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Deep immunophenotyping reveals biomarkers of MIS-C in a Latin American cohort

Emma Rey-Jurado[1,2]† PhD, Yazmin Espinosa[3]† MD, Camila Astudillo[3] MD, Lina Jimena Cortés[1,4] PhD, Juan Hormazabal[1,4] PhD, Loreani Noguera[1,2] BS, Fernanda Cofré[5] MD, Cecilia Piñera[6,7] MD, Ricardo González[6] MD, Alexander Bataszew[8] MD, Paula Muñoz[1] MD PhD, Dona Benadof[5] MD, Patricia Álvarez[3] MD, Valeria Acevedo[3] MD, Pablo Vial[1,4] MD, Cecilia Vial[1,4]‡ PhD, M. Cecilia Poli[1,2,3]‡* MD PhD

[1]Facultad de Medicina Clínica Alemana de Santiago, Universidad del Desarrollo, Santiago, Chile
[2]Programa de Inmunogenética y Inmunología Traslacional. Instituto de Ciencias e Innovación en Medicina, Facultad de Medicina, Universidad del Desarrollo, Santiago, Chile
[3]Unidad de Inmunología y Reumatología, Hospital Roberto del Río, Santiago, Chile
[4]Programa de Hantavirus y Zoonosis, Instituto de Ciencias e Innovación en Medicina, Facultad de Medicina, Universidad del Desarrollo, Santiago, Chile
[5]Unidad de Infectología, Hospital Roberto del Río, Santiago, Chile
[6]Hospital Exequiel González Cortés, Santiago, Chile
[7]Facultad de Medicina, Universidad de Chile, Santiago, Chile
[8]Unidad de Cuidados Intensivos, Hospital Roberto del Río, Santiago, Chile

*Corresponding author: Cecilia Poli. Av. La Plaza 680, Santiago de Chile, Chile.
cpoli@udd.cl

†, ‡, These authors contributed equally to this work

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ABSTRACT

Background: Multisystemic inflammatory syndrome in children (MIS-C) is a life-threatening disease that occurs 2-5 weeks after SARS-CoV-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: To characterize the immune response and clinical features of patients with acute MIS-C and determine biomarkers of disease in a cohort of 42 Latin American patients.

Methods: Immune characterization was performed using flow cytometry from peripheral mononuclear cells and SARS-CoV-2-specific humoral and cellular response was performed using flow cytometry, ELISPOT, ELISA and neutralizing antibody assays.

Results: MIS-C is characterized by robust T cell activation and cytokine storm. We uncovered that while CXCL9, 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 to Kawasaki-like MIS-C. Interestingly, MIS-C patients show an NK 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. SARS-CoV2-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.

CLINICAL IMPLICATIONS STATEMENT

MIS-C is distinguished by cytokine storm and decreased NK cell degranulation that is persistent after 6 months. Distinct biomarkers were identified for severe and mild forms of disease.

CAPSULE SUMMARY

We identify CXCL9, IL-10, CXCL8, CXCL10, IL-6 and IL-18 as biomarkers of severe MIS-C . Persistently decreased NK cell degranulation suggests the possibility of an underlying defect.

Keywords: COVID-19**,** Inflammation**,** Multisystemic inflammatory syndrome in children, biomarkers, NK cell deficiency

