+</sup> T cells that can patrol the vasculature [10–12]. CD4<sup>+</sup> and CD8<sup>+</sup> T cells from patients with MIS-C display increased usage of the T cell Receptor Beta Variable 11–2 gene (*TRBV11–2*) that is not found in pediatric COVID-19, severe adult COVID-19, pre-pandemic KD, or TSS [12–16]. This expansion of Vβ11–2<sup>+</sup> T cells resolves quickly with immunomodulatory treatment and is not found in convalescent MIS-C [14,16]. Further, greater V-beta (Vβ) repertoire skewing is associated with higher levels of circulating inflammatory cytokines and increased disease severity in patients with MIS-C [13–16]. Porritt et al. and Sacco et al. demonstrated associations between HLA class I alleles (A02, C35, C04) and increased usage of *TRBV11–2* in T cells from MIS-C patients; however, this has not been replicated in other studies [12,14–16]. Furthermore, it is not known if such *TRBV11–2* expansions occur in other pediatric hyperinflammatory syndromes such as systemic juvenile idiopathic arthritis (sJIA) or macrophage activation syndrome (MAS).

To understand T cell responses in MIS-C, we evaluated the T cell receptor (TCR) repertoire and characteristics of SARS-CoV-2-specific T cells in these patients compared to children and adults with COVID-19 and other pediatric patients with hyperinflammatory syndromes. Our results confirm the previously observed enrichment of T cells expressing *TRBV11–2* in MIS-C and further demonstrate that this expansion of Vβ11–2<sup>+</sup> T cells is not found in other hyperinflammatory conditions of childhood such as KD, sJIA, and MAS. The increased usage of *TRBV11–2* in this cohort was not associated with HLA type. SARS-CoV-2-specific T cell responses were evaluated through T cell assays and TCR sequencing. The proportion of T cells that responded to stimulation with SARS-CoV-2 peptides, the frequency of SARS-CoV-2-specific TCRs, and the distribution of viral antigens recognized by these SARS-CoV-2 specific TCRs were similar in patients with MIS-C and children with COVID-19.

## 2. Materials and methods

### 2.1. Patients and samples

Patients were recruited from Boston Children's Hospital (BCH) and provided a peripheral blood sample for the study. Children with MIS-C and COVID-19 were enrolled in the Taking on COVID-19 Together study or the Kawasaki Disease (KD) Biorepository between 5/2020–4/2021. MIS-C patients were included if they met the Centers for Disease Control (CDC) and/or World Health Organization (WHO) Case Definitions for MIS-C [17,18]. Severe MIS-C was defined by admission to the intensive care unit or development of coronary artery aneurysms (CAAs) (z-score ≥ 2.5). Children with COVID-19 presented with fever, respiratory symptoms, and/or a COVID-19 exposure along with a positive SARS-CoV-2 test by polymerase chain reaction (PCR). Blood samples obtained 2 weeks after a positive PCR for SARS-CoV-2 were classified as convalescent COVID-19.

In addition, children with complete or incomplete KD (American Heart Association criteria), sJIA (modified International League of Associations for Rheumatology criteria, without the arthritis requirement), and MAS (2016 Classification Criteria) were also studied [19–21]. Children with KD unrelated to SARS-CoV-2 had no known close contacts with COVID-19 and tested negative for SARS-CoV-2 by both serology and PCR. Children with fever not related to SARS-CoV-2 who were evaluated in the Emergency Department, served as febrile controls. Pediatric controls with non-inflammatory musculoskeletal symptoms were recruited from the Rheumatology Clinic. Adult healthy controls

also participated in the study.

The electronic medical record (EMR) was reviewed by a pediatric rheumatologist (LAH) to gather the necessary clinical data, which was entered into a REDCap database. Written informed consent and assent (when appropriate) were provided by the study subjects under the following Institutional Review Board (IRB) protocols: IRB—P00035409, X10-01-0308, and IRB—P00005723.

### 2.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by Ficoll density gradient centrifugation and cryopreserved in liquid nitrogen.

### 2.3. T cell receptor sequencing

Genomic DNA was extracted from PBMCs (QIAamp Mini Blood DNA kit, Qiagen). A multiplex PCR was used to amplify the TCRβ complementarity determining region 3 (CDR3β), using a standard quantity of DNA. Sequencing was performed with the Illumina HiSeq platform (Adaptive Biotechnologies). Correction for PCR amplification bias was previously determined by using a complete, synthetic TCR repertoire to establish an amplification baseline and adjust the assay chemistry to minimize primer bias [22]. Residual PCR bias was addressed by using barcoded, spiked-in synthetic templates to assess sequence coverage. Resulting data were filtered and clustered using the relative frequency ratio between similar clones and a modified nearest neighbor algorithm to merge closely related sequences and remove both PCR and sequencing errors, as described [22,23].

The International ImMunoGeneTics (IMGT) system was used to align the sequences to a reference genome [24]. Subsequently, productive sequences were evaluated with the immunoseq™-Analyzer set of online tools. For diversity measures, the raw immunoseq dataset was imported in Python v3.7.0 using the `import_adaptive_file()` function in the `tcridist3` library. The data was then filtered to exclude clonotypes whose CDR3β sequence length was <4 amino acids and did not start with the consensus C amino acid or end with the consensus W/F amino acid. The productive frequency of each clonotype was then recalculated to account for excluded clones. The number of templates (copies) of each remaining clonotype was then used as input for plotting rarefaction curves and estimating Shannon Entropy at a fixed sample coverage of 0.2 using `iNEXT`, to identify potential outliers and correct for differences in sample size and sequencing depth in clonality estimates [25]. Using this approach, 3 patients were excluded from the diversity analysis due to inadequate sequencing depth (pediatric control,  $n = 1$ ; MIS-C,  $n = 1$ ; MAS,  $n = 1$ ). Shannon entropy was used to assess repertoire diversity [26]. Clonality was measured by the median frequency of the 100 most abundant T cell clonotypes [27]. *VDJ* usage and *V-J* gene pairing were also determined. Chord diagrams were generated with R package `circlize` and heatmaps were created with `GraphPad-Prism` version 9 [28]. The primary TCR sequencing data are available at: [clients.adaptivebiotech.com/pub/lam-2022-ci](https://clients.adaptivebiotech.com/pub/lam-2022-ci).

The immunoseq dataset and multiplexed identification of antigen-specific T cell receptors assay (MIRA) dataset from the ImmuneCODE database were sourced to provide TCR repertoires from adults with COVID-19 and SARS-CoV-2-specific TCRs, respectively [29–31]. Briefly, the immunoseq dataset is composed of TCRβ sequences from the adults (whole blood or PBMCs) with the following: 1) exposure to SARS-CoV-2 within the prior 2 weeks; 2) active COVID-19 based on a physician diagnosis or positive PCR test; 3) recovered COVID-19 defined by resolution of symptoms, 2 negative PCR tests, or clearance by a physician. Severe COVID-19 was defined as an adult with COVID-19 and a WHO ordinal severity scale rating of ≥6. In MIRA, SARS-CoV-2-specific peptide pools were used to identify antigen-specific T cells. The TCRs of these antigen-specific T cells were then sequenced. By using different combinations of antigen peptide pools, the target peptide recognized by

a given TCR can be determined.

