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Polyclonal expansion of TCR Vbeta 21.3^{+} CD4^{+} and CD8^{+} T cells is a hallmark of Multisystem Inflammatory Syndrome in Children

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Author contributions: AB, TW, DK, JM, EM-F designed and analyzed experiments MM, K LG, AB, CM, RP, SP, SJ, ALM, MP, MV, EC, IR, FV, PB, SYZ performed and analyzed experiments. CM, GM and FV conceptualized the FACS analysis CM and RP supervised cytokine experiments. EJ performed inclusions, chair the clinical investigation and took care of all ethical committee agreement. AP, YJ and BK set up the clinical, RP, ST, MG, TL, FV, AMZ, MD, HP, LC, JCR, MM, OD, JMDG, FB provided clinical samples and clinical details for all cohorts. OT and VB explored HLA in MIS-C patients and JLC, LA supervised genetic inference exploration of HLA. IR and EC provided biobanking and help to generate material for the study TW and AB supervised, designed and funded this study. TW and AB prepared the initial draft. All authors critically reviewed the paper and agreed on the final form.

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Abstract

Objectives—Multiple Inflammatory Syndrome in Children (MIS-C) is a delayed and severe complication of SARS-CoV-2 infection that strikes previously healthy children. As MIS-C combines clinical features of Kawasaki disease and Toxic Shock Syndrome (TSS), we aimed to compare the immunological profile of pediatric patients with these different conditions.

Methods—We analyzed blood cytokine expression and T cell repertoire and phenotype in 36 MIS-C cases in comparison with 16 KD, 58 TSS, and 42 COVID-19 cases.

Results—Similarly to TSS, an increase of serum inflammatory cytokines (IL-6, IL-10, IL-18, TNF-α, IFNγ, CD25s, MCP1, IL-1RA) was observed in MIS-C contrasting with low expression of HLA-DR in monocytes. A specific expansion of T cells with an activated phenotype and expressing the Vβ21.3 T cell receptor β chain variable region and correlating with the cytokine storm was detected in both CD4 and CD8 subsets in 75% of MIS-C patients and not in any patient with TSS, KD, or acute COVID-19. TCR sequencing uncovered the polyclonal nature of the Vβ 21.3+ population. The T cell repertoire returned to normal within weeks after MIS-C resolution. Vβ21.3+ T cells from MIS-C patients expressed high levels of HLA-DR, CD38 and CX3CR1 but did not react against SARS-CoV-2 peptides *in vitro* unlike T cells from COVID-19 patients. Consistently, the T cell expansion was not associated with specific classical HLA alleles.

Conclusions: We demonstrate the existence of a specific polyclonal Vβ21.3 T cell expansion in MIS-C patients not directed against SARS-CoV-2 antigenic peptides and not observed in other related conditions, namely KD, TSS and acute COVID-19.

One Sentence Summary:

We report the expansion of a polyclonal and activated TCR Vβ21.3+ subset of CD4+ and $CD8^+$ T cells in about 75% of MIS-C patients, which are not specific of HLA-restricted SARS-CoV-2 antigenic peptides and not observed in Kawasaki disease and toxic shock syndrome.

Keywords

MIS-C; PIMS; COVID-19; SARS-CoV2; post-infectious disease; Kawasaki disease; Toxic Shock Syndrome; Immune profiling; TCR sequencing; myocarditis

Introduction

At the end of April 2020, European clinicians warned the Public Health Agencies about an abnormal increase of Kawasaki-like diseases (KLD) and myocarditis requiring critical care support in the context of the ongoing COVID-19 epidemic in children (1-3). Later on, American clinicians also reported a large outbreak of severe inflammation in children following COVID-19 infection, a condition that is now named Pediatric Inflammatory Multisystemic Syndrome (PIMS) or Multisystem Inflammatory Syndrome in children (MIS-

C) (4-6). The clinical phenotype of this emerging disease is broad and encompasses features of Kawasaki disease (KD) and toxic shock syndrome (TSS). Many cases require intensive care support, making MIS-C one of the most severe manifestation of COVID-19 in children. Of note, the temporal occurrence of MIS-C raised the hypothesis of a post-infectious disease occurring about 3 to 4 weeks after acute COVID-19 in children (3, 5-7).

To date, reports on MIS-C have shown slight differences in cytokine profiling and immunophenotype between MIS-C and KD or pediatric COVID-19 (8, 9). Analysis of T cells revealed a lower number of T cells in MIS-C with no or subtle signs of activation (10). Multi-dimensional immune profiling on small numbers of patients showed differences between acute COVID-19 or pre-pandemic KD (8, 11). Anti-SARS-CoV2 antibodies were equally produced in pediatric COVID-19 and MIS-C. Autoantibodies were uniquely found during MIS-C or KD, which supports a contribution of the humoral response to both diseases (8, 11). Finally, a role for genetic factors has been evocated in MIS-C pathogenesis as it seems to occur more frequently in children from Hispanic or African ethnicity (12-14). Despite these pioneer studies, the immunological mechanism underlying MIS-C remains unknown.

To address this question, we compared the immune profile in MIS-C patients in comparison to that of COVID-19 patients and that of patients with other clinically similar entities such as KD and TSS. For this, we explored the cytokine and cellular immune profile using different techniques. Using flow cytometry and transcriptomic analyses, we uncovered a specific Vβ21.3+ T cell expansion in 24/32 tested patients in MIS-C patients when assessed in the first month after onset. TCR sequencing revealed the polyclonal nature of the Vβ21.3+ expansion. No specific HLA bias was identified in patients but we found a specific activation profile within Vβ21.3+ T cells. This activation was transient with a normalization of the repertoire within days to weeks after the inflammatory episode.

Results:

MIS-C presentation overlaps with TSS and KD

We constituted a cohort of 36 children with MISC and compared them with 16 KD diagnosed during and before the pandemic, 58 retrospective cases of TSS patients and 42 patients with acute COVID-19 (11 children, 31 adults). This comparison was motivated by previous descriptions of MIS-C in Europe and in the US, showing a clinical overlap between staphylococcal toxin-mediated TSS and KD in patients with MIS-C. Figure 1A outlines the study flowchart and the clinical and biological parameters we evaluated. Patients included fulfilled criteria for MIS-C, classical KD or TSS. All three groups of patients were then subjected to deep immunological analyses combining cytokine profiling, TCR Vβ analysis and T cell stimulation assays (Fig. 1A). We confirmed the strong clinical overlap between MIS-C, TSS and KD. Indeed, many patients in the MIS-C group fulfilled the major criteria for TSS and KD respectively (Fig. 1B). Considering the clinical parameters, the most frequent features of MIS-C patients in our cohort were fever, cardiac dysfunction, gastrointestinal symptoms, coagulopathy and systemic inflammation (Fig. 1C). Additional clinical data are presented in Table 1 for KD, TSS and acute COVID-19., and in Table S1 for

all patients. Moreover, Table S2 gives a list of the patients analyzed in each of the following figure panels.