Abbreviations

MIS-C: Multisystemic inflammatory syndrome in children

COVID-19: Coronavirus disease

KD: Kawasaki disease

MAS: Macrophage activation syndrome

ICU: Intensive Care Unit

proBNP: pro natriuretic peptide test

IFN-γ: Interferon gamma

TNF-α: tumor necrosis factor alpha

IL-6: Interleukin 6

IL-18: Interleukin 18

IL-18BP: Interleukin 18 binding protein

IL-10: Interleukin 10

CXCL9: Chemokine (C-X-C motif) ligand 9

CXCL10: Chemokine (C-X-C motif) ligand 10

CXCL8: Chemokine (C-X-C motif) ligand 8

CCL5: Chemokine (C-C motif) ligand 5

CCL2: Chemokine (C-C motif) ligand 2

NK: Natural Killer

PBMC: peripheral mononuclear cells

PMA: phorbol 12-myristate 13-acetate

CBA: Cytometric Bead Array

ELISA: Enzyme linked-assay

ACE-2: Angiotensin-converting enzyme-2

GFP: Green Fluorescence Protein

IC50: Half-maximal inhibitory concentration

IMV: invasive mechanical ventilation

HIV: Human of immunodeficiency virus

RAAS: renin-angiotensin-aldosterone system

INTRODUCTION

Multisystemic inflammatory syndrome in children (MIS-C) is a rare but life-threating condition that occurs in children or adolescents 2-5 weeks after SARS-CoV-2 exposure (1, 2). MIS-C is defined by systemic hyperinflammation with multiple organ involvement including gastrointestinal, cardiac, dermatologic, respiratory, renal and neurological symptoms which may lead to multiorgan failure (3). 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 gastrointestinal symptoms and shock (4). Early recognition of MIS-C is key for appropriate treatment and successful outcome (5). However, diagnosis is often challenging due to clinical overlap with non-SARS-CoV-2 KD, sepsis and other infectious conditions (6). Additionally, endemic circulation of SARS-CoV-2, cessation of lockdowns and vaccination has made it harder to identify COVID 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 (7, 8). Overall, 60% of MIS-C patients require Intensive Care Unit (ICU) admission and vasoactive support (9, 10). Laboratory parameters including decreased platelets and lymphocytes, and increased C-reactive protein, D-dimer, troponin, proBNP, ferritin and IL-6 levels can predict severity, however they are still non-specific (11-13).

Multi-dimensional immune studies of MIS-C, comparing it to KD and severe COVID-19 in adults, reveal that while they are all characterized by hyperinflammation, MIS-C is a unique entity with higher IL-6, CXCL9 and CXCL10 levels (14, 15). Studies characterizing the immune response in MIS-C have shown reduced numbers of $CD4^+$ and $CD8^+$ T cells, Natural Killer (NK) cells and γδT 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 it´s different clinical

manifestations have not been clearly defined (14-19). Similarly, KD-like MIS-C patients are clinically well defined, however, immune mechanisms underlying this specific form of MIS-C are still poorly understood (20, 21).

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 that will require more complex or targeted interventions.

Comprehensive immune studies in African and Hispanic children are lacking and may contribute to understand genetic and environmental components that may explain a higher incidence observed in these populations (7, 8, 22-24). 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 three clinical centers in Chile.

METHODS

Ethical statements

This study was approved by Ethical Committee of Facultad de Medicina Clínica Alemana Universidad del Desarrollo. All participants or legal guardians gave written informed consent in accordance with the Declaration of Helsinki (25).

Subjects

A total of 67 patients admitted with suspected diagnosis of MIS-C (June 2020-June 2021) were recruited in the city of Santiago de Chile from three clinical centers: Roberto del Rio pediatric Hospital, Dr. Exequiel González Cortés pediatric hospital and Clínica Alemana de Santiago. Clinical information was uploaded into REDcap (Research Electronic Data Capture) (26-28). After recruitment, patients were re-screened according to WHO definition for MIS-C (29). SARS-CoV-2 exposure was corroborated by clinical history 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) (Sup. Table1). A sub-group of these patients were diagnosed with alternative febrile conditions and we identified them as febrile controls. In addition, 21 young adults hospitalized with COVID-19 pneumonia were recruited to compare with MIS-C. Blood and fecal 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). Three flow cytometry panels were performed: 1) NK and T cells functionality, 2) Memory and activation T cells, 3) Monocytes (Sup. methods, Sup. Figures 1-4). For T cell and NK cell functional characterization, cells were stimulated with 1ug/ml of phorbol 12-myristate 13-acetate (PMA) and 1ug/ml of Ionomycin with Brefeldin and Golgi stop (BD). 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 blinded for both, clinical features and clinical laboratory data.

Measurement of serum cytokine and chemokine levels

Serum was isolated by centrifugation, stored at -80°C and thawed for cytokine assessment using BD™ Cytometric Bead Array (CBA) human Th1/Th2 cytokine kit, human inflammatory cytokine kit and human chemokine kit according to manufacturer instructions. Samples were acquired on Cytoflex LX flow cytometer and analyzed using FlowJo software V9.1. To evaluate CXCL9, IL-18 and IL18-BP, commercially available ELISA were used according to manufacturer instructions (#DY392, #DY318-05, #DY119 R&D Systems). Free IL-18 levels were calculated considering the law of mass action as described (30, 31).

