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Deep immunophenotyping reveals biomarkers of multisystemic inflammatory syndrome in children in a Latin American cohort

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Background: Multisystemic inflammatory syndrome in children (MIS-C) is a life-threatening disease that occurs 2-5 weeks after severe acute respiratory syndrome coronavirus 2 exposure and is characterized by severe multisystemic inflammation. Early recognition of MIS-C is key to prognosis; therefore, establishing clinical and laboratory biomarkers that predict complications is urgently needed.

Objective: We characterized the immune response and clinical features of patients with acute MIS-C and determined biomarkers of disease in a cohort of 42 Latin American patients.

Methods: Immune characterization was performed using flow cytometry from peripheral mononuclear cells and severe acute respiratory syndrome coronavirus 2–specific humoral and cellular response was performed using flow cytometry, enzyme-linked immunospot, enzyme-linked immunosorbent assay, and neutralizing antibody assays.

Results: MIS-C is characterized by robust T-cell activation and cytokine storm. We uncovered that while C-X-C motif chemokine ligand (CXCL) 9, IL-10, CXCL8, CXCL10, IL-6, and IL-18 are significantly elevated in patients with shock, while CCL5 was increased in milder disease. Monocyte dysregulation was specifically associated with KD-like MIS-C. Interestingly, MIS-C patients show a natural killer cell degranulation defect that is persistent after 6 months of disease presentation, suggesting it could underlie disease susceptibility. Most MIS-C had gastrointestinal involvement, and higher levels of neopterin were identified in their stools, potentially representing a biomarker of intestinal inflammation in MIS-C. Severe acute respiratory syndrome coronavirus 2–specific cellular response and neutralizing antibodies were identifiable in convalescent MIS-C patients, suggesting sustained immunity.

Conclusion: Clinical characterization and comprehensive immunophenotyping of Chilean MIS-C cohort provide valuable insights in understanding immune dysregulation in MIS-C and identify relevant biomarkers of disease that could be used to predict severity and organ involvement. (J Allergy Clin Immunol 2022;150:1074-85.)

Key words: COVID-19, inflammation, multisystemic inflammatory syndrome in children, biomarkers, NK cell deficiency

Multisystemic inflammatory syndrome in children (MIS-C) is a rare but life-threatening condition that occurs in children or adolescents at 2 to 5 weeks after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposure. MIS-C is defined by systemic hyperinflammation with multiple organ involvement including gastrointestinal (GI), cardiac, dermatologic, respiratory, renal, and neurologic symptoms that may lead to multiorgan failure. Different yet overlapping clusters of phenotypic presentations have been defined for MIS-C; some patients present with cutaneous involvement resembling Kawasaki disease (KD), while others present with GI symptoms and shock. Early recognition of MIS-C is key for appropriate treatment and successful outcome. However, diagnosis is often challenging as a result of clinical overlap with non–SARS-CoV-2 KD, sepsis, and other infectious conditions. Additionally, endemic circulation of SARS-CoV-2, cessation of lockdowns, and vaccination has made it harder to identify coronavirus disease 2019 (COVID-19) contacts and interpret serology. In this context, identifying biomarkers for MIS-C is key to facilitate differential diagnosis.

Severity of disease is defined by the degree of myocardial involvement and shock, occurring in 80% and 50% of patients, respectively. Overall, 60% of MIS-C patients require intensive care unit (ICU) admission and vasoactive support. Laboratory parameters including decreased platelets and lymphocytes, and increased C-reactive protein, d-dimer, troponin, pronatriuretic peptide test, ferritin, and IL-6 levels can predict severity; however, they are still nonspecific.
Multidimensional immune studies of MIS-C that compare it to KD and COVID-19 in adults reveal that although they are all characterized by hyperinflammation, MIS-C is a unique entity with higher IL-6, CXCL9, and CXCL10 levels. Studies characterizing the immune response in MIS-C have shown reduced numbers of CD4⁺ and CD8⁺ T cells, natural killer (NK) cells, and γδ T cells overall in MIS-C patients. Earlier work has correlated immune profiles and clinical manifestations of pediatric and adult COVID-19, but biomarkers for of MIS-C and its different clinical manifestations have not been clearly defined. Similarly, KD-like MIS-C cases are clinically well defined; however, immune mechanisms underlying this specific form of MIS-C are still poorly understood. Characterizing the immune response in the wide disease spectrum of MIS-C is paramount for further understanding of disease, and most importantly to allow early identification of patients who will require more complex or targeted interventions. Comprehensive immune studies in African and Hispanic children are lacking and may contribute to our understanding of genetic and environmental components that may explain the higher incidence observed in these populations. We characterized the immune response and clinical features of patients with acute MIS-C and determined biomarkers of disease in a cohort of 42 Latin American patients in 3 clinical centers in Chile.