High levels of proinflammatory cytokines in MIS-C contrasting with lymphopenia and low HLA-DR expression in monocytes.

SARS-CoV2 can cause fatal acute respiratory distress syndrome in patients at risk. This manifestation is caused by delayed and poorly controlled immune responses, with a deleterious role of inflammatory cytokines. Moreover, we and others have identified a subgroup of severe COVID-19 patients with impaired type-I interferon production (15-18). Thus a regulated production of cytokines is paramount for a good control of SARS-CoV2 infection. This prompted us to investigate how cytokines could contribute to MIS-C pathogenesis. We compared the serum level of IFN-α, IFN-γ, TNF-α, IL-10, soluble CD25 (sCD25), MCP1, IL1Ra, IL-6 and IL-18 between healthy controls and MIS-C, KD, TSS and different forms of COVID-19 (mild pediatric, mild or severe adult-onset COVID-19, see Table S1 for a list of clinical features in the different patients' groups).

The expression of interferon-stimulated genes (ISGs) in blood cells was significantly higher in MIS-C compared to controls, but rather low compared to COVID-19 patients (Fig. 2A). The level of serum IFNa2 followed the same trends, while serum IFNg was variable among MIS-C patients, with very high levels in a few patients. The expression of the other cytokines measured (IL-6, IL-10, IL-18, TNFα, MCP1, IL1RA, CD25s) was very high in MIS-C patients compared to controls, and very similar to that of KD, TSS and severe COVID-19 patients (Fig. 2B-C). Of note the level of CD25s was significantly higher in TSS than in MIS-C patients, and significantly lower in severe COVID-19 patients than in MIS-C patients (Fig. 2B-C). Of note, a previous study found higher levels of serum IL-6 in KD patients than in MIS-C contrasting with our data (8).

To further explore the MIS-C immunological profile, we then quantified the number of peripheral lymphocytes of different types, as well as the expression of HLA-DR in patient's monocytes. T and NK cell counts were on average very low in MISC and KD patients while B cell counts were normal (Fig. 2D). We found a decreased expression of HLA-DR in monocytes in both KD and MIS-C patients compared to controls (Fig. 2E). Altogether our data show a strong similarity in cytokine profiles between MIS-C, KD and TSS and highlight the decreased lymphocyte counts and low HLA-DR expression in monocytes in MIS-C patients compared to controls.

Expansion of Vβ21.3+ peripheral T cells in a large fraction of MIS-C patients

TSST1-related TSS is associated with a skewing of the T cell repertoire towards Vβ2, as a result of TSST1-superantigen induced proliferation of Vβ2+ T cells (19). Every other *S. aureus* superantigenic toxins induce the expansion of specific TCR Vβ subsets, i.e. Vβ 5.2, 5.3, 7.2, 9, 16, 18, 22 for staphylococcal enterotoxin A (SEA) or Vβ 3, 12, 13.2, 14, 17, 20 for SEB (20). Given the similarities between TSS and MIS-C, we explored the possibility that MIS-C was also associated with specific T cell expansions. To explore the T cell repertoire in MIS-C, we first used flow cytometry to assess the distribution of Vβ subunits in T cells from MIS-C patients, in comparison with KD, TSS and COVID-19

patients (Fig. 3A). As expected, TSS patients displayed the hallmark expansion of the Vβ2+ subset. Interestingly, several Vβ-specific expansions were also visible in MIS-C patients, and in most cases Vβ21.3+ expansions (Fig. 3A), in both CD4 and CD8 T subsets (Fig. S1A-B). These expansions had similar amplitudes as the Vβ2+ expansions in TSS (Fig. 3A). A principal component analysis of the Vβ distribution in CD4 and CD8 T cells showed that the main parameters separating the different patients were the frequency of Vβ2+ and the frequency of Vβ21.3+ cells (Fig. S1C-D). Overall, the expansion of Vβ21.3+ T cell subsets was seen in 15/26 (58%) of MIS-C patients and in none of the other conditions analyzed by flow cytometry ie KD, TSS and COVID-19 (Fig. 3A). Next, we wanted to use a different technique to test the specificity of this expansion, and we therefore performed transcriptomic analyses of Vβ expression in PBMC using the Nanostring technology. This technique also requires much less material than flow cytometry, which allowed us to run lymphopenic samples from severe COVID-19 cases. This transcriptomic analysis firmly established that the Vβ21.3+ T cell expansion is a hallmark of MIS-C as it was seen in 18/23 MIS-C patients tested (Fig. S1E). Thus, taking together flow cytometry and Nanostring analyses, we found that 24/32 (75%) of MIS-C patients and none in the other clinical groups displayed *TRBV11-2*/Vβ21.3+ expansions.

In an effort to understand the physiopathological mechanism underlying MIS-C we then compared the level of serum cytokines between MIS-C patients with and without Vβ21.3+ T cell expansions, at the time of the acute episode. The levels of IL-18 and IL-1RA (Fig. 3C-D) were associated with the expansions, but not those of the other cytokines tested (Fig. S2B), suggesting that Vβ21.3+ T cells were associated with the cytokine storm.

TCR sequencing highlights the polyclonal nature of TCR Vβ21.3 expansions

To investigate the clonality of Vβ21.3+ expanded cells, we analyzed the TCR repertoire of 11 MIS-C patients for whom whole blood RNA was available by TCR-sequencing. We analyzed the composition of the TCR beta rearrangements involving the TRBV11-2 gene (which corresponds to Vβ21.3). First, by representing the TRBV11-2/TRBJ combination usage as chord diagrams (Fig. 3E, 3F), we confirmed the expansion of T cells using TRBV11-2 in 7 out of the 11 patients (MISC-3, 7, 8, 9, 13, 23, 26). These TRBV11-2 rearrangements were associated with multiple TRBJ genes, suggesting the polyclonal nature of the expansions. To further evaluate the polyclonality, we analyzed the CDR3 length distribution of TRBV11-2 clonotypes (barplots Fig. 3E-F-G.). The CDR3 spectratype distributions are typical of the bell-shaped Gaussian distribution expected in polyclonal repertoires. In order to evaluate the degree of polyclonality, we identified the expanded clonotypes by setting a threshold based on the binomial distribution of the clonotype frequencies per sample (see methods section and Fig. S3A). No major monoclonal expansions (red lines in the CDR3 spectratypes) explaining the global TRBV11-2 expansion were detected. Instead, most of the clonotypes were found at low frequencies (grey lines), typical of a polyclonal diverse repertoire. The percentages of expanded clonotypes were not significantly different between patients with or without TRBV11-2. We calculated the cumulative frequencies of such expanded clonotypes within the full repertoire and found that they were always far below the frequency of the full TRBV11-2 expansion in patients with expansions, representing in average 0,51 % of the total repertoire. Finally, these limited