Determination of Neopterin and ACE-2 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 ACE-2 according to manufacturer instructions (#RE59321, IBL; #DY933-05, R&D Systems). Wet stool weight was used for normalization.

SARS-CoV-2-specific cellular immune response

Patient PBMC obtained six 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 ELISPOT, human IFN-γ single-color Elispot (CTL, Immunospot®) were used according to manufacturer instructions. To determine cellular immune response in different lymphocyte subsets, we used flow cytometry (Sup. methods, Sup. Figure 4).

Measurement of IgG and neutralizing antibodies

Enzyme linked-assay (ELISA) was performed as previously described (32). Microtiter plates were coated with 1ug/mL of SARS-CoV-2 Spike protein overnight 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 VSV-GFP-Spike SARS-CoV-2 (33). Serially diluted serum previously incubated with pseudovirus VSV-GFP-Spike SARS-CoV-2 was transferred into Vero cells monolayer at a final multiplicity of infection of 0.5 and incubated at 37°C 5% CO_2 for 18-20 h. The infection was measured in each well by determining GFP fluorescence intensity using Cytation3 plate reader. Half-maximal inhibitory concentration (IC50) was calculated using nonlinear regression analysis.

Statistical analysis

Statistical analyses were performed using GraphPad Prism V9.1.0. Correlation matrix was created using nonparametric Spearman test, with confidence interval of 95%. Each correlation was done independently between two variables, with no multiple comparison correction because of the small sample size. Immune parameters were compared among MIS-C, COVID-19, febrile controls and healthy donors using non-parametric Mann-Whitney tests. To compare immune parameters between patients, volcano plots were created per 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, Perú and Chile) residing in Chile, 55% were male and mean age was 7 years old. Seventy-nine percent of patients required ICU admission (1-10 days of stay), 33% invasive mechanical ventilation (IMV) and 40% inotropic support. Most patients had fever and gastrointestinal involvement (Table 1, Sup. Table 2). Sixty percent had shock while 62% showed had cardiac involvement (Figure 1A). Sixty-seven percent had Kawasaki-like symptoms (Figure 1B), and 68% of these patients also presented with shock. Patients were treated with intravenous immunoglobulin (IVIG) (76%), oral (76%) and i.v. corticosteroids (81%), tocilizumab (7%) and infliximab (2%) (Figure 1C) according to local treatment guidelines (34). After 12 months follow-up, most patients survived with no sequalae; only one patient died during acute illness with macrophage activation (MAS) and cardiac failure, one patient shows a persistent coronary aneurism and one patient was diagnosed with Crohn´s disease immediately after MIS-C.

MIS-C patients are characterized by T cell activation, elevated inflammatory cytokines and a 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 with severe COVID-19 patients and febrile controls (Figure 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 $CD8^+CD69^+$) (Figure 2A-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 findings (16, 35) (Figure 2D). Although monocyte distribution was heterogenous, we identified a lower expression of HLA-DR in non-classical monocytes of MIS-C patients (Figure 2E) possibly contributing to impaired immune homeostasis in this acute condition (36). 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, CCL2, CXCL8, CXCL9 and CXCL10. Even though total IL-18 was higher in MIS-C, free IL-18 was significantly lower than in COVID-19 (Figure 2F). TNF-α, IL-5, IL-4 and IL-2 were undetectable in all patients (data not shown). Altogether these data suggest MIS-C is distinguished from COVID-19 by substantial activation of T cells and non-classical and intermediate monocytes, together with a pro-inflammatory cytokine and chemokine storm.