**METHODS**

**Ethical statements**

This study was approved by ethical committee of the Facultad de Medicina Clínica Alemana Universidad del Desarrollo. All participants or legal guardians provided written informed consent in accordance with the Declaration of Helsinki.

**Subjects**

A total of 67 patients admitted with suspected diagnosis of MIS-C (June 2020 to June 2021) were recruited in the city of Santiago de Chile from 3 clinical centers: Roberto del Río Pediatric Hospital, Dr Exequiel González Cortés Pediatric Hospital, and Clínica Alemana de Santiago. Clinical information was obtained 6 months after disease onset. Most patients were recruited to compare with MIS-C. Blood and stool samples were obtained from patients with alternative febrile conditions, and we identified them as febrile control subjects. In addition, 21 young adults hospitalized with COVID-19 pneumonia were recruited to compare with MIS-C. Blood and stool samples were obtained from most patients within 7 days of admission, and follow-up blood samples were obtained 6 months after disease onset.

**Immune cell phenotyping by flow cytometry**

To evaluate functionality of different immune cells, flow cytometry was performed from fresh blood or frozen peripheral mononuclear cells (PBMCs) previously isolated with Histopaque (Sigma-Aldrich, St Louis, Mo). Three flow cytometry panels were performed: (1) NK and T-cell functionality, (2) memory and activation T cells, and (3) monocytes. More information is provided in the Methods and Figs E1 to E4 in the Online Repository at www.jacionline.org. For T-cell and NK cell functional characterization, cells were stimulated with 1 μg/mL of phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL of ionomycin with

**Abbreviations used**

AC2: Angiotensin-converting enzyme 2

CCL: Chemokine (C-C motif) ligand

COVID-19: Coronavirus disease 2019

CXCL: Chemokine (C-X-C motif) ligand

ELISA: Enzyme-linked immunosorbent assay

ELISPOT: Enzyme-linked immunospot

GFP: Green fluorescent protein

GI: Gastrointestinal

ICU: Intensive care unit

KD: Kawasaki disease

MAS: Macrophage activation syndrome

MIS-C: Multisystemic inflammatory syndrome in children

NK: Natural killer

PBMC: Peripheral mononuclear cells

PMA: Phorbol 12-myristate 13-acetate

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

UMAP: Uniform manifold approximation and projection

VSV: Vesicular stomatitis virus

**TABLE I. Clinical characteristics of Chilean MIS-C cohort with 42 patients**

| Characteristic | No. | % |
|---------------|-----|---|
| Clinical manifestations | | |
| Fever | 41 | 98 |
| Shock | 25 | 60 |
| Cardiac involvement | | |
| Coronary dilation | 26 | 62 |
| Pericardial effusion | 5 | 12 |
| Myocarditis | 17 | 40 |
| Ventricular dysfunction | 15 | 36 |
| (ejection fraction <55%) | | |
| Cutaneous involvement | 22 | 52 |
| MAS | 1 | 2 |
| GI involvement | 38 | 90 |
| KD-like | 28 | 67 |
| Renal (AKI) | 11 | 26 |
| Neurologic | 16 | 38 |
| Respiratory | 8 | 19 |
| Death | 1 | 2 |
| Support | | |
| ICU | 33 | 79 |
| IMV | 14 | 33 |
| ECMO | 1 | 2 |
| Inotropes | 17 | 40 |
| Treatment | | |
| IVIG | 32 | 76 |
| Solumedrol | 34 | 81 |
| Oral prednisone | 32 | 76 |
| Tocilizumab | 3 | 7 |
| Infliximab | 1 | 2 |
| Heparin | 32 | 76 |
| Aspirin | 31 | 74 |
| SARS-CoV-2⁶ | | |
| Positive PCR | 9 | 21 |
| Indeterminate PCR | 3 | 7 |
| Positive IgG or IgM (serology) | 38 | 90 |
| Positive PCR or serology | 42 | 100 |

⁵No PCR result available for 1 patient; his mother had positive PCR results.

⁶AKI, Acute kidney injury; ECMO, extracorporeal membrane oxygenation; IMV, invasive mechanical ventilation; IVIG, intravenous immunoglobulin.

Of exposure, nasopharyngeal PCR, and specific SARS-CoV-2 spike protein IgG antibodies. If an alternative diagnosis was established during follow-up, such patients were excluded from analysis (n = 42) (see Table E1 in this article’s Online Repository at www.jacionline.org). A subgroup of these patients was diagnosed with alternative febrile conditions, and we identified them as febrile control subjects. In addition, 21 young adults hospitalized with COVID-19 pneumonia were recruited to compare with MIS-C. Blood and stool samples were obtained from most patients within 7 days of admission, and follow-up blood samples were obtained 6 months after disease onset.
After 5 hours, dead cells were stained using LIVE/DEAD Fixable Near-IR, permeabilized with BD Cytofix/Cytoperm kit (BD), and stained for intracellular markers. When analyzing flow cytometry data, investigators were unaware of both clinical features and clinical laboratory data.