expansions represented in average 4,47% of the TRBV11-2 repertoire in patients with TRBV11-2 expansions and 6,31% in patients without TRBV11-2 expansions (Table S4 and Fig. S3B). To confirm the polyclonality of the TRBV11-2 expansion, we computed the Berger-Parker index (BPI) on TRBV11-2 clonotype for MIS-C patients harboring or not TRBV11-2 expansions (Fig. S3C). This index measures the proportional abundance of the most abundant clonotypes within TRBV11-2 clonotypes. There was no significant differences when we compared the BPI on TRBV11-2 clonotypes between patients with or without TRBV11-2 expansions, further confirming that TRBV11-2 expansions in the 7 patients are not explained by monoclonal expansions.

We then asked whether the Vβ21.3+ T cell expansion persisted overtime. For this we repeated the TCR sequencing and the flow cytometry Vβ analyses in a group of patients for which blood samples were available during and after the acute inflammatory episode. As shown in Figures 3F-H, the Vb21.3/TRBV11-2 distributions for all the patients returned to normal within days to weeks after MIS-C. Interestingly, when we compared the CDR3 length distributions by calculating the perturbation score using the ISEApeaks tool between repertoires obtained during and after the acute response, we found no differences between the two groups, further supporting the polyclonal expansion profile of TRBV11-2 during the acute response (Fig. S3D). Finally, this transient expansion suggested a pro-apoptotic phenotype of Vβ21.3+ T cell. To test this hypothesis, we stained PBMCs from MIS-C patients with Annexin-V that marks early apoptotic cells. A higher fraction of Vβ21.3+ compared with Vβ21.3-T cells were stained with Annexin-V in MIS-C patients with Vβ21.3+ expansions (Fig. 3I), which substantiated our hypothesis.

Vβ21.3+ T cells have an activated phenotype but do not react against SARS-CoV2 peptides

As Vβ21.3+ T cells expand in MIS-C patients, we investigated their activation status and the mechanisms underlying their proliferation. We found that the activation markers HLA-DR and CD38 were expressed at high levels in both CD4 and CD8 T cells from MIS-C patients with Vβ21.3+ expansions compared to those without expansions and to healthy controls (Fig. 4A-4B). This was due to a specific up regulation of CD38 and HLA-DR in Vβ21.3+ CD4 and CD8 T cells in MIS-C patients with expansions compared to those without expansions (Fig. 4C, 4D). A recent paper reported a specific activation of CX3CR1+ CD4 and CD8 T cells in MIS-C patients, as assessed by HLA-DR/CD38 levels(22). This prompted us to measure CX3CR1 levels in Vβ21.3+ T cells. As shown in Figure 4E, Vβ21.3+ were in majority CX3CR1 positive both in CD4 and CD8 T cells in MIS-C patients with Vβ21.3+ expansions compared to those without expansions, even though the percentage of CX3CR1 positive cells was not higher in MIS-C than in control patients (Fig. S4A). Moreover, in MIS-C patients, a large frequency of CX3CR1+ CD4 and CD8 T cells had an activated phenotype in terms of HLA-DR and CD38 expression (Fig. S4B).

Given that MIS-C followed COVID-19, we wondered if Vβ21.3+ T cells were raised against SARS-CoV-2 antigens. To test this possibility, we stimulated PBMCs from MIS-C or convalescent COVID-19 patients with a commercial cocktail of SARS-CoV2 peptides spanning S, N and M viral proteins. T cells from MIS-C patients responded poorly to stimulation with viral peptides, regardless of Vβ21.3 expansion, compared to T cells from

convalescent COVID-19 patients that responded well (Fig. 4F, 4E, S3). This was not due to a lack of adaptive anti-SARS-CoV-2 response, because all MIS-C patients tested had high SARS-CoV-2-specific antibody levels (Fig. S4C-E). Finally, we could not identify any specific allele nor mutations of classical HLA class I or class II genes associated with TRBV11-2 expansions by genomic sequencing of the HLA loci of 13 MIS-C patients (Table S3). Together with the lack of Vβ21.3+ expansion in COVID-19 patients, these data show that Vβ21.3+ T cells are not specific for HLA-restricted SARS-CoV-2 peptides.

Discussion

Here, we first confirmed the strong overlap in clinical phenotype between KD, MIS-C, and TSS. MIS-C and TSS were even more similar to each other with cardiac dysfunction, hypotension, maculo-papular skin rash and conjunctivitis as defining features. Moreover, we have recently identified the critical importance of early steroid therapy in the management of MIS-C, similarly to what has been previously shown in TSS (23, 24). MIS-C and TSS are obviously linked to infections, while many KD features suggest an infectious cause for KD as well (25). In particular, the efficacy of IV immunoglobulins, the occurrence in young children, the acute nature of the disease and the absence of relapse suggest that an infectious agent is driving the pathogenesis. The epidemic of a novel coronavirus in 2005 (New Haven) was associated to KD and linked the viral infection to vascular inflammation(26).

We found important similarities in terms of cytokine expression between MIS-C, TSS and KD such as high TNF-α, IL6, IL18 and IL1Ra levels. A previous study noted that a subgroup of severe MIS-C patients had higher levels of IFN-γ, IL-18, GM-CSF, RANTES, IP-10, IL-1α, and SDF-1 than mild MISC or KD patients (27). We also observed a subset of MIS-C patients with high serum IFN-γ, IL-18 and CD25s. These observations confirm previous reports showing a clinical and biological overlap between MIS-C and macrophage activation syndrome(3), and suggest the importance of IFN-γ in the disease. The suppressor of cytokine signaling protein-1 SOCS1 is a major negative regulator of IFN-γ signaling and SOCS1 haploinsufficiency is associated to an increase of IFN-γ sensitivity(28, 29). A recent paper reported MIS-C development in a child with heterozygous loss-of-function SOCS1 mutations (1), perhaps suggesting a pathological role for IFN-γ. However there are also important differences between immune profiles of MIS-C and hemophagocytic syndrome. Indeed, the latter is characterized by very high HLA-DR expression in monocytes (30) while we observed low expression of HLA-DR in monocytes in MIS-C patients. This observation is suggestive of immune unresponsiveness, as seen following TSS or septic shock (30-33). Indeed, low HLA-DR expression in monocytes is considered as a very good marker of sepsis-induced immunosuppression (33).