Differentiating children with MIS-C from other inflammatory conditions is challenging, given the wide range of differential diagnosis in pediatric patients and the often-unclear history of COVID exposure. Furthermore, with vaccination, positive serology becomes difficult to interpret. To address this point, we compared MIS-C with other febrile conditions. We identified that, while both patient groups were characterized by strong T cell activation, MIS-C was distinguished by higher

248 expression of IFN-γ in $CD4^+$ T cells and higher levels of IL-10, CXCL8, CCL2 and distinctively even
249 higher levels of CXCL9, CXCL10, suggesting an IFN-γ signature as a biomarker of MIS-C as previously
250 suggested (Figure 2F) (37, 38).
251 Interestingly, MIS-C patients showed decreased NK cell numbers and NK cell degranulation
252 measured by CD107a expression after PMA/ionomycin stimulation (Figure 2G). This reduction was
253 independent of NK cell numbers. While degranulation improved after 6 months, convalescent MIS-
254 C patients still exhibit lower CD107a expression than healthy controls, suggesting these children
255 may have an underlying functional NK cell defect.
256
257 ***Cytokine storm correlates with lower platelets and disease severity in MIS-C***
258 As we and others have observed, MIS-C is characterized by increased proinflammatory cytokines
259 including IL-6, IL-18, IFN-γ and IL-17A (14, 15). However, comprehensive understanding of the
260 correlation between immune perturbations, cytokines and clinical or laboratory parameters has not
261 been clearly elucidated (15). To determine biomarkers for severity, we studied the correlation of
262 chemokine and cytokine profiles with clinical manifestations, clinical laboratory parameters and
263 multiparametric immune cell characterization, establishing one on one comparisons using non-
264 parametric Spearman correlation. Furthermore, these results were contrasted with COVID-19 and
265 febrile controls to determine whether the identified associations were unique to MIS-C.
266 As expected, we identified T lymphocyte activation markers ($CD4^+CD69^+$, $CD8^+CD69^+$, $CD4^+HLA\text{-}DR^+$,
267 $CD8^+HLA\text{-}DR^+$), correlated with increased lymphocyte-cytokine expression ($CD4^+IFN\text{-}\gamma^+$, $CD8^+IFN\text{-}\gamma^+$,
268 $CD4^+TNF\text{-}\alpha^+$, $CD8^+TNF\text{-}\alpha^+$) (Figure 3A). Interestingly, we observed a correlation between higher levels
269 of CCL5, a lower cytokine milieu and higher platelet numbers and WBC which was not identified in
270 febrile controls or COVID-19 suggesting this is a unique feature of MIS-C (Figure 3A, Sup. Fig 5,6).
271 Patients with shock showed significantly higher levels of CXCL9, IL-10, CXCL8, CXCL10, IL-6 and IL-

18, and lower levels of free IL-18 (Figure 3A-B), Sup. Fig 7E). Overall, this data suggests 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 pediatric COVID revealed lower platelet numbers in MIS-C patients (39). We observed MIS-C patients with shock had significantly lower platelet numbers than patients without shock, despite they were mostly within the normal range (normal range:140,000-400,000) (Sup. table 3). This is in line with previous observations showing an association between reduced platelets and disease severity in MIS-C as well as in COVID-19 (13, 40). While higher IL-6, IL-10, CXCL9, and CXCL10 correlated with pericardial effusion (Figure 4A), no significant associations were identified for heart failure, defined as ejection fraction below 55%. Neurologic and renal involvement were associated with higher IL-1β and higher perforin levels in NK cells, respectively. We did not identify biomarkers for respiratory involvement, probably due to the low frequency of respiratory symptoms in our cohort (Figure 4B). Biomarkers of neurologic, and renal involvement found in MIS-C differ from COVID-19 and febrile controls, suggesting these correlations are exclusive for MIS-C (Sup. Fig 5,6).

We identified that KD-like MIS-C is characterized by higher HLA-DR$^+$ expression in non-classical 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 (Figure 4C, Sup. Fig 7F).

Gastrointestinal (GI) involvement was present in 90% of our cohort, however we did not find correlation between any immune parameter and GI involvement. ACE-2 serves as a receptor for

