Polyclonal expansion of TCR Vβ 21.3+ CD4+ and CD8+ T cells is a hallmark of multisystem inflammatory syndrome in children

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Multisystem inflammatory syndrome in children (MIS-C) is a delayed and severe complication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection that strikes previously healthy children. As MIS-C combines clinical features of Kawasaki disease (KD) and toxic shock syndrome (TSS), we aimed to compare the immunological profile of pediatric patients with these different conditions. We analyzed blood cytokine expression and the T cell repertoire and phenotype in 36 MIS-C cases, which were compared with 16 KD, 58 TSS, and 42 coronavirus disease 2019 (COVID-19) cases. We observed an increase of serum inflammatory cytokines (IL-6, IL-10, IL-18, TNF-α, IFN-γ, sCD25, MCP1, and IL-1RA) in MIS-C, TSS, and KD, contrasting with low expression of HLA-DR in monocytes. We detected a specific expansion of activated T cells expressing the Vβ21.3 T cell receptor β chain variable region in both CD4 and CD8 subsets in 75% of patients with MIS-C and not in any patient with TSS, KD, or acute COVID-19; this correlated with the cytokine storm detected. The T cell repertoire returned to baseline within weeks after MIS-C resolution. Vβ21.3+ T cells from patients with MIS-C expressed high levels of HLA-DR, CD38, and CX3CR1 but had weak responses to SARS-CoV-2 peptides in vitro. Consistently, the T cell expansion was not associated with specific classical HLA alleles. Thus, our data suggested that MIS-C is characterized by a polyclonal Vβ21.3 T cell expansion not directed against SARS-CoV-2 antigenic peptides, which is not seen in KD, TSS, and acute COVID-19.

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INTRODUCTION
At the end of April 2020, European clinicians warned public health agencies about an abnormal increase of Kawasaki-like diseases and myocarditis requiring critical care support in the context of the ongoing coronavirus disease 2019 (COVID-19) epidemic in children (1–3). American clinicians also reported a large outbreak of severe inflammation in children after COVID-19 infection, a condition that is now named pediatric inflammatory multisystemic syndrome or 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. MIS-C occurs 3 to 4 weeks after acute COVID-19 in children (3, 5–7).

To date, reports on MIS-C show slight differences in cytokine profiling and immunophenotype between MIS-C and KD or pediatric COVID-19 (8, 9). Analysis of T cells reveals 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 shows differences between acute COVID-19 and pre-pandemic KD (8, 11). A subset of activated CD8 T cells expressing the CX3C chemokine receptor (CX3CR1) is observed in MIS-C (12), and both CD8 and natural killer (NK) cells demonstrate an elevated expression of cytotoxicity genes (13). Anti–severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies (Abs) are equally produced in pediatric COVID-19 and MIS-C. Autoantibodies are uniquely found during MIS-C or KD, which supports the contribution of the humoral response to both diseases (8, 11). Last, a role for genetic factors is evoked in MIS-C pathogenesis because it occurs more frequently in Hispanic or African children (14–16). Despite these pioneer studies, the immunological mechanism underlying MIS-C remains unknown.

To address this question, we compared the immune profile of patients with MIS-C with that of patients with COVID-19 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 of 32 tested patients with MIS-C when assessed in the first month after onset. T cell receptor (TCR) sequencing revealed the polyclonal nature of the Vβ21.3+ expansion. No specific human leukocyte antigen (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. Together, our findings provide an immunological signature in MIS-C with potential implication in the diagnosis and treatment of this rare disease.

RESULTS
MIS-C presentation overlapped with TSS and KD
We took a cohort of 36 children with MIS-C and compared them with 16 KD cases diagnosed during and before the pandemic, 58 retrospective cases of patients with TSS, and 42 patients with acute COVID-19 (11 children and 31 adults). This comparison was motivated by previous descriptions of MIS-C in Europe and in the United States, showing a clinical overlap between staphylococcal toxin–mediated TSS and KD in patients with MIS-C (1–3). Figure 1A outlines the study flowchart and the clinical and biological parameters we evaluated. Patients diagnosed for MIS-C, classical KD, TSS, or acute COVID-19 were included. 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. Many patients in the MIS-C group also fulfilled some of the five major criteria for TSS and KD, respectively (Fig. 1B). Considering the clinical parameters, the most frequent features of patients with MIS-C in our cohort were fever, cardiac dysfunction, gastrointestinal symptoms, coagulopathy, and systemic inflammation (table S1). Additional clinical data are presented in table S2 for KD, TSS, and acute COVID-19 and in table S3 for all patients. Moreover, table S4 gives a list of the patients analyzed in each of the following figure panels.