We report the expansion of a TCR Vβ21.3+ T cell subset with an activated phenotype in as many as 75% of MIS-C patients. These expansions were identified using flow cytometry experiments taking advantage of available antibodies against the different Vβ chains, and with a new dedicated Nanostring panel allowing the measurement of mRNA encoding for these chains in whole blood in a large number of MIS-C patients. Vβ21.3+ T cell expansions were also reported in smaller numbers of MIS-C patients in two recent studies, that used either TCR sequencing or single cell RNA-seq (34, 35). In both Porritt and our study

Vβ21.3+ T cell expansions appeared polyclonal as judged by the large number of TRBJ gene segments associated with TRBV11.2 and by the even distribution of the CDR3 domain. Our study is however the only one to show that Vβ21.3+ CD4 and CD8 T cell expansions are a feature of MIS-C that well discriminates them from KD, TSS and COVID-19 patients.

We observed a correlation between Vβ21.3+ T cell expansions and the level of serum cytokines IL-18 and IL-1RA from matching samples, confirming a previous study (36) and indicating that Vβ21.3+ T cell expansions are associated with the cytokine storm. Our data also show that Vβ21.3+ T cells have an activated phenotype, with high HLA-DR and CD38 expression and that activated Vβ21.3+ T cells expressed high levels of CX3CR1, a marker of patrolling monocytes and of cytotoxic lymphocytes. CX3CR1 binds to CX3CL1, a membrane-bound chemokine induced on vascular endothelial cells upon inflammation. The CX3CL1-CX3CR1 axis is thought to have an important role in vascular inflammation in different inflammatory diseases(37), and could contribute to MIS-C pathogenesis. This interaction could promote the cytotoxic action of different lymphocyte populations, which fits with the reported elevated expression of cytotoxicity genes in NK and CD8+ T cells in MIS-C patients(38).

We demonstrate that both TSS and MIS-C are marked by the polyclonal proliferation of a specific Vβ subset i.e. Vβ2+ cells for TSS related to TSST1, and Vβ21.3+ cells for MIS-C. We found that the amplitude of the expansion was also similar in both syndromes. Considering the other similarities between MIS-C and TSS shown in this study in terms of clinical phenotype, cytokine production and treatment, this raises the hypothesis that Vβ21.3+ cell expansions are caused by a superantigen structure in MIS-C. The term superantigen has been coined by Kappler and Marrack as an operational definition of various T-cell activating substances with specificity for T cell antigen receptors Vβ subunits regardless of the rearrangement and antigen-specificity (39). Superantigens bind external regions of T cell receptor and MHC molecules (40) and can induce massive expansions of T cells expressing one specific TCR Vβ chain while classical antigens induce the expansion of T cells bearing different Vβ. Previous papers have suggested that the SARS-CoV2 Spike protein could behave as a superantigen structure (41). Using *in silico* modelling, Porritt et al identified a putative interaction between Vβ21.3 and a superantigen-like motif on the spike of SARS-CoV2. However, Vβ21.3+ T cell expansions occur in a delayed manner relative to SARS-CoV-2 infection, and the virus is often undetectable in MIS-C patients at the time of the acute inflammation. The kinetics of MIS-C relative to COVID-19 is compatible with a causal role of anti-SARS-CoV-2 antibodies. One can hypothesize that immune complexes composed of SARS-CoV-2 bound to antibodies may act as superantigen structures. However, a previous study failed to detect such immune complexes in MIS-C patients(27). In addition, Vβ restricted T cells have been shown to adhere to endothelial cells following superantigen activation(42) and thus the CX3CR1+ Vb21.3 expanded T cells may play a role in vascular injury in MIS-C. Alternative mechanisms may be put forward, such as secondary autoimmune reactions. Several studies have indeed reported the appearance of autoantibodies in MIS-C patients, some of which directed against endothelial antigens(8, 11), while others have reported immune events consistent with autoimmunity such as the expansion of proliferating plasmablasts(38) or the persistence of functional SARS-CoV-2-specific monocyte-activating antibodies(43). How B cell mediated autoimmunity would

be linked to Vβ specific T cell expansions is however unclear. One could speculate that immune complexes composed of autoantibodies and endogenous antigens could behave as superantigens. Finally, given the rarity of MIS-C, there could be a genetic susceptibility to this post-infectious disease promoting hyperinflammatory reaction of adaptive immunity in response to SARS-CoV2(14). We limited our analysis to classical HLA alleles, but did not find any significant association, even though a previous study reported an HLA-I bias in a smallest group of MIS-C patients(36).

Limitations of the study:

All samples from MIS-C patients were obtained after anti-inflammatory treatments (see supplementary table I), and it is likely that those treatments affect the level of serum cytokines, which could have impacted the comparisons we made between clinical conditions, and the associations between cytokines and T cell expansions.

Methods

Study design

The aim of the study was to compare MIS-C features with those of Kawasaki disease and Toxic Shock Syndrome (TSS). The immunological profile of 36 MIS-C cases, 16 KD and 58 TSS cases and 42 non-MISC COVID-19 were included (Fig. 1A). Samples were collected within the first week of symptoms and analyzed for cytokine immunoprofiling, standard immunophenotyping, Vβ expression, TCR sequencing, SARS-CoV2-dependent T cell response. Because of low volume sampling of pediatric patients, we did not have the same availability for research blood draws. The samples used for each experiments are detailed in table S2.

Patients and ethics

Four distinct cohorts were used for the data collection and analyses registered in ClinicalTrial.gov and consents were obtained from parents. The main clinical features are summarized in Fig. 1C, Table 1, Table S2. Written informed consent was obtained for all data collection and blood sampling as detailed in supplemental material. The 36 MIS-C patients were included since the beginning of the pandemics, from April 2020 from French participating centers (HPI COVID). We took advantage of previous collection of Kawasaki from Necker's Hospital and additional patients with KD or TSS previously included into a study on toxic shock syndrome (approved by the Ethical review board Sud Est IV, DC-2008-176). Acute COVID-19 patients were derived from either HPI project on pediatric COVID-19 (HPI COVID), n=11 or from two ongoing project on mild adult COVID-19 in health care providers (COVID-SER), n=21 or severe adult COVID-19 in critical care unit (COVID-Rea), n=10. All details for ethical agreement are listed in Supplemental material. Clinical information of KD, COVID-19 and TSS patients are detailed in Table 2 and Table S2. All patients could not be included in all analysis, this information is provided in Table S1.