296 SARS-CoV-2 entry and although a higher expression of ACE-2 in children's GI tract has been
297 proposed, the mechanisms underlying GI involvement in MIS-C are poorly understood. Neopterin is
298 released by macrophages upon IFN stimulation and is involved in redox reactions (41). Increased
299 neopterin levels in patient´s serum and feces are associated with severity in COVID-19 (42, 43). Our
300 results show that, MIS-C patients have significantly higher neopterin levels and a trend to higher
301 ACE-2 levels in their stools than COVID-19 patients. We identified that higher levels of neopterin
302 correlated with lower levels of ACE-2 in MIS-C patients, which could be associated with a
303 downregulation of ACE-2 after SARS-CoV-2 exposure (45). In addition, we found that higher
304 neopterin levels in stools, were associated with mixed cardiac shock suggesting a link between
305 intestinal inflammation and cardiac dysfunction (Figure 4D).
306
307 ***Convalescent MIS-C patients show lower IFN-γ^{+} memory T cells and higher titer of neutralizing***
308 ***antibodies than convalescent COVID-19 patients.***
309 Cellular immunity is crucial to provide long-term protection, thus it is important to determine if
310 convalescent MIS-C patients develop sustained cellular immune responses to SARS-CoV-2. To
311 determine T cell-specific responses, we performed ELISPOT and flow cytometry in PBMC stimulated
312 with SARS-CoV-2 protein and compared with convalescent COVID-19 pneumonia patients after 6
313 months of disease presentation. Because memory T cell subsets change with age, we included
314 unvaccinated age-matched controls who were recruited at the beginning of the pandemic (most
315 probably naïve to SARS-Co V2). While we identified SARS-CoV-2 specific memory T cells in both MIS-
316 C and COVID-19 convalescent patients (Figure 5A), MIS-C patients showed consistently lower levels
317 of IFN-γ by flow cytometry and Elispot (Figure 5B-C). We used UMAP to compare convalescent MIS-
318 C and COVID-19 patients, age matched individuals were used as controls. This analysis determined
319 that most UMAP differences between convalescent MIS-C and COVID-19 patients were attributable

to age (Figure 5C). Interestingly, we identified a cluster of interferon gamma secreting $CD3^+CD4^-CD8^-$ $CD45RA^-CCR7^+$ T cells that were exclusive to MIS-C convalescent patients (Sup. Fig. 8). Nevertheless, neutralizing antibody levels were higher in convalescent MIS-C than COVID-19 patients (Figure 5E-G). All together, these results suggest convalescent MIS-C patients could have lower SARS-CoV-2-specific IFN-γ^+ memory T cell responses, despite strong T cell activation in the acute setting, however they still have neutralizing antibodies 6 months after presentation.

DISCUSSION

Immune characterization of MIS-C patients and correlation with distinct clinical features and outcome is crucial to further understand this recently identified disease. In this study, immunophenotyping and clinical characterization of 42 Latin American MIS-C patients revealed biomarkers that differentiate MIS-C from acute COVID-19 and other febrile conditions in pediatrics. We also identified biomarkers for severity and for specific clinical features illuminating underlying immune mechanisms of disease.

Immune signatures of MIS-C overlap with severe adult COVID-19 (15, 16). In accordance with previous studies, we observed that MIS-C is characterized by a marked elevation of a milieu of inflammatory cytokines and chemokines (14, 15, 17), identifying CXCL9, IL-10, CXCL8, CXCL10, IL-6 and IL-18 as biomarkers of disease severity. As proposed by Rivas et al, spike protein has a structural similarity with staphylococcal enterotoxin B and has been postulated to act as a superantigen, interacting with MHC class II and TCR molecules to trigger a cytokine storm and subsequent inflammation, proposing this as a driver of cytokine storm in MIS-C (46, 47). While CCL5 elevation is not specific to MIS-C, and it is also elevated in COVID-19 and other febrile conditions, we identified that higher levels of CCL5 in the context of MIS-C were associated to a lower cytokine milieu, higher platelet levels and WBC. This is in line with previous observations associating higher CCL5 levels with

milder acute COVID-19 (48). CCL5 induces recruitment of basophils, neutrophils, T cells, NK cells and dendritic cells, promotes sustained $CD8^+$ T cell responses, potentially contributing to enhance viral clearance in the acute setting (49-51). Furthermore, CCL5 has been suggested to participate in the increase of platelet counts in other diseases including idiopathic thrombocytopenic purpura and aplastic anemia, however, the mechanism for this finding is yet to be elucidated (52, 53). In this context, our results suggest that CCL5 could have a homeostatic role in MIS-C.