**Measurement of serum cytokine and chemokine levels**

Serum was isolated by centrifugation, stored at $-80^\circ$C, and thawed for cytokine assessment using BD Cytometric bead array human Th1/Th2 cytokine kit, human inflammatory cytokine kit, and human chemokine kit according to
the manufacturer’s instructions. Samples were acquired on Cytoflex LX flow cytometer and analyzed by FlowJo 9.1 software (Treestar, Ashland, Ore). To evaluate CXCL9, IL-18, and IL-18 binding protein, commercially available enzyme-linked immunosorbent assays (ELISAs) were used according to the manufacturer’s instructions (catalog DY392, DY318-05, and DY119; R&D Systems, Minneapolis, Minn). Free IL-18 levels were calculated considering the law of mass action, as described elsewhere.29,30

**Determination of neopterin and ACE2 in stool samples**

Stool samples were stored at −80°C and later thawed, vortexed with 0.9% saline, and centrifuged. The supernatant was used to assess fecal concentrations of neopterin and angiotensin-converting enzyme 2 (ACE2) according to the manufacturer’s instructions (RE59321, IBL International, Hamburg, Germany; DY933-05, R&D Systems). Wet stool weight was used for normalization.

**SARS-CoV-2–specific cellular immune response**

Patient PBMC samples obtained 6 months after MIS-C or acute COVID-19 presentation were thawed and stimulated with 50 ng/mL of SARS-CoV-2 spike protein for 24 hours. PMA/ionomycin and diluent of spike protein were added as positive and negative controls, respectively. For enzyme-linked immunosorbent spot (ELISPOT) human IFN-γ single-color ELISPot analyses (ImmunoSpot, Cellular Technology Limited [CTL], Shaker Heights, Ohio) were performed according to the manufacturer’s instructions. To determine cellular immune response in different lymphocyte subsets, we used flow cytometry (see the Methods and Fig E4 in the Online Repository at www.jacionline.org).

**Measurement of IgG and neutralizing antibodies**

ELISA was performed as previously described.31 Microtiter plates were coated with 1 μg/mL of SARS-CoV-2 spike protein overnight at 4°C. Each sample was analyzed in duplicate, and the cutoff was set as the mean value of negative controls (healthy donor pre-pandemic serum specimens) plus 3 standard deviations.

Neutralizing antibodies were measured using vesicular stomatitis virus (VSV)-green fluorescent protein (GFP)-Spike SARS-CoV-2.32 Serially diluted serum previously incubated with pseudovirus VSV-GFP-Spike SARS-CoV-2 was transferred into a Vero cell monolayer at a final multiplicity of infection of 0.5 and incubated at 37°C 5% CO₂ for 18 to 20 hours. The infection was measured in each well by determining GFP fluorescence intensity using a Cytomics plate reader (Agilent, Santa Clara, Calif). Half-maximal inhibitory concentration was calculated by nonlinear regression analysis.

**Statistical analysis**

Statistical analyses were performed by GraphPad Prism 9.1.0 software (GraphPad Software, La Jolla, Calif). A correlation matrix was created by nonparametric Spearman test, with a confidence interval of 95%. Each correlation was done independently between 2 variables, with no multiple comparison correction because of the small sample size. Immune parameters were compared among MIS-C patients, COVID-19 patients, febrile controls, and healthy donors by nonparametric Mann-Whitney tests. To compare immune parameters between patients, volcano plots were created for each relevant clinical manifestation representing all parameters simultaneously. The volcano plots represent Mann-Whitney tests performed separately for each parameter; we did not apply multiple comparison correction because of the small sample size.

**RESULTS**

Forty-two MIS-C patients were included for analysis. All patients were Latin American (Venezuela, Peru, and Chile) residing in Chile, 55% were male, and mean age was 7 years. Seventy-nine percent of patients required ICU admission (1-10 days of stay), 33% invasive mechanical ventilation, and 40% inotropic support. Most patients had fever and GI involvement (Table I; see Table E2 in the Online Repository at www.jacionline.org). Sixty percent had shock, while 62% showed cardiovascular involvement (Fig 1, A). Sixty-seven percent had KD-like symptoms (Fig 1, B), and 68% of these patients also presented with shock. Patients were treated with intravenous immunoglobulin (76%), oral (76%) and intravenous corticosteroids (81%), tocilizumab (7%) and infliximab (2%) according to local treatment guidelines.33 After 12 months’ follow-up, most patients survived with no sequelae; only 1 patient died during acute illness with macrophage activation (MAS) and cardiac failure, 1 patient showed a persistent coronary aneurism, and 1 patient was diagnosed with Crohn disease immediately after MIS-C.