Cytokines and IFN score assessment

Whole blood was sampled on EDTA tubes and plasma was frozen at −20°C within 4 hours following blood collection. Plasma concentrations of IL-6, TNF-a, IFN-g, IL-10, MCP-1, IL-1ra and CD25s were measured by Simpleplex technology using ELLA instrument (ProteinSimple), following manufacturer's instructions. Plasma IFN-α concentrations were determined by single-molecule array (Simoa) on a HD-1 Analyzer (Quanterix) using a commercial kit for IFN-α2 quantification (Quanterix). Whole blood was collected on PAXgene blood RNA tubes (BD Biosciences) or on EDTA tubes for IFN signature, RNA extraction was performed with the kit maxwell 16 LEV simply RNA blood associated with the Maxwell extractor (Promega) and quantified by absorbance (Nanovue). IFN score was obtained using nCounter analysis technology (NanoString Technologies) by calculating the mediane of the normalized count of 6 ISGs as previously described(44)

T-cell Vβ repertoire analysis and immunophenotyping

The phenotypic analysis of T-cell Vβ repertoire was performed on whole blood sample using the IOTest Beta Mark kit (Beckman-Coulter) containing 24 monoclonal antibodies (mAbs) identifying ~ 70% of the T cell repertoire. Whole blood cells were stained with APC-Alexa Fluor 750-conjugated anti-CD3, Pacific Blue-conjugated anti-CD4, Krome Orange-conjugated anti-CD8 and each combination of 3 FITC-, PE- and FITC/PE-conjugated ant-Vβ mAbs (Beckman-Coulter) in 8 sample tubes. Whole blood sample were lysed with OptiLyse C Lysing Solution (Beckman-Coulter), washed and fixed in 0.5% formaldehyde in PBS. 0.5 to 10^4 T cells were acquired on a NAVIOS flow cytometer and data were analyzed using NAVIOS software. Lymphocytes were first gated according to FSC/SSC parameter, then by selection of CD3+, CD4+ and CD3+CD4− positive cells. The proportion of each Vβ family was compared to the minimum and the mean+2SD of each reference values obtained from data from IOTest Beta Mark® kit to evaluate expanded or restricted Vβ family. Expansions or restrictions were defined respectively for values above the mean+2SD or below the minimum reference values of the corresponding family.

Lymphocytes immunophenotyping

CD3, CD4 and CD8 T lymphocyte subsets were enumerated on EDTA-anticoagulated peripheral whole blood by single-platform the fully automated volumetric single plateforme technology flow cytometer AQUIOS CL (Beckman-Coulter) as previously described(45). The phenotypic characterization of B, NK and T activated lymphocyte subsets were performed on EDTA-anticoagulated whole blood using the following combination of monoclonal antibodies: APC-Alexa Fluor 750-conjugated anti-CD3, Pacific Blue-conjugated anti-CD4, Krome Orange-conjugated anti-CD8, FITC-conjugated anti-HLA-DR, APC-conjugated anti-CD19, Krome Orange-conjugated anti-CD16 and ECD-conjugated anti-CD56 (Beckman-Coulter). The preparations were lysed and fixed by thoroughly mixing and incubating for 10 min successively with 500μL of OptiLyse C reagent (Beckman-Coulter) and 1mL of PBS. The cells were centrifuged for 5min at 400g, resuspended in 500μl of PBS and acquired on a NAVIOS flow cytometer (Beckman-Coulter).

Nanostring TCR expression analysis

Total RNA was extracted from PAXgene™ tubes using the Maxwell® 16 LEV simplyRNA Blood kit (Promega), following the manufacturer's guidelines. The RNA quantity was determined using a Nanodrop (Thermo Scientific). 200 ng total RNA were hybridized with the nCounter® T cell repertoire panel (Nanostring©, #LBL-10805-01) and counted on an nCounter® FLEX platform according to the manufacturer's guidelines. Raw counts were normalized using internal positive standards and 12 housekeeping genes. Raw counts of *TRBV* genes were expressed as a proportion among total *TRBV* gene counts for each patient and normalized using the median value from the healthy control group.

Monocyte HLA-DR expression assessment

Monocyte HLA-DR expression was determined on EDTA-anticoagulated peripheral whole blood as previously described(46).

TCR-sequencing

RNA was extracted from whole blood as reported above. T cell receptor (TCR) alpha/beta libraries were prepared from 300ng of RNA from each sample with SMARTer Human TCR a/b Profiling Kit (Takarabio) following provider protocol as previously described (32). Briefly, the reverse transcription was performed using a mixture of TRBC and TRAC reverse primers and further extended with a template-switching oligonucleotide (SMART-Seq v4). cDNAs were then amplified following two semi-nested PCR: a first PCR with TRBC and TRAC reverse primers as well as a forward primer hybridizing to the SMART-Seq v4 sequence added by template-switching and a second PCR targeting the PCR1 amplicons with reverse and forward primer including Illumina Indexes allowing for sample barcoding. PCR2 are then purified using AMPUre XP beads (Beckman-Coulter). The sequencing was then carried out on a MiSeq Illumina sequencer using the MiSeq v3 PE300 protocol at the Biomics Platform (Institut Pasteur, Paris, France). Single end sequences were aligned and annotated using MiXCR 3.0.13 (47), providing a list of clonotypes, each of which is defined as a unique combination of one TRBV gene with one CDR3 amino-acid sequence and one TRBJ gene.

TCR-Seq repertoire analysis

Analyses were performed in R 4.0.3 on the TRB clonotype lists obtained with MiXCR. For each clonotype, read count was recorded. Frequencies for TRBV, TRBJ and clonotypes were calculated based on the total read counts per sample. Chord diagrams were made using the circlize package(48) on TRBVBJ frequencies, CDR3 length spectratypes were made using ggplot2 (49) using clonotype frequencies. To identify TRBV11-2 expanded clonotypes, first (Q1) and third (Q3) quartiles and the interquartile range (IQR) were computed for all patients without TRBV11-2 expansion. Expanded clonotypes are defined as those with counts superiors to Q3+(1.5*IQR).