High levels of proinflammatory cytokines in MIS-C contrasted with lymphopenia and low HLA-DR expression in monocytes
SARS-CoV-2 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 (IFN) production (17–20). Thus, a regulated production of cytokines is paramount for control of SARS-CoV-2 infection. This prompted us to investigate how cytokines could contribute to MIS-C pathogenesis. We compared the serum levels of IFN-α, IFN-γ, tumor necrosis factor–α (TNF-α), interleukin 10 (IL-10), soluble CD25 (sCD25), monocyte chemoattractant protein 1 (MCP1), IL-1 receptor antagonist (IL-1Ra), 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 S2 for a list of clinical features in the different patients’ groups).

The expression of IFN-stimulated genes (ISGs) in blood cells was significantly higher in MIS-C compared with controls but rather low compared with patients with COVID-19 (Fig. 2, A to C). The level of serum IFN-α2 followed the same trends, whereas serum IFN-γ was variable among patients with MIS-C, with very high levels in a few patients. The expression of the other cytokines measured (IL-6, IL-10, IL-18, TNF-α, MCP1, IL-1RA, and sCD25) was very high in patients with MIS-C compared with controls and very similar to that of patients with KD, TSS, and severe COVID-19 (Fig. 2, B and C). The level of sCD25 was significantly higher in patients with TSS than in patients with MIS-C and significantly lower in patients with severe COVID-19 than in patients with MIS-C (Fig. 2, B and C). A previous study found higher levels of serum IL-6 in patients with KD than in patients with MIS-C, contrasting with our data (8).

To further explore the MIS-C immunological profile, we quantified the number of peripheral lymphocytes of different types and the expression of HLA-DR in patients’ monocytes. T and NK cell counts were, on average, very low in patients with MIS-C and KD, whereas B cell counts were normal (Fig. 2D and fig. S1). We found a decreased expression of HLA-DR in monocytes in both patients with KD and MIS-C compared with controls (Fig. 2E and fig. S1). Together, 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 patients with MIS-C compared with controls.
Expansion of V\(\beta\)21.3\(^+\) peripheral T cells in a large fraction of patients with MIS-C

TSS toxin 1 (TSST1)–related TSS is associated with a skewing of the T cell repertoire toward V\(\beta\)2 as a result of TSST1 superantigen–induced proliferation of V\(\beta\)2\(^+\) T cells (21). Every other *Staphylococcus aureus* superantigenic toxin induces the expansion of specific TCR V\(\beta\) subsets, i.e., V\(\beta\)5.2, 5.3, 7.2, 9, 16, 18, and 22 for staphylococcal enterotoxin A (SEA) or V\(\beta\)3, 12, 13.2, 14, 17, and 20 for SEB (22). 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 V\(\beta\) subunits in T cells from patients with MIS-C, in comparison with patients with KD, TSS, and COVID-19 (Fig. 3A and fig. S2A). As expected, patients with TSS displayed the hallmark expansion of the V\(\beta\)2\(^+\) subset. Several V\(\beta\)–specific expansions were also visible in patients with MIS-C and, in most cases, V\(\beta\)21.3\(^+\) expansions (Fig. 3A) in both CD4 and CD8 T subsets (fig. S3A). These expansions had similar amplitudes as the V\(\beta\)2\(^+\) expansions in TSS (Fig. 3A). A principal components analysis (PCA) of the V\(\beta\) distribution in CD4 and CD8 T cells showed that the main parameters separating the different patients were the frequency of V\(\beta\)2\(^+\) and of V\(\beta\)21.3\(^+\) cells (fig. S3, B and C). Overall, the expansion of V\(\beta\)21.3\(^+\) T cell subsets was seen in 15 of 26 (58%) patients with MIS-C and in none of the other conditions analyzed by flow cytometry, i.e., 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 V\(\beta\) expression in peripheral blood mononuclear cells (PBMCs) 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 V\(\beta\)21.3\(^+\) T cell expansion is a hallmark of MIS-C because it was seen in 18 of 23 patients with MIS-C tested (fig. S3D). Thus, taking together flow cytometry and NanoString analyses, we found that 24 of 32 (75%) patients with MIS-C and none in the other clinical groups displayed *T cell receptor beta variable genes* 11-2 (TRBV11-2)/V\(\beta\)21.3\(^+\) expansions.