#### 2.4. HLA typing

DNA was extracted from either whole blood or PBMCs (QIAamp Blood Midi/Mini Kit, Qiagen) from children with MIS-C SARS-CoV-2<sup>pos</sup> and pediatric COVID-19. High resolution sequencing of 11 HLA loci (A, B, C, DRB1/3/4/5, DQA1, DQB1, DPA1, DPB1) was performed by the HLA Research Testing Services of the American Red Cross. HLA allele frequencies in children with SARS-CoV-2 infections were compared to those in the National Marrow Donor Program, which contains HLA typing on millions of individuals in the United States [32].

#### 2.5. SARS-CoV-2 peptide pool assays

PBMCs were stimulated with either water (negative control) or peptide pools covering the N-terminal S1 domain of the spike (S), nucleocapsid (N), and membrane (M) proteins of SARS-CoV-2 (Milteny Biotec) for 16 h. The cells were harvested and stained for CD154 (CD40 ligand, eBioscience) as a measure of TCR activation. CD154 expression was evaluated by flow cytometry.

#### 2.6. Statistical analysis

One-way ANOVA with correction for multiple comparisons (Turkey correction) or unpaired, two-tailed *t*-tests were used to compare study groups. For *TRBV* gene usage analysis, two-way ANOVA with Dunnett's multiple comparison test was performed to compare study group and the frequency of each V $\beta$  gene. Spearman's non-parametric rank correlation was used to test for a monotonic association between two variables. Statistical analysis was performed with GraphPad-Prism version 9 or R, including the package immunarch version 0.6.7 [33]. We ran the *geneUsage* function from immunarch to calculate gene segment frequencies for each *TRBV* gene per sample. We used Wilcoxon test to compare *TRBV11-2* gene usage of patients with MIS-C SARS-CoV-2<sup>pos</sup> versus ImmuneCODE groups. Principal component analysis on *TRBV* gene usage fractions was performed using the *prcomp()* function from the *stats* package without centering and scaling the data. To assess whether gene usage frequency of *TRBV11-2* was dependent on read depth sequencing, we down sampled clones per repertoire to the size of the smallest repertoire using the *repSample* function from immunarch and we calculated gene usage as described previously. To test whether the viral antigen profile recognized by SARS-CoV-2-specific TCRs was more similar between children with MIS-C and COVID-19 than expected by chance, we calculated the sum of differences in frequency of antigen-specific TCRs for each position of the viral genome between the two groups (observed), and then performed 1 million permutations of the genomic positions, re-calculating the sum of differences for each position at each iteration, creating an expected null distribution.

**Table 1**  
Clinical Characteristics of the Study Subjects.

	MIS-C n = 24	Peds COVID-19 n = 10	KD n = 5	sJIA n = 7	MAS n = 3	Peds Febrile Controls n = 6	Peds Controls n = 7	Adult Controls n = 5
Age-yrs (median, IQR)	9.0, 6.3–13.8	11.0, 1.2–13.5	7.0, 1.5–10.5	6.0, 5.0–16.0	1.67, 1.2–12.0	4.5, 1.9–9.0	9.0, 6.0–10.0	26.5, 25.0–28.8
Sex (#, % female)	15, 63%	7, 70%	2, 40%	3, 43%	2, 67%	2, 34%	4, 57%	4, 80%
Race & Ethnicity (#, %)								
White, non-Hispanic	4, 17%	2, 20%	3, 60%	5, 71%	2, 67%	1, 17%	2, 29%	2, 40%
Black, non-Hispanic	4, 17%	0, 0%	0, 0%	0, 0%	0, 0%	0, 0%	1, 14%	0, 0%
Hispanic	9, 38%	5, 50%	1, 20%	1, 14%	0, 0%	3, 50%	1, 14%	0, 0%
Asian	0, 0%	1, 10%	1, 20%	0, 0%	0, 0%	1, 17%	0, 0%	1, 20%
Other	2, 8%	0, 0%	0, 0%	1, 14%	1, 33%	0, 0%	0, 0%	0, 0%
Unknown	5, 21%	2, 20%	0, 0%	0, 0%	0, 0%	1, 17%	3, 43%	2, 40%

### 3. Results

#### 3.1. Study cohorts

Children with MIS-C (*n* = 24), COVID-19 (*n* = 10), KD (*n* = 5), sJIA (*n* = 7), and MAS (*n* = 3) were studied along with pediatric febrile controls (*n* = 6), healthy children (*n* = 7), and adult controls (*n* = 5) (Tables 1, S1-S3).

#### 3.2. Children with SARS-CoV-2 infections

Patients with MIS-C met either the CDC or WHO case definitions for MIS-C (Table S1) [17,34]. Fever was present in 100% of patients with MIS-C and gastrointestinal (GI) symptoms (92%), conjunctivitis (71%), and rash (38%) were also frequent. Children with MIS-C demonstrated evidence of systemic inflammation (median CRP 16.6 mg/dL, IQR 6.9–26.0), lymphopenia (median absolute lymphocyte count  $0.92 \times 10^3$  [3]/mL, IQR 0.42–1.72) and coagulopathy (median D-dimer 3.1  $\mu$ g/mL, IQR 1.4–6.2). Approximately 80% of MIS-C patients had positive SARS-CoV-2 serologies. Five patients tested negative for SARS-CoV-2 by PCR and antibody levels but met the CDC or WHO Case Definitions for MIS-C and were thought to have MIS-C by their treating providers. Of the total MIS-C cohort, 11/24 (46%) were considered to have severe disease defined by admission to the intensive care unit (ICU) and/or development of CAAs. At the time of sampling, 79% of MIS-C patients had received immunomodulatory therapy, most commonly IVIG and/or glucocorticoids.

Most children with COVID-19 in this cohort had mild disease: 40% of patients were admitted to the hospital, 20% were treated with supplemental oxygen or non-invasive mechanical ventilation, and 10% required ICU level care (Table S1). Children with COVID-19 were frequently febrile (70%) with respiratory symptoms (60%), and all were positive for SARS-CoV-2 by PCR. In contrast to MIS-C, children with COVID-19 mostly lacked GI and mucocutaneous symptoms.