Immunophenotyping of Vβ 21.3+ T cells

Thawed PBMC were labeled labeled using Fixable Viability Dye eFluor™ 506 from Thermo Fisher. PBMCs were stained with surface markers, APC-conjugated anti-

CD3, BUV486- conjugated anti-CD4, PE-Cy7-conjugated anti-CD8, APC-Cy7-conjugated anti-CD14, APC-Cy7-conjugated anti-CD16, APC-Cy7-conjugated anti-CD19, BV711-conjuagetd anti-CCR7 and BV421-conjugated anti-CD38 (BioLegend), FITC-conjugated anti-Vb21.3 (Miltenyi), Biotin-conjugated anti-HLA-DR, APC-conjugated CX3CR1 (Ebiosciences), BV605-conjugated anti-CD45RA and streptavidine-conjugated PE-texas Red (BD). Cells were then washed, fixed with PBS/Formalin 2%(Sigma-Aldrich). Cell apoptosis was assessed by annexin V staining with the PE Annexin V Apoptosis Detection Kit I (BD). All samples were acquired on a BD LSRFortessa (BD Biosciences) flow cytometer and analyzed using FlowJo version 10 software.

Stimulation with SARS-CoV-2 overlapping peptide pools and flow cytometry

Briefly, overnight-rested PBMCs were stimulated with SARS-CoV-2 PepTivator pooled peptides (Miltenyi Biotec) at a final concentration of 2 μg ml–1 for 1 h in the presence of 2 μg ml–1 monoclonal antibodies CD28 and CD49d, and then for an additional 5 h with GolgiPlug and GolgiStop (BD Biosciences). Dead cells were labeled using LIVE/DEAD Fixable near IR dye from Invitrogen. Surface markers, including APC-conjugated anti-CD3, BUV486- conjugated anti-CD4, PE-Cy7-conjugated anti-CD8, APC-Cy7-conjugated anti-CD14, APC-Cy7-conjugated anti-CD16 and APC-Cy7-conjugated anti-CD19 (BioLegend) and FITC-conjugated anti-Vb21.3 (Miltenyi) were stained. Cells were then washed, fixed with Cytofix/Cytoperm (BD Biosciences) and stained with V450- conjugated anti-IFNγ (eBioscience). Negative controls without peptide stimulation were run for each sample. All samples were acquired on a BD LSRFortessa (BD Biosciences) flow cytometer and analyzed using FlowJo version 10 software.

Serology

Serum samples were tested with three commercial assays: the Wantai Ab assay detecting total antibodies against the receptor binding domain (RBD) of the S protein, the bioMérieux Vidas assay detecting IgG to the RBD and the Abbott Architect assay detecting IgG to the N protein.

Statistical analyses

PCA analysis was made in R with stats package and visualized with ggplot2 (49) for Vbeta frequencies obtained by flow cytometry. All statistical analyses were performed using GraphPad with the help of a trained biostatistician.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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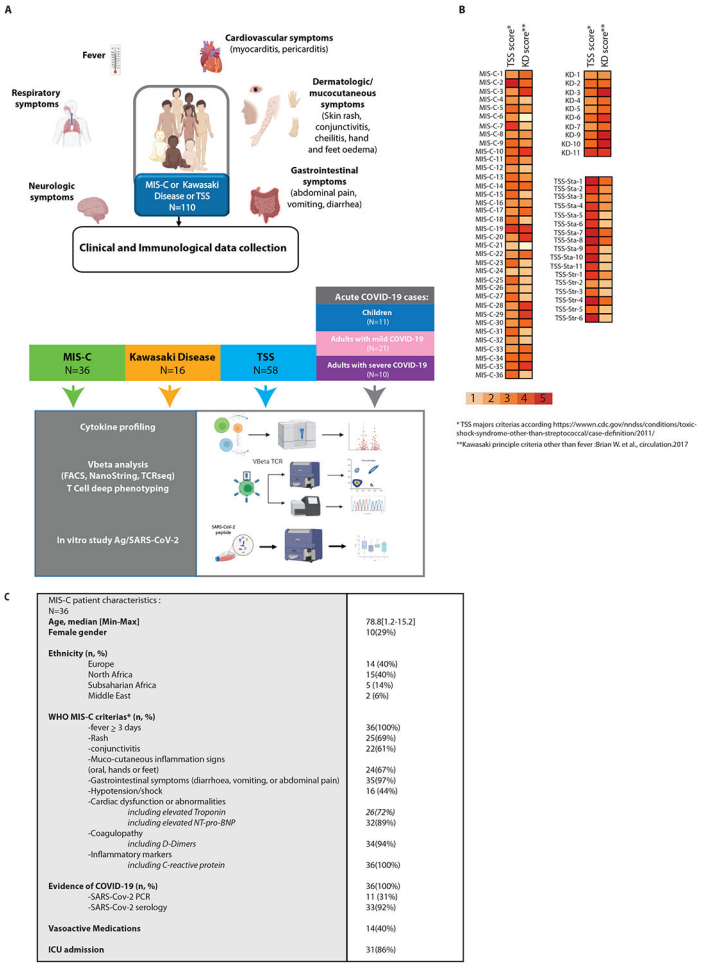

C

MIS-C patient characteristics : N=36	
Age, median [Min-Max]	78.8[1.2-15.2]
Female gender	10(29%)
Ethnicity (n, %)	
Europe	14 (40%)
North Africa	15(40%)
Subsaharian Africa	5 (14%)
Middle East	2 (6%)
WHO MIS-C criterias* (n, %)	
-fever ≥ 3 days	36(100%)
-Rash	25(69%)
-conjunctivitis	22(61%)
-Muco-cutaneous inflammation signs (oral, hands or feet)	24(67%)
-Gastrointestinal symptoms (diarrhoea, vomiting, or abdominal pain)	35(97%)
-Hypotension/shock	16 (44%)
-Cardiac dysfunction or abnormalities	
including elevated Troponin	*26(72%)*
including elevated NT-pro-BNP	32(89%)
-Coagulopathy	
including D-Dimers	34(94%)
-Inflammatory markers	
including C-reactive protein	36(100%)
Evidence of COVID-19 (n, %)	36(100%)
-SARS-Cov-2 PCR	11 (31%)
-SARS-Cov-2 serology	33(92%)
Vasoactive Medications	14(40%)
ICU admission	31(86%)

Figure 1. Study design and clinical features of MIS-C patients

(**A**) Outline of the study including MIS-C, KD, TSS and acute COVID-19 patients and the immunological investigation workflow. (**B**) Heatmap showing the TSS or KD clinical score for TSS, MIS-C and KD patients included in our study, calculated as the number of major criteria reached for each disease. (C) Clinical description of all MIS-C patients included in the study.