We then compared the level of serum cytokines between MIS-C patients with and without V\(\beta\)21.3\(^+\) T cell expansions at the time of the acute episode. The levels of IL-18 and IL-1RA (Fig. 3, C and D) were associated with the polyclonal V\(\beta\)21.3 expansions, but not those of the other cytokines tested (fig. S4, A and B), suggesting that V\(\beta\)21.3\(^+\) T cells were associated with the cytokine storm.

**TCR sequencing highlighted the polyclonal nature of TCR V\(\beta\)21.3 expansions**

To investigate the clonality of V\(\beta\)21.3\(^+\) expanded cells, we analyzed the TCR repertoire of 11 patients with MIS-C for whom whole-blood
**Fig. 2.** Systemic inflammation and signs of immune paralysis in patients with MIS-C. (A) Left: IFN score calculated as the normalized mean expression of six ISGs measured using the NanoString technology, as previously described (38, 45). Middle: Serum IFN-α, as measured with the Simoa technology. Right: Serum IFN-γ level measured by enzyme-linked immunosorbent assay (ELISA). (B) Serum levels of the indicated cytokines as measured by automated ELISA. (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. (E) HLA-DR expression in T cells and monocytes, as measured by flow cytometry in MIS-C. Gray shading indicates the derived central 95% HD (healthy donor) reference interval (D and E). See table S4 for subject numbers per panel. Statistical test: Kruskal-Wallis test between healthy donors and all other groups with adjustment for multiple comparisons using Benjamini-Hochberg correction (A and B) or between MIS-C and all other groups (C) with the same strategy. *P < 0.05, **P < 0.01, and ***P < 0.001. ns, not significant.
Most of the clonotypes are not abundant within each repertoire. Because the frequency of the clonotype type is represented as a gray line. Clonotypes using TRBV11-2 is shown (G), the CDR3 length distribution of with the TRBV11-2 gene. In (E) to (G) the same four patients and Methods are shown in red. In (C and D) Serum IL-18 (C) and IL-1RA (D) levels in MIS-C patients with or without (wo) Vβ21.3+ T cell expansions (exp). (E to G) Chord diagrams of the TRBV (bottom, gray) and TRBJ (top, blue) combinations assessed by TCR sequencing of TCR chains αβ. (A) Frequency of total CD3+ T cells expressing the indicated Vβ chains, as measured by flow cytometry using specific Abs against the corresponding Vβ within PBMCs of patients of the indicated group. Patients with TSS, mild COVID-19, pediatric COVID-19 (ped-COVID), KD, and MIS-C are colored in blue, pink, dark blue, orange, and green, respectively. Bubbles represent the normalized individual Vβ frequency reported to the mean frequency for each Vβ in the general adult population. (B) Normalized frequency of Vβ21.3+ T cells in different clinical conditions, as indicated. (C and D) Statistical test: Kruskal-Wallis test for each Vβ in the general adult population. (B) ***P < 0.001, ****P < 0.0001. (E) 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. (I) Annexin-V staining of T cells in the indicated patients’ groups. Results show the ratio of the annexin-V fluorescence in Vβ21.3+ versus Vβ21.3− T cells. See table S4 for subject numbers per panel. (B) Statistical test: Kruskal-Wallis test between MIS-C and all other groups with adjustment for multiple comparisons using Benjamini-Hochberg correction and (C, D, and I) unpaired Wilcoxon test comparing two groups. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
RNA was available by TCR sequencing. We analyzed the composition of the TCR β rearrangements involving the TRBV11-2 gene (which corresponds to Vβ21.3). First, by representing the TRBV11-2/ T cell receptor beta joining genes (TRBJ) combination usage as chord diagrams (Fig. 3, E and F), we confirmed the expansion of T cells using TRBV11-2 in 7 of 11 patients. These TRBV11-2 rearrangements were associated with multiple TRBJ genes, suggesting the polyclonal nature of the expansions. To further evaluate polyclonality, we analyzed the hypervariable sequence CDR3 length distribution of TRBV11-2 clonotypes (bar plots, Fig. 3, E to G). The CDR3 size distributions showed a bell-shaped Gaussian distribution as expected in polyclonal repertoires (23–25). 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 Materials and Methods and fig. S5A). No major monoclonal expansions (red lines in the CDR3 spectra) explaining the global TRBV11-2 expansion were detected. Instead, most of the clonotypes were found at low frequencies (gray 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 these 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, on average, 0.51% of the total repertoire. Last, these limited expansions represented, on average, 4.47% of the TRBV11-2 repertoire in patients with TRBV11-2 expansions and 6.31% in patients without TRBV11-2 expansions (table S6 and fig. SSB). To confirm the polyclonality of the TRBV11-2 expansion, we computed the Berger-Parker index (BPI) on TRBV11-2 clonotype for patients with MIS-C harboring or not TRBV11-2 expansions (fig. S5C). This index measures the proportional abundance of the most frequent clonotypes within TRBV11-2 clonotypes. There were 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 seven patients were not explained by monoclonal expansions.