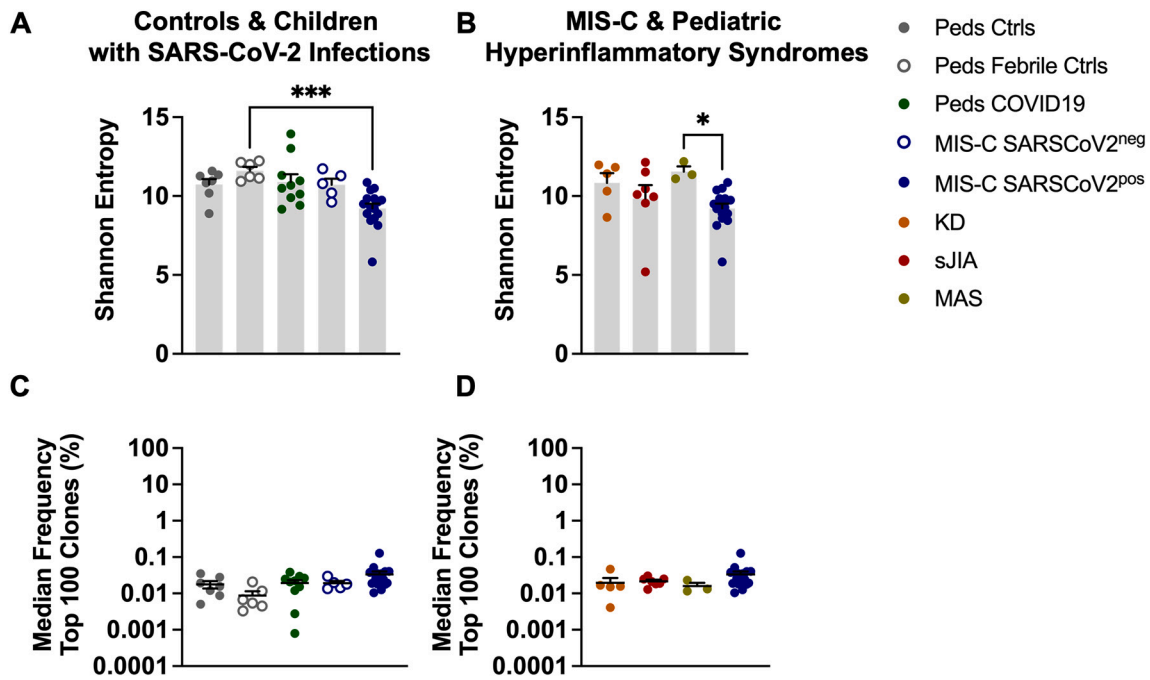
#### 3.3. Pediatric hyperinflammatory syndromes

To compare MIS-C to other pediatric hyperinflammatory syndromes, pediatric patients with KD, sJIA, and MAS were also studied. Of the 5 children with KD, 60% met criteria for complete KD and 40% had CAAs (Table S2). None of the patients with KD presented with shock or were admitted to the ICU. All children with sJIA and MAS had active disease with fever, rash, and/or elevated inflammatory markers (Table S3).

#### 3.4. TCR repertoire diversity and clonality in MIS-C

Shannon entropy was used to quantify TCR repertoire diversity in children with MIS-C compared to pediatric controls and pediatric COVID-19 (Fig. 1A) [26]. Children with MIS-C had the lowest levels of





**Fig. 1.** TCR Repertoire Diversity and Clonality in MIS-C. A–B) Shannon Entropy, a measure of repertoire diversity, estimated at fixed sample coverage. C–D) Median frequency (percent) among the top 100 most frequent clones with T cell clone defined as amino acid sequence in CDR3 $\beta$  of the TCR. Summary data on bar graphs is mean  $\pm$  standard error. Statistical testing for A–D: Kruskal-Wallis test with Dunn's multiple comparisons test.  $P$  value \*  $\leq$  0.05, \*\*\*  $\leq$  0.001. CDR3, complementarity determining region 3; TCR, T cell receptor; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MIS-C, multisystem inflammatory syndrome in children; peds, pediatric; ctrls, controls; KD, Kawasaki disease; sJIA, systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome.

repertoire diversity but a statistically significant difference was noted only when comparing MIS-C patients with confirmed SARS-CoV-2 infection to pediatric febrile controls (Fig. 1A) ( $p \leq 0.001$ ). Children diagnosed with MIS-C who tested negative for SARS-CoV-2 by PCR and antibody tests (MIS-C SARS-CoV-2<sup>neg</sup>), were grouped separately from those children who had laboratory evidence of infection (MIS-C SARS-CoV-2<sup>pos</sup>) to determine if there were differences in TCR repertoire characteristics across these two groups. There were no significant differences in Shannon entropy in the MIS-C SARS-CoV-2<sup>pos</sup> vs. MIS-C SARS-CoV-2<sup>neg</sup> patients. Next, we compared the TCR repertoire diversity of children with MIS-C to other pediatric hyperinflammatory syndromes (Fig. 1B). There was a trend towards reduced diversity in the MIS-C patients that only reached statistical significance when compared to patients with MAS (Fig. 1B) ( $p \leq 0.05$ ). TCR repertoire clonality was measured by determining the median frequency of the 100 most abundant T cell clones in each sample. There were no differences in T cell clonal expansion across the study groups (Fig. 1C–D, S1). In summary, these findings suggest that children with MIS-C have a slight decrease in TCR repertoire diversity but no major T cell clonal expansions.