**MIS-C patients are characterized by T-cell activation, elevated inflammatory cytokines, and functional NK cell defect**

We sought to understand immunopathogenesis and identify biomarkers for MIS-C using multiparametric flow cytometry and serum cytokines and chemokines in the acute phase of disease and compared to severe COVID-19 patients and febrile controls (Fig 2).

While acute COVID-19 and MIS-C both have reduced proportions of CD4⁺ and CD8⁺ memory T cells, MIS-C showed a higher proportion of activated T cells (CD4⁺CD69⁺ and CD8⁺CD69⁺) (Fig 2, A-C). Characterization of monocytes in our cohort revealed a heterogenous distribution of monocyte subsets in MIS-C, while COVID-19 patients showed significantly higher proportions of classical monocytes, in line with previous findings16,34 (Fig 2, D). Although monocyte distribution was heterogenous, we identified a lower expression of HLA-DR in nonclassical monocytes (MIS-C patients (Fig 2, E), possibly contributing to impaired immune homeostasis in this acute condition.35 While cytokine dysregulation has been identified in MIS-C,14 we found that MIS-C is distinguished from COVID-19 by significantly higher levels of IL-6, IFN-γ, IL-10, chemokine ligand (CCL) 2, CXCL8, CXCL9, and CXCL10. Even though total IL-18 was higher in MIS-C, free IL-18 was significantly lower than in COVID-19 (Fig 2, F). TNF-α, IL-5, IL-4, and IL-2 were undetectable in all patients (data not shown). Taken together, these data suggest MIS-C is distinguished from COVID-19 by substantial activation of T cells and nonclassical and intermediate monocytes, together with a proinflammatory cytokine and chemokine storm.

Differentiating MIS-C from other inflammatory conditions in children is challenging, given the wide range of differential diagnosis in pediatric patients and the often unclear history of COVID-19 exposure. Furthermore, with vaccination, positive serology becomes difficult to interpret. To address this point, we compared MIS-C with other febrile conditions. We found that although both patient groups were characterized by strong T-cell activation, MIS-C was distinguished by higher expression of IFN-γ in CD4⁺ T cells and higher levels of IL-10, CXCL8, and CCL2, as well as distinctively even higher levels of CXCL9 and CXCL10, suggesting that IFN-γ signature is a biomarker of MIS-C, as previously suggested (Fig 2, F).16,37

Interestingly, MIS-C patients showed decreased NK cell numbers and NK cell degranulation measured by CD107a
FIG 2. T-cell activation, NK cell defect, and elevated inflammatory cytokines in MIS-C patients. (A) Memory T cells evaluated in blood using flow cytometry in MIS-C (n = 28), COVID-19 (n = 21), HD (n = 6), and FC (n = 14). (B) T-cell activation evaluated in blood using flow cytometry using HLA-DR T marker for MIS-C (n = 28), COVID-19 (n = 21), HD (n = 6), and FC (n = 14). (C) T-cell cytokine expression evaluated using flow cytometry in MIS-C (n = 19), COVID-19 (n = 21), HD (n = 6), and FC (n = 12). (D) Monocyte subsets evaluated in blood using flow cytometry. (E) HLA-DR T cell subsets: Classical, Intermediate, and Non-classical. (F) Inflammatory cytokines: IL-18, Free IL-18, IL-6, IL-10, IFN-γ, CXCL8, CXCL9, CXCL10, CCL2, CCL5. (G) NK cell cytotoxicity and cytokines: Perforin, CD107a, IFN-γ, TNF-α.
expression after PMA/ionomycin stimulation (Fig 2, G). This reduction was independent of NK cell numbers. While degranulation improved after 6 months, convalescent MIS-C patients still exhibited lower CD107a expression than healthy controls, suggesting that these children may have an underlying functional NK cell defect.

Cytokine storm correlates with lower platelets and disease severity in MIS-C

As we and others have observed, MIS-C is characterized by increased proinflammatory cytokines including IL-6, IL-18, IFN-γ, and IL-17A. However, comprehensive understanding of the correlation between immune perturbations, cytokines, and clinical or laboratory parameters has not been clearly elucidated. To determine biomarkers for severity, we studied the correlation of chemokine and cytokine profiles with clinical manifestations, clinical laboratory parameters, and multiparametric immune cell characterization, establishing one-on-one comparisons using nonparametric Spearman correlation. Furthermore, these results were contrasted with COVID-19 patients and febrile controls to determine whether the identified associations were unique to MIS-C.