Inflammatory biomarkers to distinguish MIS-C from other diseases with similar pathophysiology such as KD or MAS are crucial. Higher levels of CXCL9 are found in MIS-C as compared to KD or MAS (54). While increased $CD14^+$ monocytes counts have been previously proposed as biomarkers of severe KD, we identified activated non-classical monocytes ($CD14^-CD16^+HLA\text{-}DR^+$) distinguish Kawasaki-like MIS-C (55). Monocyte-derived cytokines can activate endothelial cells, recruit lymphocytes and monocytes contributing to endothelitis (56) and classical monocyte differentiation has been described in KD immunopathogenesis (57). 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 (58).

Fecal neopterin is elevated in patients with active intestinal inflammation including Crohn's disease and acute viral infection (44). We found higher neopterin levels in fecal samples of MIS-C patients, most frequently in those with gastrointestinal involvement, suggesting an inflammatory nature for this clinical manifestation. A trend to higher ACE-2 levels was identified in fecal samples of MIS-C patients. ACE-2 converts angiotensin I to angiotensin II and is key for homeostasis in renin-angiotensin-aldosterone system (RAAS) (59). In this context, down-regulation of ACE-2 could imbalance RAAS, resulting in enhanced inflammation (45, 60). Our results showing a negative

correlation between ACE-2 and neopterin levels are in line with this observation and suggest the possibility of RAAS dysregulation contributing to gut inflammation in MIS-C.

Interestingly, we observed consistently lower NK cell degranulation in MIS-C patients in agreement with a previous study showing a dysregulation of cytotoxic cells characterized by exhausted $CD8^+$ lymphocytes and $CD56^{dim}CD57^+$ NK cells (61). 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 or KD (61-64). Although we did not test viral 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 post-infectious inflammation as previously suggested (65, 66). 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 and although MIS-C patients had lower levels of specific IFN-γ production, to date none of the patients in our cohort has suffered a second episode of clinically evident COVID 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 HIV infection (67). Patients with autoimmunity such as systemic lupus erythematosus or autoimmune lymphoproliferative syndrome are also characterized by high circulant levels of pro-inflammatory double negative T cells (68, 69). The role

of these cells in triggering an inflammatory environment in convalescent MIS-C and further characterization of this cluster requires 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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AUTHOR CONTRIBUTIONS

ERJ designed and performed experiments, analyzed data, and wrote and reviewed the manuscript. JE coordinated collection samples, provided and analyzed clinical metadata and reviewed manuscript. LN, JC, JH designed and performed experiments, analyzed data, and reviewed manuscript. CA, FC, CP, RG, AB, PM, DB, PA, PV and VA provide clinical specimens, clinical data and scientific input. CP and CV supervised the research project, wrote and reviewed the manuscript.

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609 **Table 1. Clinical characteristics of Chilean MIS-C cohort with 42 patients.**
610

	COUNT	PERCENTAGE (%)
Clinical manifestations		
Fever	41	98
Shock	25	60
Cardiac Involvement	26	62
- Coronary dilation	5	12
- Pericardial effusion	17	40
- Myocarditis	15	36
- Ventricular dysfunction (EF<55%)	11	21
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-2*		
Positive PCR	9	21
Indeterminate PCR	3	7
Positive IgG or IgM (Serology)	38	90
Positive PCR or serology	42	100
6 months follow-up		
No sequalae	40	95
Persistent coronary aneurism	1	2
Chron's disease	1	2

611 * No PCR available in one patient, his mother had positive PCR. Abbreviations as follows: IVIG,
612 intravenous immunoglobulin; MAS, macrophage activation syndrome

613

FIGURE LEGENDS

Figure 1. Patient characteristics and clinical follow-up. A) Percentage of patients that presented shock and specific types of shock. **B)** Percentage of patients with Kawasaki-like symptoms. **C)** Treatment of patients and follow-up.