Next, to address whether the Vβ21.3+ T cell expansion persisted overtime, 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 Fig. 3 (F to H), the Vb21.3/TRBV11-2 distributions for all the patients returned to normal within days to weeks after MIS-C. When we compared the CDR3 length distributions by calculating the perturbation score using the ISEApeaks tool between reper- tories 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. SSD). Last, this transient expansion suggested a proapoptotic phenotype of Vβ21.3+ T cell. To test this hypothesis, we stained PBMCs from patients with MIS-C 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 and fig. S2B), which substantiated our hypothesis.

Vβ21.3+ T cells had an activated phenotype but did not react against SARS-CoV-2 peptides

Because Vβ21.3+ T cells expand in patients with MIS-C, 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 with those without expansions and to healthy controls (Fig. 4, A and B). 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 Vβ21.3 expansions compared with those without Vβ21.3 expansions (Fig. 4, C and D). A recent paper reports a specific activation of CX3CR1+ CD4 and CD8 T cells in patients with MIS-C, as assessed by HLA-DR/CD38 levels (12). This prompted us to measure CX3CR1 levels in Vβ21.3+ T cells. As shown in Fig. 4E and fig. S6A, Vβ21.3+ T cells overexpressed CX3CR1 in both CD4 and CD8 T cells in MIS-C patients with Vβ21.3+ expansions compared with those without expansions, although the percentage of CX3CR1+ cells was not higher in MIS-C than in control patients (fig. S7A). Moreover, in patients with MIS-C, a large frequency of non-naïve CX3CR1+ CD4 and CD8 T cells had an activated phenotype as previously reported (fig. S7B) (12).

Given that MIS-C came about weeks after COVID-19, we wondered whether Vβ21.3+ T cells were raised against SARS-CoV-2 antigens. To test this possibility, we stimulated PBMCs from patients with MIS-C or convalescent COVID-19 with a commercial cocktail of SARS-CoV-2 peptides spanning S, N, and M viral proteins. T cells from patients with MIS-C responded poorly to stimulation with viral peptides, regardless of Vβ21.3 expansion, compared with cells from patients with convalescent COVID-19 that responded well (Fig. 4, F and G, and fig. S6, B and C). This was not due to a lack of adaptive anti–SARS-CoV-2 response, because all patients with MIS-C tested had high SARS-CoV-2–specific Ab levels (fig. S7, C and E). Last, 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 patients with MIS-C (table S4). Together with the lack of Vβ21.3+ expansion in patients with COVID-19, these data showed that Vβ21.3+ T cells were not specific for HLA-restricted SARS-CoV-2 peptides. Together, these data revealed that the Vβ21.3+ CD4 and CD8 T cell expansion were highly activated and expressed CX3CR1 but had poor responsiveness to SARS-CoV-2 antigens.

DISCUSSION

Here, we confirmed the strong overlap in clinical phenotype between KD, MIS-C, and TSS; MIS-C and TSS had similar defining features, specifically cardiac dysfunction, hypotension, maculopapular skin rash, and conjunctivitis. We recently identified the critical importance of early steroid therapy in the management of MIS-C, similarly to what has been previously shown in TSS (26, 27). MIS-C and TSS are obviously linked to infections, whereas many KD features suggest an infectious cause for KD as well (28). The epidemic of a novel coronavirus in 2005 (New Haven) was associated to KD and linked the viral infection to vascular inflammation (29).