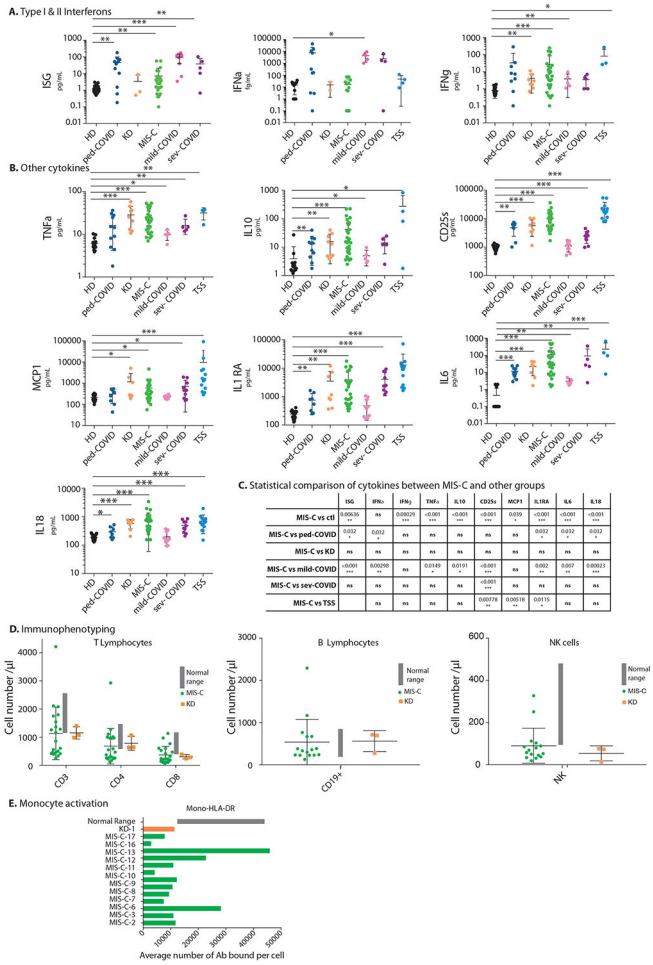

Figure 2. Systemic inflammation and signs of immune paralysis in MIS-C patients

(**A**) Left panel: Interferon score calculated as the normalized mean expression of six ISGs measured using the Nanostring technology, as previously described (44, 50). Middle panel: Serum IFN-α, in different groups of patients, as measured with the Simoa technology. Right panel: Serum IFN-γ level measured by Elisa. N=3 to 30 per group, as indicated in Table S2; Statistical test: multiple comparisons and correction using Benjamini-Hochberg procedure. (**B**). Serum levels of the indicated cytokines as measured by automated ELISA. N=5 to 30 per group, as indicated in Table S2; Statistical test: multiple comparisons and correction using Benjamini-Hochberg procedure. (**C**) Table showing the statistical results of the comparison of cytokine levels between MIS-C and other groups, as indicated. (**D**) T, B and NK lymphocyte counts measured by flow cytometry in MIS-C and KD. The grey bar indicates the normal range in healthy donors . N=3 to 13 per group, as indicated in Table S2. (**E**) HLA-DR expression in T cells and monocytes, as measured by flow cytometry in MIS-C. The grey bar indicates the normal range in healthy donors. *P < 0.05, **P < 0.01, ***P < 001.

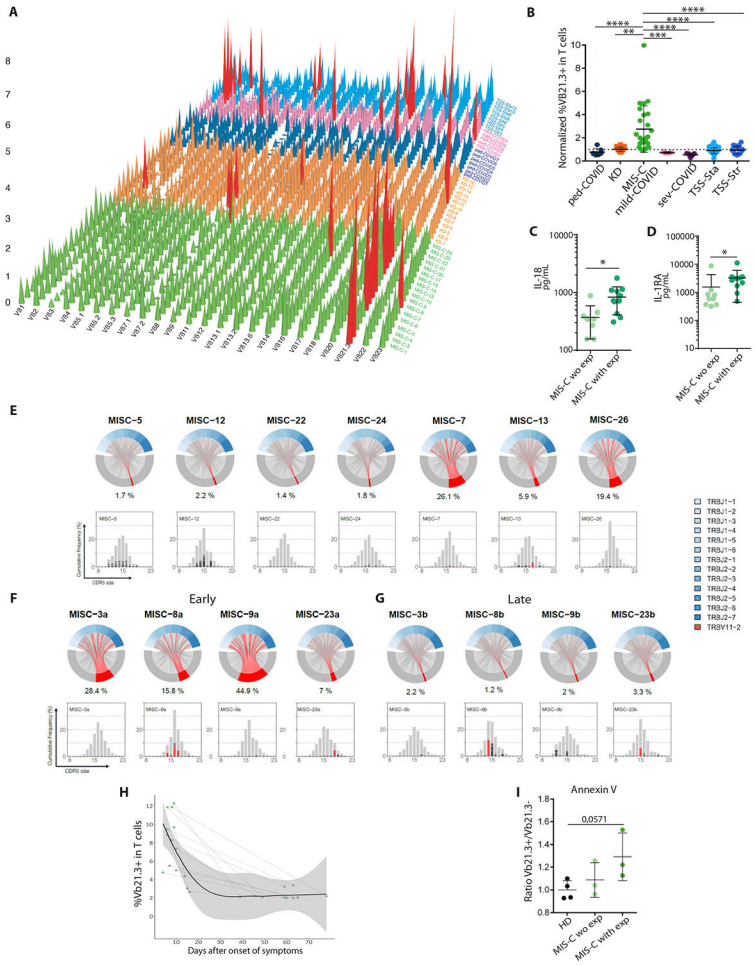

Figure. 3. Polyclonal Vb21.3+ T cell expansion in MIS-C patients

(**A**) Frequency of total CD3+ T cells expressing the indicated V-beta (Vβ) chains, as measured by flow cytometry using specific antibodies against the corresponding Vβ within PBMCs of patients of the indicated group. TSS, mild COVID-19, pediatric COVID-19 (ped-COVID), KD and MIS-C patients are colored in blue, pink, dark blue, orange and green respectively. The red color highlights values at least twice higher as the mean frequency in the general adult population. (**B**) Normalized frequency of Vβ21.3+ T cells in different clinical conditions, as indicated. N=5 to 26 per group, as indicated in Table S2; Statistical test: Mann-Withney using FDR adjustment. (**C-D**) Serum IL-18 (C) and IL-1RA (D) levels in MIS-C patients with or without Vβ21.3+ T cell expansions (exp). N=6 to 11 per group, as indicated in Table S2; Statistical test: Mann-Withney. (**E-G**) Chord diagrams of the TRBV (bottom, grey) and TRBJ (top, blue) combinations assessed by TCR sequencing of TCRab chains in whole blood of MIS-C patients. The relative frequency of all TRBVBJ combinations have been calculated per sample on the full TRB repertoire data. Combinations using TRBV11-2 are highlighted in red. Each red line indicates pairing with a given TRBJ, the thickness indicates the frequency of this pairing. The percentage values under each chart indicate the percentage of clonotypes composed with the TRBV11-2 gene. In (**E-G**) the CDR3 length distribution of clonotypes using TRBV11-2 is shown as