Figure 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=21, HD=6 and FC=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 and CD69$^+$ T cells: 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=6 and FC n=12. (D) Monocyte subsets evaluated in blood using flow cytometry in MIS-C n=13, COVID-19 n=20, HD n=6 and FC n=7 (E) Activated monocytes evaluated in blood using flow cytometry in same individuals as in (D). (F) Cytokines levels in serum measured by ELISA in MIS-C n=19, COVID-19 n=21, HD=6 and FC=12 (G) NK cells cytotoxicity and cytokines evaluated in blood using flow cytometry in MIS-C=19, COVID-19=21, HD=6 and FC=12. HD: healthy donors, MIS-C: acutely ill MIS-C patients, COVID-19: acute adult COVID-19 patients, FC: febrile controls, MIS-C-Conv: Convalescent MIS-C samples after 6 months of disease onset. Mann-Whitney comparisons, *$p<0.05$.

Figure 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 Figure 2. B) Volcano plot showing differences of parameters evaluated with MIS-C patients with and without shock, each dot represents one parameter. Number of MIS-C samples tested for each parameter is shown in Figure 2. Significant p-values are shown above the blue line with red dots. Comparison of each parameter was done with

637 Mann-Whitney test, significant p-value was considered: $p<0.05$. Abbreviations: HD: healthy donors,
638 MIS-C: acutely ill MIS-C patients, COVID-19: acute adult COVID-19 patients

639 **Figure 4.**

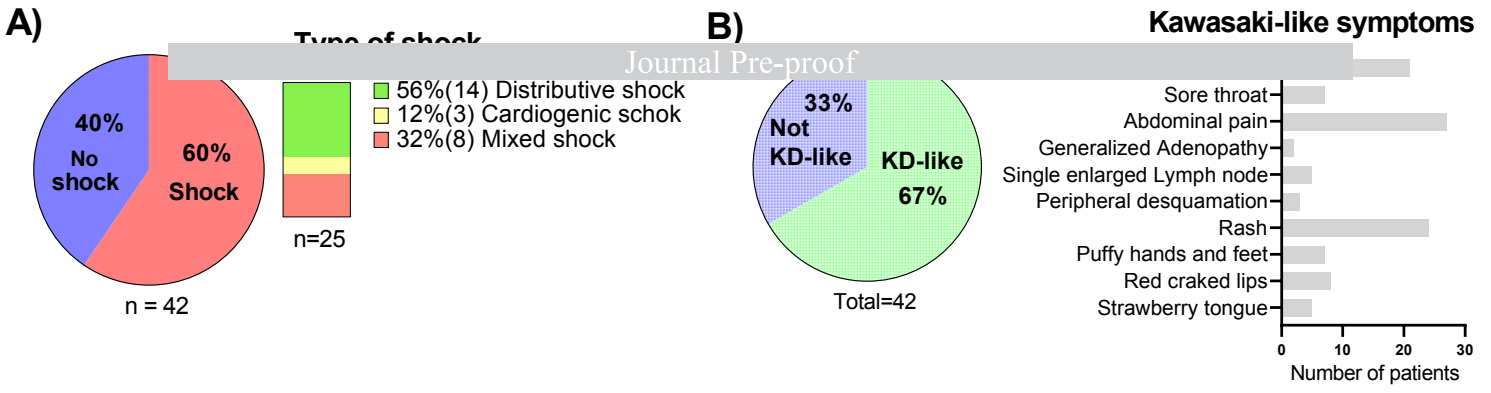
640 **Biomarkers of organ involvement and KD features in MIS-C.** Volcano plots showing correlation of
641 parameters evaluated with clinical manifestations of MIS-C patients, each dot represents one
642 parameter. Number of MIS-C samples tested for each parameter is shown in Figure 2. Significant
643 correlations are shown above the blue line with red dots. Volcano plots of differences between
644 parameters of patients with or without A) heart involvement: pericardial effusion and heart failure.
645 B) Neurological, renal and respiratory involvement C) Volcano plot comparing differences between
646 parameters of MIS-C patients with or without Kawasaki-like symptoms. Violin plots comparing T-
647 cells, monocytes and CXCL8 in HD, and MIS-C patients with or without KD-like symptoms. (volcano
648 plots and violin graphs comparing with HD D) Volcano plot comparing differences between
649 parameters of MIS-C patients with or without GI involvement. Violin plots comparing ACE-2 and
650 neopterin levels from feces samples using ELISA in MIS-C n=30 and COVID-19 n=10 patients. Volcano
651 plot comparing differences between parameters of MIS-C patients with distributive shock and
652 mixed/cardiac shock. . Abbreviations: HD: healthy donors, MIS-C: acutely ill MIS-C patients, COVID-
653 19: acute adult COVID-19 patients, KD-like: Kawasaki-like, GI: Gastrointestinal, All comparisons were
654 performed using Mann-Whitney test, significance $p<0.05$.