As expected, we identified T-lymphocyte activation markers (CD4+CD69+, CD8+CD69+, CD4+HLA-DR+, CD8+HLA-DR+), correlated with increased lymphocyte–cytokine expression (CD4+IFN-γ+, CD8+IFN-γ+, CD4+TNF-α+, CD8+TNF-α+) (Fig 3, A). Interestingly, we observed a correlation between higher levels of CCL5, a lower cytokine milieu, and higher platelet numbers and white blood cell count that was not identified in febrile controls or patients with COVID-19, suggesting that this is a unique feature of MIS-C (Fig 3, A; see Fig E5 and E6 in the Online Repository at www.jacionline.org). Patients with shock showed significantly higher levels of CXCL9, IL-10, CXCL8, CXCL10, IL-6, and IL-18, as well as lower levels of free IL-18 (Fig 3, A and B; see Fig E7 in the Online Repository). Overall, these data suggest MIS-C is characterized by T-cell activation and cytokine storm that determines severity.

Biomarkers of different clinical features and organ involvement in MIS-C

To identify biomarkers of different MIS-C phenotypes, we studied the correlation between immune parameters and specific clinical manifestations including specific organ involvement or KD-like symptoms. A previous study comparing MIS-C with Kawasaki disease revealed lower platelet numbers in MIS-C patients, which could be associated with a downregulation of ACE2 levels in their stools than COVID-19 patients. We found that higher levels of neopterin correlated with lower levels of ACE2 in MIS-C patients, which could be associated with a downregulation of ACE2 after SARS-CoV-2 exposure. In addition, we found that higher neopterin levels in stools were associated with mixed cardiac shock, suggesting a link between intestinal inflammation and cardiac dysfunction (Fig 4, D).

Convalescent MIS-C patients show lower IFN-γ+ memory T cells and higher titer of neutralizing antibodies than convalescent COVID-19 patients

Cellular immunity is crucial to provide long-term protection; it is thus important to determine if convalescent MIS-C patients develop sustained cellular immune responses to SARS-CoV-2. To determine T-cell–specific responses, we performed ELISpot and flow cytometry in PBMC stimulated with SARS-CoV-2 protein and compared it to convalescent COVID-19 pneumonia patients 6 months after disease presentation. Because memory T-cell subsets change with age, we included unvaccinated age-matched controls who were recruited at the beginning of the pandemic (most probably naïve to SARS-CoV-2). While we identified SARS-CoV-2–specific memory T cells in both MIS-C and COVID-19 patients, we did not find a correlation between any immune parameter and GI involvement. ACE2 serves as a receptor for SARS-CoV-2 entry, and although a higher expression of ACE2 in children’s GI tract has been proposed, the mechanisms underlying GI involvement in MIS-C are poorly understood. Neopterin is released by macrophages upon IFN stimulation and is involved in redox reactions. Increased neopterin levels in patient serum and feces are associated with severity in COVID-19. Our results show that MIS-C patients have significantly higher neopterin levels and trend to higher ACE2 levels in their stools than COVID-19 patients. We found that higher levels of neopterin correlated with lower levels of ACE2 in MIS-C patients, which could be associated with a downregulation of ACE2 after SARS-CoV-2 exposure. In addition, we found that higher neopterin levels in stools were associated with mixed cardiac shock, suggesting a link between intestinal inflammation and cardiac dysfunction (Fig 4, D).

**Observations showing an association between reduced platelets and disease severity in MIS-C as well as in COVID-19.**

While higher IL-6, IL-10, CXCL9, and CXCL10 correlated with pericardial effusion (Fig 4, A), no significant associations were identified for heart failure, defined as ejection fraction below 55%. Neurologic and renal involvement were associated with higher IL-1B and higher perforin levels in NK cells, respectively. We did not identify biomarkers for respiratory involvement, probably as a result of the low frequency of respiratory symptoms in our cohort (Fig 4, B). Biomarkers of neurologic and renal involvement found in MIS-C differed between COVID-19 patients and febrile controls, suggesting that these correlations are exclusive for MIS-C (see Figs E5 and E6 in the Online Repository at www.jacionline.org).

We identified that KD-like MIS-C is characterized by higher HLA-DR+ expression in nonclassical monocytes, higher CD69+ and IFN-γ+ expression in CD8+ T cells, and higher CXCL8, a chemokine with a potent chemotactic activity for monocytes and neutrophils (Fig 4, C; see Fig E7 in the Online Repository at www.jacionline.org).