We found important similarities in terms of cytokine expression between MIS-C, TSS, and KD, such as high TNF-α, IL-6, IL-18, and IL-1RA levels. A previous study noted that a subgroup of patients with severe MIS-C had higher levels of IFN-γ, IL-18, GM-CSF (granulocyte-macrophage colony-stimulating factor), RANTES (regulated upon activation, normal T cell expressed and secreted), IP-10 (interferon gamma-induced protein 10), IL-1α, and SDF-1 (stromal cell–derived factor 1) than patients with mild MIS-C or KD (30). We also observed a subset of patients with MIS-C with high serum...
IFN-γ, IL-18, and sCD25. 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.

Here, we reported the expansion of a TCR Vβ21.3+ T cell subset with an activated phenotype in as many as 75% of patients with MIS-C. Vβ21.3+ T cell expansions were also reported in smaller numbers of patients with MIS-C in two recent studies (13, 31). In both Porritt et al. (31) 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 further showed that Vβ21.3+ CD4 and CD8 T cell expansions are a discriminating feature of patients with MIS-C compared with patients with KD, TSS, and COVID-19.
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 (31) and indicating that Vβ21.3+ T cell expansions were associated with the cytokine storm. Our data also showed 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 (12). The CX3CL1–CX3CR1 axis is thought to have an important role in vascular inflammation in different inflammatory diseases (32) 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 patients with MIS-C (13).

We demonstrated that both TSS and MIS-C were 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. The amplitude of the expansion was also similar in both syndromes. Considering the additional clinical phenotype similarities between MIS-C and TSS shown in this study, 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 (33). Superantigens bind external regions of TCR and MHC molecules (34) and can induce massive expansions of T cells expressing one specific TCR Vβ chain, whereas classical antigens induce the expansion of T cells bearing different Vβ. Previous papers have suggested that the SARS-CoV-2 spike protein could behave as a superantigen structure (35). Using in silico modeling, Porritt et al. identified a putative interaction between Vβ21.3 and a superantigen-like motif in spike. However, Vβ21.3+ T cell expansions occur in a delayed manner relative to SARS-CoV-2 infection, and the virus is often undetectable in patients with MIS-C 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 Abs. One can hypothesize that immune complexes composed of SARS-CoV-2 bound to Abs may act as superantigen structures. However, a previous study failed to detect these immune complexes in patients with MIS-C (30). In addition, Vβ-restricted T cells adhere to endothelial cells after superantigen activation (36), and, thus, the CX3CR1+ Vβ21.3–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 patients with MIS-C, some of which directed against endothelial antigens (8, 11), whereas others have reported immune events consistent with autoimmunity, such as the expansion of proliferating plasmablasts (13) or the persistence of functional SARS-CoV-2–specific monocyte-activating Abs (37). 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.

Last, given the rarity of MIS-C, there could be a genetic susceptibility to this postinfectious disease, promoting hyperinflammatory reaction of adaptive immunity in response to SARS-CoV-2 (16). We limited our analysis to classical HLA alleles but did not find any significant association, although a previous study reported an HLA-I bias in a smallest group of patients with MIS-C (31). Our MIS-C samples were obtained in most of cases after anti-inflammatory treatments (see table S3), and it is likely that those treatments affect the level of serum cytokines, which could have affected the comparisons we made between clinical conditions and the associations between cytokines and T cell expansions. Together, MIS-C shared clinical and immunological anomalies with KD and TSS but was specifically characterized by a polyclonal Vβ21.3 expansion in CD4 and CD8+ T cells associated to activation and CX3CR1 expression.

**MATERIALS AND METHODS**

More information for all of these protocols can be found in the Supplementary Materials.

**Study design and human subjects**

The immunological profiles of 36 MIS-C, 16 KD, 58 TSS, and 42 non–MIS-C COVID-19 cases were included (Fig. 1A). Samples were collected within the first week of symptoms and analyzed for cytokine immunoprofiling, standard immunophenotyping, Vβ expression, TCR sequencing, and SARS-CoV-2–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 experiment are detailed in table S4. The main clinical features are summarized in tables S1 to S3. Written informed consent was obtained for all data collection and blood sampling as detailed in the Supplementary Materials.