### 3.5. Increased usage of *TRBV11-2* in T cells from children with MIS-C

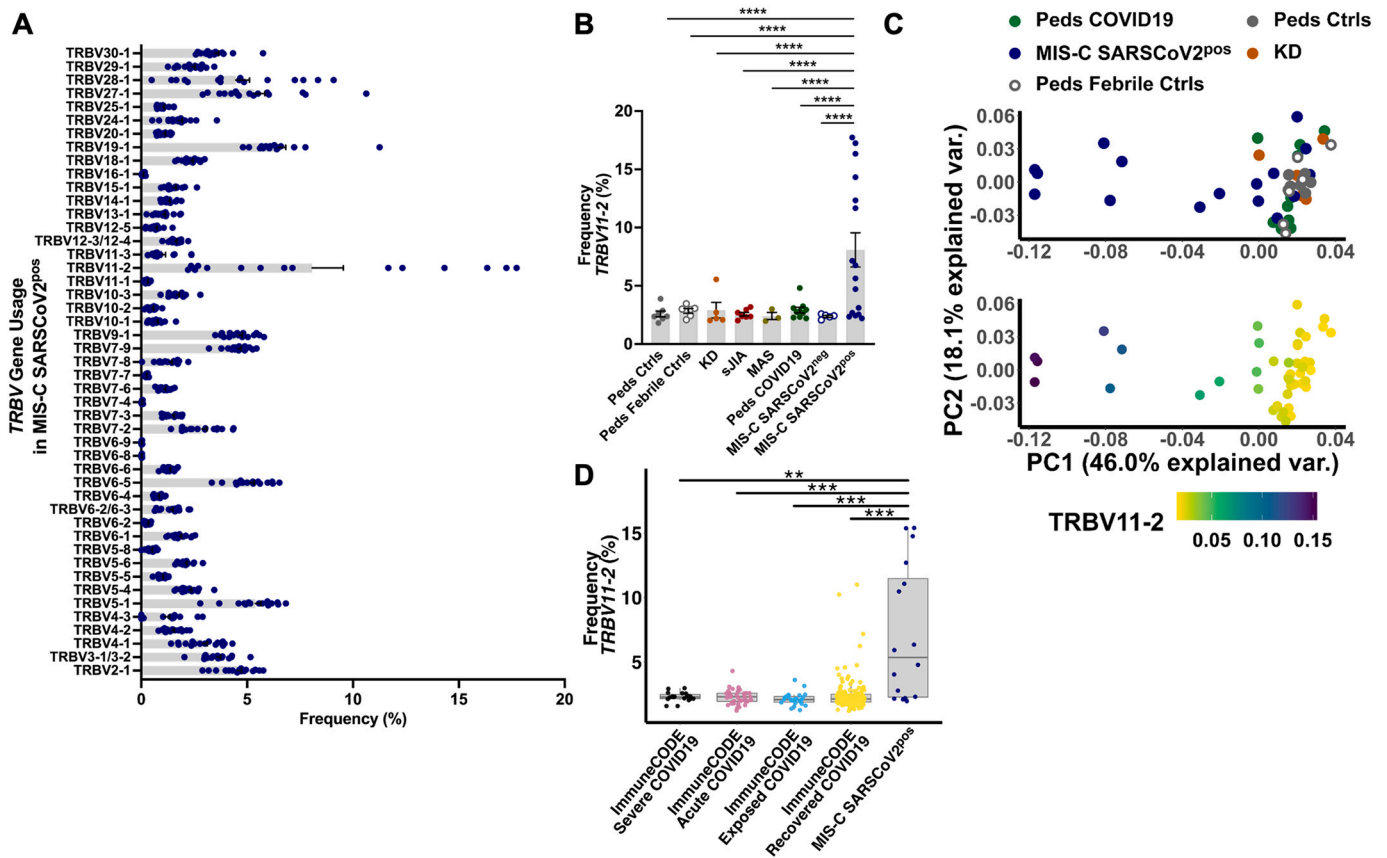
As described previously, T cells that express the *TRBV11-2* gene in the CDR3 $\beta$  domain of the TCR were markedly increased in children with MIS-C compared to pediatric controls and pediatric COVID-19 (Fig. 2A, B) ( $p \leq 0.0001$ ) [12–16]. Skewed usage of *TRBV11-2* was also significantly different in patients with MIS-C compared to children with other hyperinflammatory syndromes (KD, sJIA, MAS) (Fig. 2B) ( $p \leq 0.0001$ ). This finding was also confirmed by a principal components analysis (PCA) of *TRBV* gene usage. MIS-C SARS-CoV-2<sup>pos</sup> patients were separated from the remaining study groups by PC1, which explained 46% of the variance and included *TRBV11-2* as the main driver of PC1 (Fig. 2C) (Spearman rho =  $-0.88$ ,  $P < 2.2e-16$ ). Interestingly, children who met the case definition for MIS-C and were diagnosed with MIS-C by their treating providers but tested negative for SARS-CoV-2 did not display

such V $\beta$  skewing (Fig. 2B). Given this difference in MIS-C SARS-CoV-2<sup>pos</sup> vs. MIS-C SARS-CoV-2<sup>neg</sup> patients, we continued to analyze these groups separately.

Next, we leveraged the ImmuneCODE database to compare *TRBV11-2* usage in children with MIS-C SARS-CoV-2<sup>pos</sup> and adults with severe acute COVID-19 ( $n = 19$ ), acute COVID-19 ( $n = 40$ ), recovered COVID-19 ( $n = 159$ ), or known SARS-CoV-2 exposure ( $n = 26$ ). T cell clonotypes expressing *TRBV11-2* were significantly more frequent in MIS-C SARS-CoV-2<sup>pos</sup> patients compared to all of the adult groups evaluated in ImmuneCODE, even after controlling for read depth differences between the datasets (Fig. 2D, S2A). The frequency of V $\beta$ 11-2<sup>+</sup> T cells did not differ in adults based on the severity of COVID-19 illness. A small number of adults (4/159) in the COVID-19 recovered group had *TRBV11-2* expansions, defined as a frequency  $>2$  standard deviations (SD) above the mean.

There was a bimodal distribution of *TRBV11-2* usage in MIS-C SARS-CoV-2<sup>pos</sup> patients. In some children, the frequency of T cell clonotypes expressing *TRBV11-2* was comparable to controls ( $n = 6$ ) while others had profound expansions of these T cells ( $n = 10$ ) (Fig. 2B). *TRBV11-2* expansions were defined as frequencies  $>2$  SD above the mean of pediatric controls. We sought to determine what factors may explain this distribution of *TRBV11-2* expression in MIS-C SARS-CoV-2<sup>pos</sup> patients. There was a non-statistically significant trend towards increased frequencies of V $\beta$ 11-2<sup>+</sup> T cells in children with severe MIS-C, defined by admission to the ICU or development of CAAs (Fig. S2B). The number of children in the MIS-C SARS-CoV-2<sup>pos</sup> group who provided a blood sample prior to immunomodulatory treatment was small ( $n = 3$ ), thus limiting the comparison of V $\beta$ 11-2<sup>+</sup> T cell pre- vs. post-treatment (Fig. S2C). There was no statistically significant correlation between *TRBV11-2* usage in T cells and patient age, body mass index (BMI), or days from admission to biosample collection (Fig. S2D–F).

In some prior studies, expansions of V $\beta$ 11-2<sup>+</sup> T cells in children with MIS-C were associated with HLA class I alleles, specifically A02, B35, and C04 [15,16]. Through HLA typing, the frequencies of HLA alleles in children with MIS-C SARS-CoV-2<sup>pos</sup> and COVID-19 were compared to



**Fig. 2.** Expansion of T cells expressing *TRBV11-2* in MIS-C. **A**) The frequency of T cell clonotypes expressing each *TRBV* gene in patients with MIS-C SARS-CoV-2<sup>pos</sup>. **B**) The frequency of T cell clonotypes expressing *TRBV11-2* in each study group. Statistical testing: two-way ANOVA comparing study group and V $\beta$  gene with the Dunnett's multiple comparisons test.  $P$  value \*\*\*\*  $\leq 0.0001$ . **C**) Principal components analysis (PCA) of *TRBV* gene usage. **D**) The frequency of T cell clonotypes expressing *TRBV11-2* in MIS-C SARS-CoV-2<sup>pos</sup> and adults in the ImmuneCODE database. Statistical testing: Wilcoxon test. Summary data on bar graphs is mean  $\pm$  standard error in A-B and median with IQR in D.  $P$  value, \*\*\*\*  $\leq 0.0001$ , \*\*\* = 0.001, \*\* = 0.01, \* = 0.05.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MIS-C, multisystem inflammatory syndrome in children; peds, pediatric; ctrl, control; KD, Kawasaki disease; sJIA, systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome; var., variance.

the National Marrow Donor Program (NMDP), which contains HLA typing on millions of individuals in the United States [32]. Children with MIS-C SARS-CoV-2<sup>pos</sup> did not demonstrate increased usage of HLA-I A02, B35, or C04 compared to children with COVID-19 and the larger population represented in the NMDP registry (Fig. 3). There appeared to be an increased usage of HLA-I A30 in children with MIS-C and expanded V $\beta$ 11-2<sup>+</sup> T cells; however, this allele is more commonly found in Black and Hispanic compared to White and Asian individuals in the United States based on the NMDP registry (Fig. 3B). Given the increased incidence of MIS-C in Black and Hispanic children, the observed enrichment of HLA-I A30 may reflect these racial and ethnic differences. No other HLA class I or II associations were noted in MIS-C patients with expanded T cells that expressed *TRBV11-2* (Fig. 3, S3 and Table S4).