GI involvement was present in 90% of our cohort; however, we did not find a correlation between any immune parameter and GI involvement. ACE2 serves as a receptor for SARS-CoV-2 entry, and although a higher expression of ACE2 in children’s GI tract has been proposed, the mechanisms underlying GI involvement in MIS-C are poorly understood. Neopterin is released by macrophages upon IFN stimulation and is involved in redox reactions. Increased neopterin levels in patient serum and feces are associated with severity in COVID-19. Our results show that MIS-C patients have significantly higher neopterin levels and trend to higher ACE2 levels in their stools than COVID-19 patients. We found that higher levels of neopterin correlated with lower levels of ACE2 in MIS-C patients, which could be associated with a downregulation of ACE2 after SARS-CoV-2 exposure. In addition, we found that higher neopterin levels in stools were associated with mixed cardiac shock, suggesting a link between intestinal inflammation and cardiac dysfunction (Fig 4, D).
**Fig 3.** Cytokine storm correlates with lower platelets and disease severity in MIS-C. (A) Heat map of all parameters evaluated in MIS-C patients using Spearman correlation. Number of MIS-C samples tested for each parameter is shown in Fig 2. (B) Volcano plot showing differences of parameters evaluated with MIS-C patients with and without shock; each dot represents 1 parameter. Number of MIS-C samples tested for each parameter is shown in Fig 2. Significant P values are shown above the blue line with red dots. Comparison of each parameter was done using Mann-Whitney test; P < .05 was considered significant. COVID-19, Acute adult COVID-19 patients; HD, healthy donors; MIS-C, acutely ill MIS-C patients.
COVID-19 convalescent patients (Fig 5, A), MIS-C patients showed consistently lower levels of IFN-γ by flow cytometry and ELISpot (Fig 5, B and C). We used uniform manifold approximation and projection (UMAP) to compare convalescent MIS-C and COVID-19 patients; age-matched individuals were used as controls. This analysis determined that most UMAP differences between convalescent MIS-C and COVID-19 patients were attributable to age (Fig 5, C). Interestingly, we identified a cluster of...
FIG 5. Cellular immune response in PBMCs from convalescent COVID-19 and MIS-C patients. (A) CD4¹ and CD8¹ memory T-cell subsets of HD, convalescent COVID-19, and MIS-C patients on stimulation with SARS-CoV-2 spike protein normalized by unstimulated PBMCs. (B) IFN-γ¹, CD4¹, and CD8¹ memory T-cell subsets of convalescent COVID-19 and MIS-C patients on stimulation with SARS-CoV-2 spike protein, normalized by unstimulated PBMCs. Dotted line represents value of 1. (C) Violin plot of IFN-γ-secreting cells using ELISpot. (D) UMAPs with 4 adult healthy donors, 3 healthy child controls sampled at the beginning of pandemic (unvaccinated, no COVID-19 contact documented), 13 convalescent MIS-C, and 12 convalescent COVID-19 concatenated samples. (E) Neutralizing antibody titration of convalescent COVID-19 (n = 20) patients. (F) Neutralizing antibody titration of convalescent MIS-C (n = 18) patients. (G) Neutralizing antibodies 1/IC₅₀ comparison between convalescent MIS-C and COVID-19 patients. COVID-19-Conv, convalescent COVID-19 sampled 6 months after disease onset; HD, healthy donor; IC₅₀, drug concentration causing 50% inhibition; MIS-C-Conv, convalescent MIS-C sampled 6 months after disease onset. Mann-Whitney comparisons, *P < .05.
KD or MAS. While increased CD14+ monocytes count have been previously proposed as biomarkers of severe KD, we identified activated nonclassical monocytes (CD14+ CD16+ HLA-DR+) distinguish KD-like MIS-C. Monocyte-derived cytokines can activate endothelial cells as well as recruit lymphocytes and monocytes contributing to endothelitis and classical monocyte differentiation has been described in KD immunopathogenesis. Further exploration of monocytes in the context of MIS-C could contribute to understand KD immunopathogenesis. Unfortunately, we did not measure HLA-DR expression in monocytes of convalescent MIS-C patients to evaluate whether this dysregulation is persistent. Similar to previous reports, we did not identify an association between KD-like MIS-C and disease severity or cardiac involvement.

Fecal neoepitope is elevated in patients with active intestinal inflammation, including Crohn disease and acute viral infection. We found higher neoepitope levels in fecal samples of MIS-C patients, most frequently in those with GI involvement, suggesting that this clinical manifestation has an inflammatory nature. A trend to higher ACE2 levels was identified in fecal samples of MIS-C patients. ACE2 converts angiotensin I to angiotensin II and is key for homeostasis in the renin–angiotensin–aldosterone system. In this context, downregulation of ACE2 could imbalance this system, resulting in enhanced inflammation. Our results showing a negative correlation between ACE2 and neoepitope levels are in line with this observation and suggest the possibility of dysregulation of the renin–angiotensin–aldosterone system contributing to gut inflammation in MIS-C.

Interestingly, we observed consistently lower NK cell degranulation in MIS-C patients, a result in agreement with a previous study showing a dysregulation of cytotoxic cells characterized by exhausted CD8+ lymphocytes and CD56dimCD57+ NK cells. Persistently lower NK cell degranulation in convalescent MIS-C patients suggests the possibility of an underlying NK cell defect as a predisposing factor for MIS-C, similar to what has been described for other diseases, including hemophagocytic lymphohistiocytosis and KD. Although we did not test virus clearance in this study, we hypothesize that the identified defect in NK cell degranulation could lead to ineffective viral clearance promoting sustained T-cell stimulation triggering postinfectious inflammation, as previously suggested. To our knowledge, this is the first report identifying a persistent NK cell defect in MIS-C, and further research is required to clarify the role of NK cells on immunopathogenesis of this disease.