**Immunological analyses**

**Cytokines and IFN score assessment**

Plasma concentrations of IL-6, TNF-α, IFN-γ, IL-10, IL-18, MCP-1, IL-1RA, and sCD25 were measured by Simple Plex technology using an enzyme-linked lectin assay instrument (ProteinSimple). Plasma IFN-α concentrations were determined by single-molecule array (Simoa) on an HD-1 analyzer (Quanterix) using a commercial kit for IFN-α2 quantification (Quanterix). RNA was extracted from whole blood, and IFN score was obtained using nCounter analysis technology (NanoString Technologies) by calculating the median of the normalized count of six ISGs as previously described (38).

**T cell Vβ repertoire analysis and immunophenotyping**

PBMCs were stained with surface markers, CD3, CD4, CD8, CD14, CD16, CD19, CCR7, CD38, Vβ21.3, HLA-DR, CX3CR1, and CD45RA (further details on these stains are included in the Supplementary Materials). Cell apoptosis was assessed by annexin-V staining. All samples were acquired on a BD LSRFortessa (BD Biosciences) flow cytometer and analyzed using FlowJo version 10 software. Monocyte HLA-DR expression was determined on EDTA-anticoagulated peripheral whole blood as previously described (39). The phenotypic analysis of T cell Vβ repertoire was performed on whole blood sample using the IOTest Beta Mark kit (Beckman Coulter). Whole-blood cells were stained for CD3, CD4, CD8, and each combination of three fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, and FITC/PE–conjugated anti–Vβ monocolonal Abs (mAbs) (Beckman Coulter) in eight sample tubes. Expansions were defined, respectively, for values above the mean ± 2 SD or below the minimum reference values of the corresponding family. Additional samples were analyzed for TRBV from total RNA with NanoString technology (Supplementary Materials).
Stimulation with SARS-CoV-2 overlapping peptide pools and flow cytometry

PBMCs were stimulated with SARS-CoV-2 PepTivator pooled S, N, and M peptides (Miltenyi Biotec) at a final concentration of 2 μg ml⁻¹ for 1 hour in the presence of 2 μg ml⁻¹ of mAbs CD28 and CD49d and then for an additional 5 hours with GolgiPlug and GolgiStop (BD Biosciences). Similar surface markers were stained. Cells were then washed, fixed with Cytofix/Cytoperm (BD Biosciences), and stained with V450-conjugated anti–IFN-γ. All samples were acquired on a BD LSFortessa (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 Abs against the receptor binding domain (RBD) of the S protein, the bioMérieux VIDAS assay detecting antibody responses to the bioPDCD and bioPEPdcD, and the Abbott ARCHITECH assay detecting IgG to the RBD, and the Abbott ARCHITECH assay detecting IgG to the RBD.

Statistical analyses

All tests were performed two sided with a nominal significance threshold of P < 0.05. We used nonparametric tests appropriated to the low number of observations in each of our experimental conditions, i.e., the Wilcoxon or Kruskal-Wallis test depending on whether we have two or more conditions to compare, respectively. Multiple comparisons performed with the Dunn’s all-pairs comparison for Kruskal-type ranked data were corrected by the false discovery rate method of Benjamini-Hochberg (44). PCA was made in R with stats package and visualized with ggplot2 (43) for Vβ frequencies obtained by flow cytometry. All statistical analyses were performed using GraphPad with the help of a professional biostatistician.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/6/59/eabh1516/DC1

Methods

Figs S1 to S7
Tables S1 to S7

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES


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Multisystem inflammatory syndrome in children previously infected with SARS-CoV-2, with similar features to Kawasaki disease (KD) and toxic shock syndrome (TSS). It is still unclear what immunologic correlates differentiate MIS-C from KD and TSS. Here, Moreews et al. looked at the circulating T cell repertoire and phenotype of 36 patients with MIS-C, 16 with KD, 58 with TSS, and 42 with COVID-19. They found that 75% of patients with MIS-C, and none from the other groups, expressed the Vβ21.3 T cell receptor β chain variable region in both CD4 and CD8 T cells. These cells had an activated and vascular patrolling phenotype but were not specific to SARS-CoV-2. Together, this work shows unique T cell responses in patients with MIS-C after convalescence.