Children with MIS-C SARS-CoV-2<sup>pos</sup> had decreased usage of *TRBV7-2* and *TRBV28-1* compared to some study groups, but these effect sizes were much less profound compared to the differences documented in *TRBV11-2* (Fig. S2G–H) and not consistent across all study groups.

Thus, increased expression of *TRBV11-2* in T cells was specific to MIS-C patients and was not found in children or adults with COVID-19 or pediatric patients with hyperinflammatory syndromes. Children in the MIS-C SARS-CoV-2<sup>neg</sup> did not have expansions of V $\beta$ 11-2<sup>+</sup> T cells, suggesting distinct T cell responses in patients who tested positive for SARS-CoV-2 by either PCR or serology.

### 3.6. Diverse *TRBJ* gene usage in MIS-C

In patients with MIS-C, there was no observed skewing of T Cell Receptor Beta Joining (*TRBJ*) genes in the TCR (Fig. 4A). The usage of *TRBJ* genes was similar across all study groups (Fig. 4A). In V $\beta$ 11-2<sup>+</sup> T cells from MIS-C SARS-CoV-2<sup>pos</sup> patients, pairing with J $\beta$  genes remained polyclonal and similar to children with COVID-19 (Fig. 4B, C).

### 3.7. SARS-CoV-2-specific T cells in MIS-C

We next sought to characterize SARS-CoV-2-specific T cells in patients with MIS-C. Since MIS-C occurs several weeks after an initial SARS-CoV-2 infection, this group was compared to children with convalescent COVID-19. After stimulation with peptide pools covering the S1, N, and M viral proteins, antigen-specific T cells were identified by their expression of CD154 (CD40 ligand), a T cell activation marker. The frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that recognized SARS-CoV-2 in patients with MIS-C SARS-CoV-2<sup>pos</sup> did not differ significantly from children with convalescent COVID-19 (Fig. 5A, B). Similarly, there was no statistical difference in the frequency of CD40L<sup>+</sup> T cells in mild vs. severe MIS-C SARS-CoV-2<sup>pos</sup> or in MIS-C patients with vs. without expansions of T cells expressing *TRBV11-2* (Fig. 5C, D). These results suggested that MIS-C patients had similar frequencies of SARS-CoV-2-specific T cells as children with convalescent COVID-19.

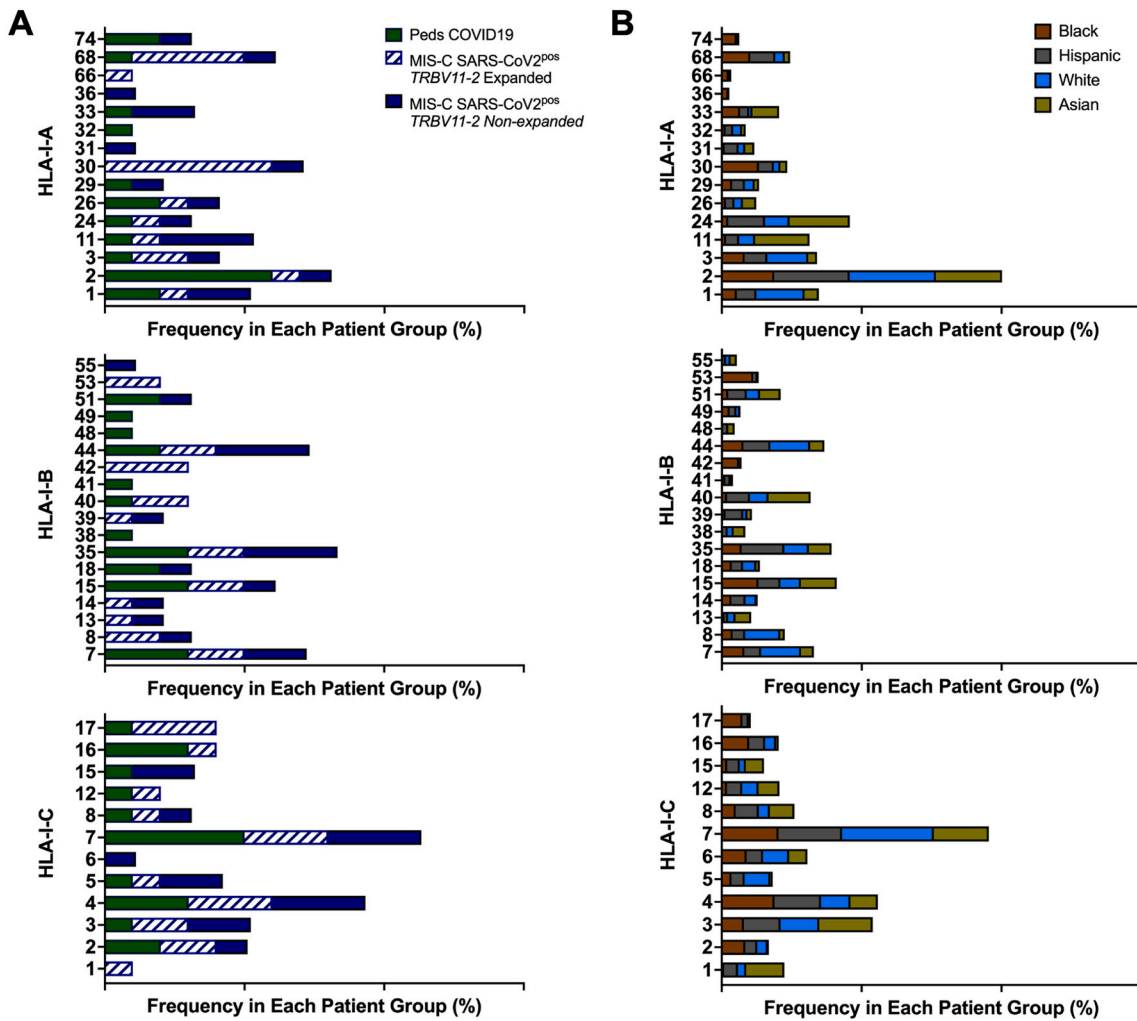


Fig. 3. HLA Class I Alleles in MIS-C. A) The frequency of each HLA class I genotype in children with pediatric COVID-19 and MIS-C. B) The of frequency each HLA class I genotype in the National Marrow Donor Program database shown by race and ethnicity as defined by US census categories. Peds, pediatric; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MIS-C, multisystem inflammatory syndrome in children.