an histogram graph. Each clonotype is represented as a grey line. The thickness of the line represents the frequency of the clonotype within each repertoire. Since most of the clonotypes are not abundant, all the grey lines are stacked together and appear as a unique grey bar, which reflect the lack of expansion. Expanded clonotypes identified as detailed in the method section are shown in red. In (F-G) the same four patients are shown during the MIS-C episode (F) and after resolution (G). (**H**) Frequency of Vβ21.3+ T cells at different time points during and after the MIS-C episode in different patients, as assessed by flow cytometry. N=11, as indicated in Table S2. (**I**) Annexin-V staining of T cells in the indicated patients groups. Results show the ratio of the Annexin-V fluorescence in Vβ21.3+ vs Vβ21.3- T cells. N=3-4 in each group. Statistics were calculated using the Mann-Withney test.

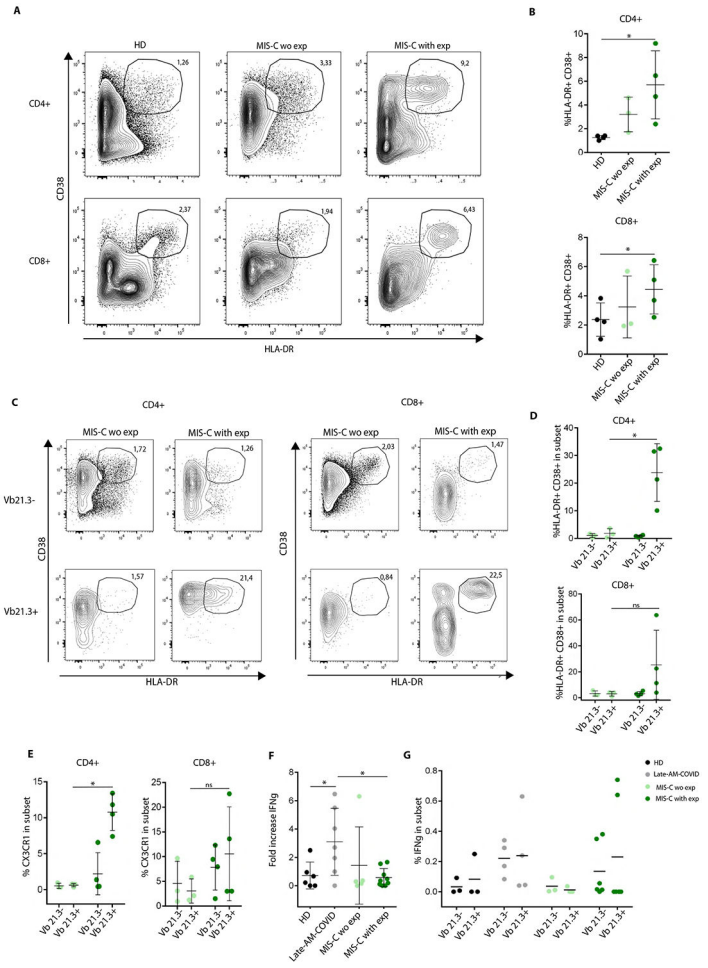

Figure 4. T cell activation within Vβ21.3 and stimulation of T cells with viral peptides in vitro.

(**A-D**) Flow cytometry analysis of CD38 and HLA-DR expression in CD4 or CD8 T cells from the indicated patients' groups (exp: Vβ21.3+ T cell expansion). (**A**) shows a representative staining, and (**B**) shows the mean +/−SD frequency of CD38+HLA-DR+ CD4 (top) and CD8 (bottom) T cells. N=3 to 4 per group, as indicated in Table S2; Statistical test: Mann-Withney using FDR adjustment. (**C-D**) A Vβ21.3+ antibody was also included in the flow cytometry panel used in (A-B) allowing a specific comparison of the Vβ21.3− and Vβ21.3+ T cells in MIS-C patients. (C) shows a representative dot plot of CD38 and HLA-DR expression in the indicated subsets; (D) mean +/−SD frequency of CD38+HLA-DR+ in the indicated CD4 (top) and CD8 (bottom) T cell subsets. N=3 to 4 per group, as indicated in Table S2; Statistical test: Mann-Withney. (**E**) Frequency of CX3CR1+ cells in gated Vβ21.3− and Vβ21.3+ CD4+ (left) and CD8+ (right) T cells in MIS-C without and MIS-C with expansion. (**F**) PBMCs from control, COVID-19 (adults, 6 months post infection) or MIS-C patients (with or without Vβ21.3+ T cell expansions) were stimulated for 6h with a commercial cocktail of synthetic peptides from S, N, and M SARS-CoV2 proteins in the presence of Golgi secretion inhibitors. Intracellular IFNγ expression was then measured in T cells by flow cytometry. The fold increase was calculated as the ratio between the stimulated and the unstimulated conditions. N=5 to 9 per group, as indicated in Table S2;

Statistical test: Mann-Withney using FDR adjustment. (**G**) shows the frequency of Vβ21.3+ and Vβ21.3− T cells expressing IFN-γ after stimulation with S, N, M SARS-CoV2 peptides in the different patient groups as indicated (one dot: one patient).

Table 1:

Demographic and clinical data of pediatric patients with Kawasaki Disease or Toxic shock syndrome (*S.aureus or S. pyogenes*) and adult patients with mild or severe COVID-19

	pediatric COVID-19	**children with Kawasaki Disease**	**children with Toxic shock syndrome (*S.aureus*)**	**children with Toxic shock syndrome (S.pyogenes)**	**adult with mild COVID-19**	**adult severe COVID-19**
	N=11	N=16	N=39	N=19	N=21	N=10
Age (y), **median [Min-Max]**	2,5 [0,1-17,6]	2,9 [0,1-15,8]	14,7 [0.4-18]	4,1 [0,7-18]	42 [29.2-57,3]	60,8 [42,3-78,8]
Female gender	3 (27%)	9 (56%)	29 (74%)	10 (67%)	18 (86%)	5 (50%)
ICU admission	3 (27%)	3 (18%)	36 (100%) (n=36)	19 (100%)	0 (0%)	10 (100%)
Vasoactive medications	0 (0%)	1 (6%)	19 (65%) (n=29)	15 (83%)	0 (0%)	3 (30%)