We observed that MIS-C patients mounted protective immune responses to SARS-CoV-2 showing specific memory T cells and neutralizing antibodies against SARS-CoV-2. Once a positive cellular immune response is identified, it is unclear if different IFN-γ levels measured in vitro correlate with different levels of clinical protection. Although MIS-C patients had lower levels of specific IFN-γ production, to date, none of the patients in our cohort has experienced a second episode of clinically evident COVID-19 infection or MIS-C. Surprisingly, we identified a cluster of double-negative T cells in convalescent MIS-C patients, similar to the expansion of double-negative T cells after human immunodeficiency virus infection. Patients with autoimmune such as systemic lupus erythematosus or autoimmune lymphoproliferative syndrome are also characterized by high circulating levels of proinflammatory double-negative T cells. The role of these cells in triggering an inflammatory environment in convalescent MIS-C, as well as further characterization of this cluster, require further exploration.

Immune characterization of our MIS-C cohort provides valuable insights in understanding immune dysregulation in MIS-C and allowed the identification of biomarkers for disease severity and specific clinical features.

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FIG E1. Gating strategy of T and NK functionality panel. Singlets and alive cells were selected. For NK cells, CD3^-CD56^+ cells and then perforin^- and CD107a^- were selected. For T cells, CD3^+ cells, CD4^+ or CD8^+ cells, and IFN-γ^+, TNF-α^+, or HLA-DR^+ were selected.
FIG E2. Gating strategy of memory/activation T-cell panel. Singlets and CD3⁺ cells, CD4⁺ or CD8⁺ cells, CD45RA⁺ CD27⁺ for memory T cells, and CD69⁺ cells were selected.
FIG E3. Gating strategy of monocyte panel. Singlet cells were selected. For nonclassical monocytes, CD16^+CD14^-, CCR2^intermediate^ CD36^+^ cells were selected. For intermediate monocytes, CD16^+CD14^+, CCR2^high^ CD36^+^, CD11c^- cells were selected. For nonclassical monocytes, CD16^+CD14^-, CD11c^- cells were selected. HLA-DR^+^ cells were selected for each monocyte subset.
FIG E4. Gating strategy of SARS-CoV-2–specific cellular immune response. For each SARS-CoV-2–stimulated PBMC sample, singlets, alive, and CD3+ cells were selected. For each study group, CD3+ cell files were concatenated. UMAPs of concatenated files were generated. CD4+ or CD8+ from UMAPs were selected, then central memory, TCM (CCR7+CD45RA-), effector memory, TEM (CCR7-CD45RA-), effector memory CD45RA+, TEMRA (CCR7-CD45RA+), and naive T cells (CCR7+CD45RA+) for CD4+ or CD8+ T cells. For each subset T cell, IFN-γ+ cells were selected.
FIG E5. Correlation matrix of COVID-19 and febrile controls. (A) COVID-19. IFN-γ and CXCL10 were removed because values were zero and data for neutralizing antibodies were not available. (B) Febrile controls. IFN-γ was removed because values were zero and data for neutralizing antibodies and monocytes were not available.
**A** Correlations between platelet numbers and cytokines

![Graphs showing correlations between platelet numbers and cytokines](image)

**B** COVID-19 Controls

![Graphs showing analysis of organ involvement in COVID-19 controls](image)

**C** Febrile Controls

![Graphs showing analysis of organ involvement in febrile controls](image)