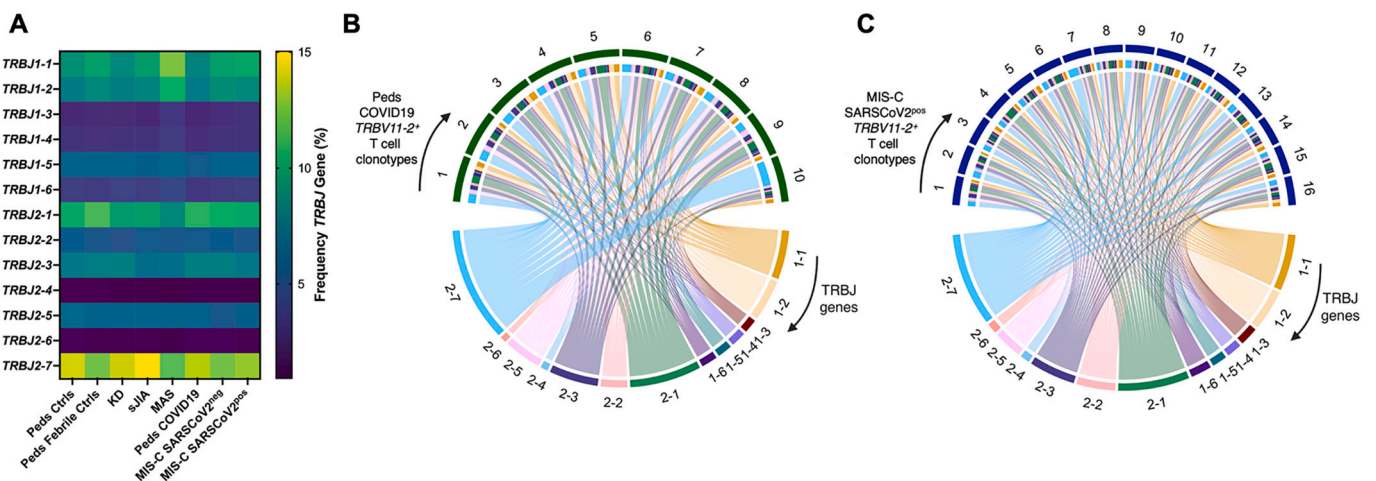
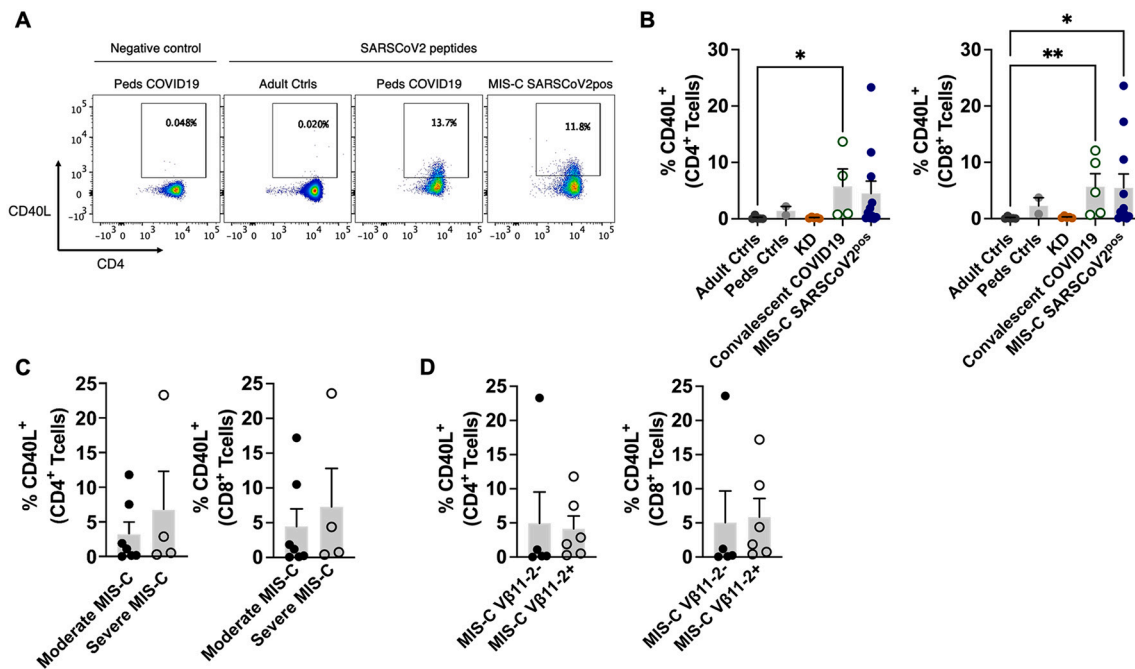


Fig. 4. Diverse *TRBJ* Gene Usage in MIS-C: A) Heatmap depicting the mean frequency of T cell clonotypes expressing each *TRBJ* gene in the given study groups. B—C) Chord diagram depicting the V $\beta$ -J $\beta$  pairing of total T cell clonotypes expressing *TRBV11-2* in B) children with COVID-19 ( $n = 10$ ) and C) MIS-C SARS-CoV2<sup>POS</sup> patients ( $n = 16$ ). SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MIS-C, multisystem inflammatory syndrome in children; peds, pediatric; ctrl, control; KD, Kawasaki disease; sJIA, systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome.



**Fig. 5.** SARS-CoV-2-Specific T Cells in MIS-C. Peripheral blood mononuclear cells were incubated with either water (negative control) or peptide pools covering the S1, N, and M viral proteins. SARS-CoV2-antigen-specific T cells were identified by their expression of CD154 (CD40 ligand). **A)** Flow cytometry dot plots depicting CD40L expression on T cells from a healthy control, child with convalescent COVID-19, and child with MIS-C SARS-CoV-2<sup>Pos</sup>. **B)** Frequency of CD4<sup>+</sup>CD40L<sup>+</sup> or CD8<sup>+</sup>CD40L<sup>+</sup> T cells in the given study group. **C)** Frequency of CD4<sup>+</sup>CD40L<sup>+</sup> or CD8<sup>+</sup>CD40L<sup>+</sup> T cells in children with moderate or severe MIS-C SARS-CoV-2<sup>Pos</sup>. **D)** Frequency of CD4<sup>+</sup>CD40L<sup>+</sup> or CD8<sup>+</sup>CD40L<sup>+</sup> T cells in MIS-C SARS-CoV-2<sup>Pos</sup> patients with and without expansions of T cell clonotypes expressing *TRBV11-2*. Statistical testing: Kruskal-Wallis test with Dunn's multiple comparisons test for panel B and for Mann Whitney tests for panels C–D. *P* value \*  $\leq 0.05$ , \*\*  $\leq 0.01$ . Summary data on bar graphs is mean  $\pm$  standard error.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MIS-C, multisystem inflammatory syndrome in children; peds, pediatric; ctrl, control; KD, Kawasaki disease.

### 3.8. SARS-CoV-2-specific T cell receptors in MIS-C

Using the MIRA dataset, we evaluated SARS-CoV-2-specific TCR $\beta$  sequences in children with MIS-C. There was a trend towards expanded SARS-CoV-2-specific TCRs in the MIS-C SARS-CoV-2<sup>Pos</sup> patients; however, this difference only reached statistical significance when compared to febrile controls ( $p = 0.02$ ) (Fig. 6A). There was a trend towards increased frequency of SARS-CoV-2-specific TCRs in patients with severe vs. moderate MIS-C that did not reach statistical significance (Fig. 6B). TCRs that expressed *TRBV11-2* and recognized SARS-CoV-2 were not enriched in MIS-C patients (Fig. 6C). The distribution of viral antigens recognized by SARS-CoV-2-specific TCRs were similar in children with MIS-C and COVID-19 (Fig. 6D-E) (permutation test). There was an increase in frequency of TCRs that recognize ORF1 of SARS-CoV-2 in MIS-C SARS-CoV-2<sup>Pos</sup> patients; however this difference was driven primarily by expanded TCRs in only 2 of the MIS-C patients. Thus, characteristics of SARS-CoV-2-specific TCRs were similar in children with MIS-C and COVID-19.

## 4. Discussion

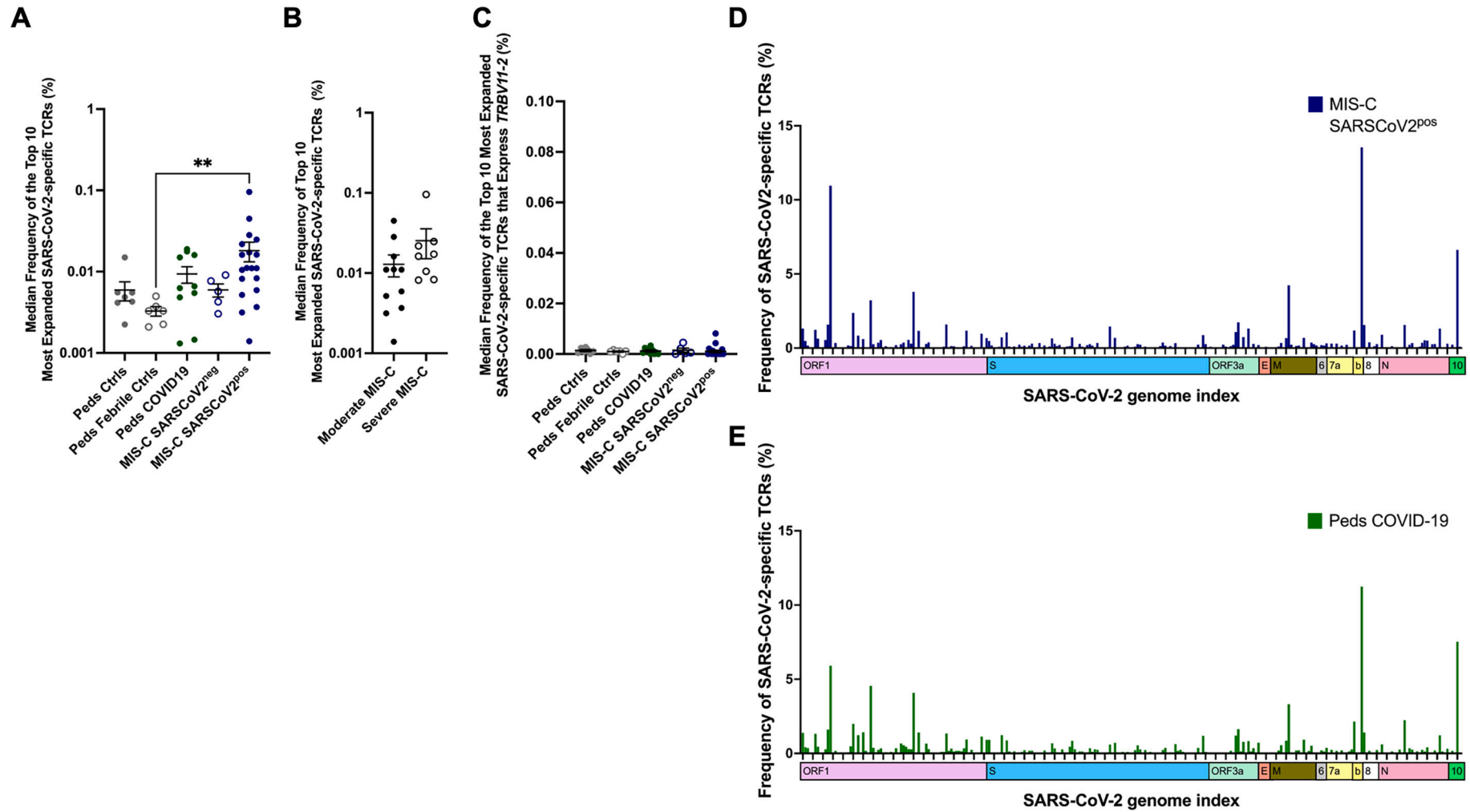
We sought to characterize T cell responses in MIS-C compared to COVID-19 and pediatric hyperinflammatory syndromes by leveraging high-throughput TCR sequencing and T cell immune assays. The most distinctive feature of the TCR repertoire in patients with laboratory-confirmed MIS-C was the skewed usage of *TRBV11-2*. In addition, children with MIS-C had robust antigen-specific T cell responses to SARS-CoV-2 that were comparable to pediatric patients with COVID-19.

The expansion of T cell clonotypes expressing *TRBV11-2* documented in our cohort of children with MIS-C adds to the substantial literature supporting this observation [12–16]. Previously, increased

usage of *TRBV11-2* was found to be unique to patients with MIS-C compared to patients with COVID-19, KD, TSS, and pediatric febrile illnesses [14–16]. Our results expand upon these findings and demonstrate that MIS-C is also differentiated from sJIA and MAS by this V $\beta$  skewing. By leveraging the publicly available ImmuneCODE database, we also show that increased *TRBV11-2* usage is not observed in a large number of adults with active COVID-19, regardless of disease severity; however, it is occasionally seen in adults who have recovered from the infection. Interestingly, increased V $\beta$ 11-2<sup>+</sup> T cells were not observed in the 5 children who met case definitions for MIS-C but tested negative for SARS-CoV-2 by PCR and serology. These patients presented during the first wave of the COVID-19 pandemic when the epidemiologic link to SARS-CoV-2 infection was relatively easy to establish. They were also diagnosed with MIS-C by their treating providers. The lack of skewed V $\beta$  usage in the MIS-C SARS-CoV-2<sup>neg</sup> patients suggests that these children may have presented with an alternate condition that mimicked the findings typically observed in MIS-C. If true, quantifying the frequency of V $\beta$ 11<sup>+</sup> T cells, which can be achieved relatively quickly by flow cytometry, may prove to be a useful diagnostic tool for MIS-C.