**FIG E6.** Biomarkers of organ involvement in (A) COVID-19 and (B) febrile controls. (A) Spearman correlation between platelet numbers and cytokine levels. (B) Analysis of shock or of renal or cardiac involvement was not performed because in the COVID-19 group, only 1 patient presented with shock, and none experienced renal or cardiac involvement. (C) Analysis of heart failure, type of shock, and pericardial effusion was not performed because only 1 patient had these clinical manifestations.
FIG E7. Functionality of T cells, monocytes, NK cells, and memory T cells were evaluated in adults hospitalized with acute COVID-19 and HD using flow cytometry and levels of cytokines in serum using ELISA. HD, Healthy donors; MIS-C KD-like, patients with KD-like symptoms; MIS-C No KD-like, patients without KD-like symptoms; NS-MIS-C, acute MIS-C without shock; S-MIS-C, acute MIS-C with shock.
FIG E8. Characterization of lymphocyte cluster identified exclusively in convalescent MIS-C patients evaluated 6 months after disease onset. Histograms show different markers of selected clusters. Cluster in blue that is exclusive to convalescent MIS-C patients corresponds to double-negative T cells secreting mainly IFN-γ (n = 13).
| Patient no. | Age | PCR COVID-19 result | Serology IgG result | Reason for ruling out MIS-C diagnosis |
|------------|-----|---------------------|---------------------|---------------------------------------|
| 1          | 3y  | Negative            | Negative            | Negative COVID-19 PCR and serology; no myocardial compromise or KD features |
| 2          | 13y | Negative            | Negative            | GATA2, medullary aplasia, candidate for bone marrow transplant |
| 3          | 1y  | Negative            | Positive            | Acute nephrotic syndrome |
| 4          | 1mo | Negative            | Negative            | Pericardial biopsy indicated nonspecific lymphocytic pericardial infiltration |
| 5          | 2mo | Positive            | Negative            | COVID-19 pneumonia |
| 6          | 2y  | Negative            | Positive            | Acute mild COVID-19, no inflammatory markers |
| 7          | 1y  | Positive            | Positive            | Mild COVID-19 |
| 8          | 4y  | Negative            | Negative            | Pyelonephritis |
| 9          | 3y  | Negative            | Negative            | Non–COVID-19–associated KD |
| 10         | 11y | Negative            | Positive            | Cryptosporidium and Escherichia coli infection |
| 11         | 10y | Undetermined        | Negative            | Parvovirus infection, serology negative for SARS-CoV-2, heart failure |
| 12         | 11y | Negative            | Negative            | PCR and serology negative for SARS-CoV-2 |
| 13         | 1y  | Positive            | Positive            | Pyelonephritis |
| 14         | 1y  | Negative            | Negative            | Salmonella infection and PCR and serology negative for SARS-CoV-2 |
| 15         | 14y | Negative            | Positive            | Salmonella infection |
| 16         | 11y | Negative            | Positive            | Pyelonephritis/urosepsis |
| 17         | 6y  | Negative            | Negative            | PCR and serology negative for SARS-CoV-2, only 1 day of fever |
| 18         | 8y  | Negative            | Negative            | PCR and serology negative for SARS-CoV-2 |
| 19         | 7y  | Negative            | Negative            | PCR and serology negative for SARS-CoV-2, shock toxic, scarlet fever |
| 20         | 1y  | undetermined        | Negative            | Cytomegalovirus systemic |
| 21         | 6y  | Negative            | Negative            | PCR and serology negative for SARS-CoV-2, sickle cell disease; coronary dilation attributable to KD; pulmonary infarct |
| 22         | 1y  | Negative            | Negative            | Classic KD |
| 23         | 8mo | Negative            | Negative            | Classic KD |
| 24         | 1y  | Positive            | Positive            | COVID-19 pneumonia |
**TABLE E2.** Sex and age of cohort subjects

| Characteristic                | MIS-C (n = 42) | COVID-19 (n = 21) | Febrile controls (n = 25) | HD adult (n = 6) | HD children (n = 3) |
|------------------------------|----------------|-------------------|---------------------------|-----------------|---------------------|
| **Sex (%)**                  |                |                   |                           |                 |                     |
| Male                         | 55             | 59                | 50                        | 50              | 67                  |
| Female                       | 45             | 41                | 50                        | 40              | 33                  |
| **Age (years), median**      | 7 (0-15)       | 35 (18-45)        | 4 (0-14)                  | 32 (22-50)      | 8 (5-11)            |
| (range)                      |                |                   |                           |                 |                     |

*HD,* Healthy donor.
### TABLE E3. Laboratory parameters of MIS-C patients with and without shock

| Laboratory parameter                  | Mean ± SD (n) of: |  |  |
|---------------------------------------|-------------------|--|--|
|                                                      | Patients with shock | Patients without shock | P value |
| Whole blood count (×1000 cells/mm³)     | 292.76 ± 579.27 (25) | 947.24 ± 1505.96 (17) | .054916 |
| Absolute neutrophil count (×1000 cells/mm³) | 4909.68 ± 5410.13 (25) | 5339.71 ± 5876.58 (17) | .482236 |
| Absolute leucocyte count (×1000 cells/mm³) | 748.76 ± 2095.58 (25) | 926.59 ± 1095.91 (17) | .750261 |
| Absolute monocyte count (×1000 cells/mm³) | 277 ± 2095.58 (25) | 691.29 ± 1281.38 (17) | .209826 |
| Hemoglobin (g/dL)                      | 108.09 ± 30.41 (25) | 104.06 ± 35.76 (17) | .697335 |
| Platelets (×1000 cells/mm³)           | 149 ± 59.22 (25) | 304.82 ± 116.92 (17) | .000001 * |
| Fibrinogen (mg/dL)                    | 493.8 ± 152.06 (25) | 497.44 ± 209.93 (16) | .949025 |
| Albumin (g/dL)                        | 48.5 ± 68.28 (25) | 61.13 ± 110.78 (16) | .657579 |

Groups were compared by Student t test.
*Significant at P < .05.