An expansion of T cells expressing *TRBV11-2* was observed in more than half of the patients with MIS-C in this study. Others have also observed that V $\beta$  skewing is not universal in MIS-C [12–14]. Factors associated with an increased frequency of V $\beta$ 11-2<sup>+</sup> T cells include severe MIS-C and high levels of inflammation [13–16]. In our cohort, there was a trend towards increased *TRBV11-2* usage in patients with severe disease compared to moderate MIS-C, which did not reach statistical significance. Sacco et al. and Moreews et al. found that immunomodulatory treatment led to rapid contraction of V $\beta$ 11-2<sup>+</sup> T cells in patients with MIS-C [14,16]. Thus, treatment-related effects may have contributed to the bimodal distribution of V $\beta$  gene usage that was observed in this study, although the paucity of pre-treatment samples did not permit





**Fig. 6.** SARS-CoV-2-Specific T Cell Receptors in MIS-C. SARS-CoV-2-specific TCR sequences were obtained from the ImmuneCODE database. Antigen-specific T cells were identified by the expression of activation markers following exposure to SARS-CoV-2 peptides. These T cells were then isolated for TCR sequencing. A) The median frequency of the top 10 most abundant SARS-CoV-2-specific TCRs for each patient in each study group. Statistical analysis Kruskal-Wallis test with Dunn's multiple comparison test. B) The median frequency of the top 10 most abundant SARS-CoV-2-specific TCRs in patients with moderate and severe MIS-C. Statistical analysis: Mann-Whitney test C) The median frequency of the top 10 most abundant SARS-CoV-2-specific TCRs that express *TRBV11-2* in each patient and each study group. Statistical analysis Kruskal-Wallis test with Dunn's multiple comparison test. D-E) Viral antigens recognized by SARS-CoV-2-specific TCRs in D) MIS-C SARS-CoV-2<sup>pos</sup> and E) pediatric COVID-19. Statistical analysis by permutation test with no difference between MIS-C SARS-CoV-2<sup>pos</sup> and peds COVID-19. *P* value, \*\*  $\leq 0.01$ .

Summary data on bar graphs is mean  $\pm$  standard error.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MIS-C, multisystem inflammatory syndrome in children; peds, pediatric; ctrl, control; TCR, T cell receptor.

a robust assessment. Finally, some studies have demonstrated HLA class I associations with *TRBV11–2* expansions in MIS-C, while other reports have not reproduced these findings [12,14,15]. To date, our cohort of 19 MIS-C patients with HLA typing represents the largest group of patients with such data and we did not observe HLA class I or II associations with *TRBV11–2* expansions.

The functional significance of *TRBV11–2* expansions in MIS-C remains to be determined.  $V\beta$  and  $J\beta$  genes are spliced together to create diversity in the antigen recognition domain of TCR $\beta$ . Our study and others show that the *TRBV11–2* gene in MIS-C is paired with polyclonal  $J\beta$  genes [12,14–16]. Prior studies have leveraged single cell RNA sequencing (scRNA-seq) to confirm that there is no bias in the pairing of TCR $\beta$  chains expressing *TRBV11–2* with specific TCR $\alpha$  chains [12]. These TCR repertoire features indicate a non-clonal expansion of a particular  $V\beta$  gene and are suggestive of superantigen-mediated pathology. Indeed, computational modeling indicates that the sequence and structure of the SARS-CoV-2 spike protein has features of a superantigen and *TRBV11–2* may interact with this superantigen-like motif [15,35]. Further, flow cytometry and scRNA-seq show that  $V\beta 11–2^+$  T cells are activated and pro-inflammatory [12,14,16]. Yet, these findings are descriptive with no functional studies confirming the role of  $V\beta 11–2^+$  T cells in MIS-C. The delay in the presentation of MIS-C compared to the primary SARS-CoV-2 infection is not typical of diseases driven by superantigens, although some reports indicate that SARS-CoV-2 may persist in some hosts outside of the respiratory tract [16,36]. Superantigens are typically HLA class II restricted. HLA associations, including those observed in our cohort, have not been clearly demonstrated in MIS-C. Further, the *TRBV11–2* expansions observed in adults with convalescent COVID-19 raises questions about the pathogenicity of these T cells. A MIS-C-like illness (MIS-A) can occur in adults, but it is even more rare than in children. It seems unlikely that 4/159 patients with *TRBV11–2* expansions in the ImmuneCODE database developed such a syndrome. Thus, it seems reasonable to conclude that adults can have increased frequencies of  $V\beta 11–2^+$  T cells without hyperinflammation. It is also possible that this dissociation occurs in the pediatric population, although we did not have sufficient number of children with convalescent COVID-19 to explore this possibility.

We utilized two approaches to explore antigen-specific T cell responses in MIS-C. First, we characterized the frequency of T cells activated by stimulation with SARS-CoV-2 peptide pools. These T cells occurred at similar frequencies in patients with MIS-C SARS-CoV-2<sup>pos</sup> and children with convalescent COVID-19. Further, the frequency of these antigen-specific T cells did not correlate with disease severity or *TRBV11–2* expansions. Second, we leveraged the publicly available MIRA dataset to identify SARS-CoV-2-specific TCRs. There was a non-statistically significant trend towards expanded SARS-CoV-2 TCRs in children with MIS-C SARS-CoV-2<sup>pos</sup>, particularly in children with severe MIS-C. Further studies in a larger number of children are needed to determine if the frequency of SARS-CoV-2-specific TCRs is associated with disease severity. The distribution of viral antigens recognized by these TCRs was similar in MIS-C SARS-CoV-2<sup>pos</sup> and pediatric COVID-19. There was no evidence that TCRs that express *TRBV11–2* compared to other  $V\beta$  genes were more likely to recognize SARS-CoV-2. In total, children with MIS-C generated SARS-CoV-2-specific T cells at levels that were comparable to children with COVID-19. In keeping with our prior findings that *TRBV11–2* expansions are non-clonal, T cells expressing this  $V\beta$  gene were not enriched in the SARS-CoV-2-specific T cell subset.

Our work furthers the understanding of T cell responses in MIS-C; however, we acknowledge limitations to our study. While the number of MIS-C patients ( $n = 24$ ) in our study is sizable compared to many other published cohorts, these children were recruited from a single center, which may limit generalizability. Since children with MIS-C can decompensate quickly and develop shock, they were treated rapidly at our center, often in the emergency department, with intravenous immunoglobulin (IVIG) and glucocorticoids. Thus, many children with

MIS-C in this cohort had received immunomodulatory treatment prior to sampling. Arguably, the TCR repertoire is impacted less by such treatment compared to studies that evaluate protein and gene expression in T cells. Finally, antigen-specific T cell responses were identified by T cell assays and SARS-CoV-2 specific TCRs found in a publicly available dataset; however, we cannot absolutely confirm that these T cells recognize SARS-CoV-2 in vivo.

## 5. Conclusions

Expansions of  $V\beta 11–2^+$  T cells was a specific biomarker of MIS-C patients with laboratory-confirmed SARS-CoV-2 infections and was not found in children and adults with COVID-19 or pediatric patients with hyperinflammatory syndromes. Children with MIS-C appeared to generate appropriate antigen-specific T cell responses to SARS-CoV-2, and these antigen-specific T cells were not associated with *TRBV11–2* expansions.

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## Declaration of Competing Interest

LAH has received salary support from CARRA; consulting fees from Sobi, Pfizer, and Adaptive Biotechnologies; and investigator-initiated research grants from Bristol-Myers Squibb.

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MIS-C, multisystem inflammatory syndrome in children; peds, pediatric; COVID-19, coronavirus disease 2019; KD, Kawasaki disease; sJIA, systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2022.109106>.

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