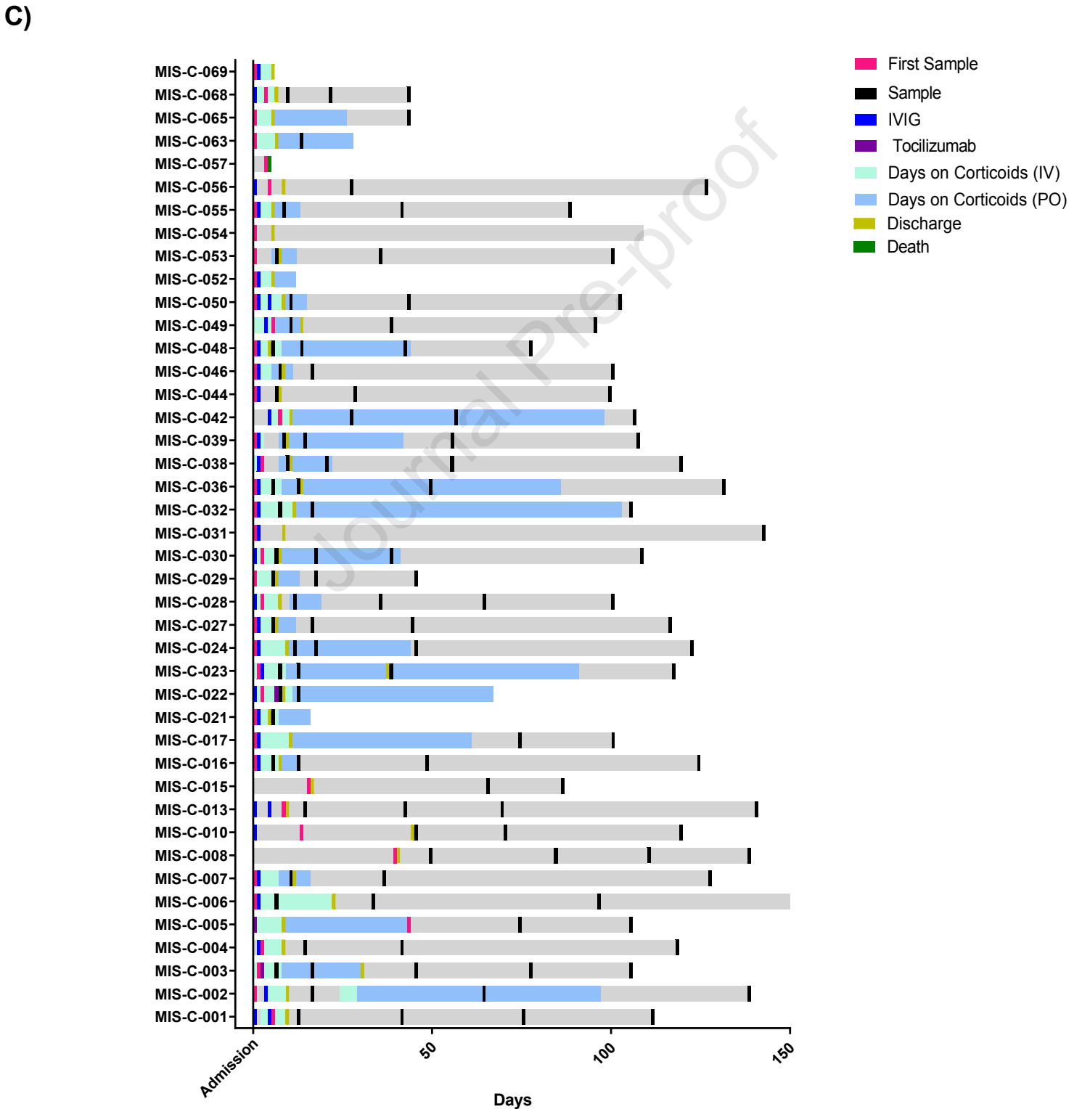
655

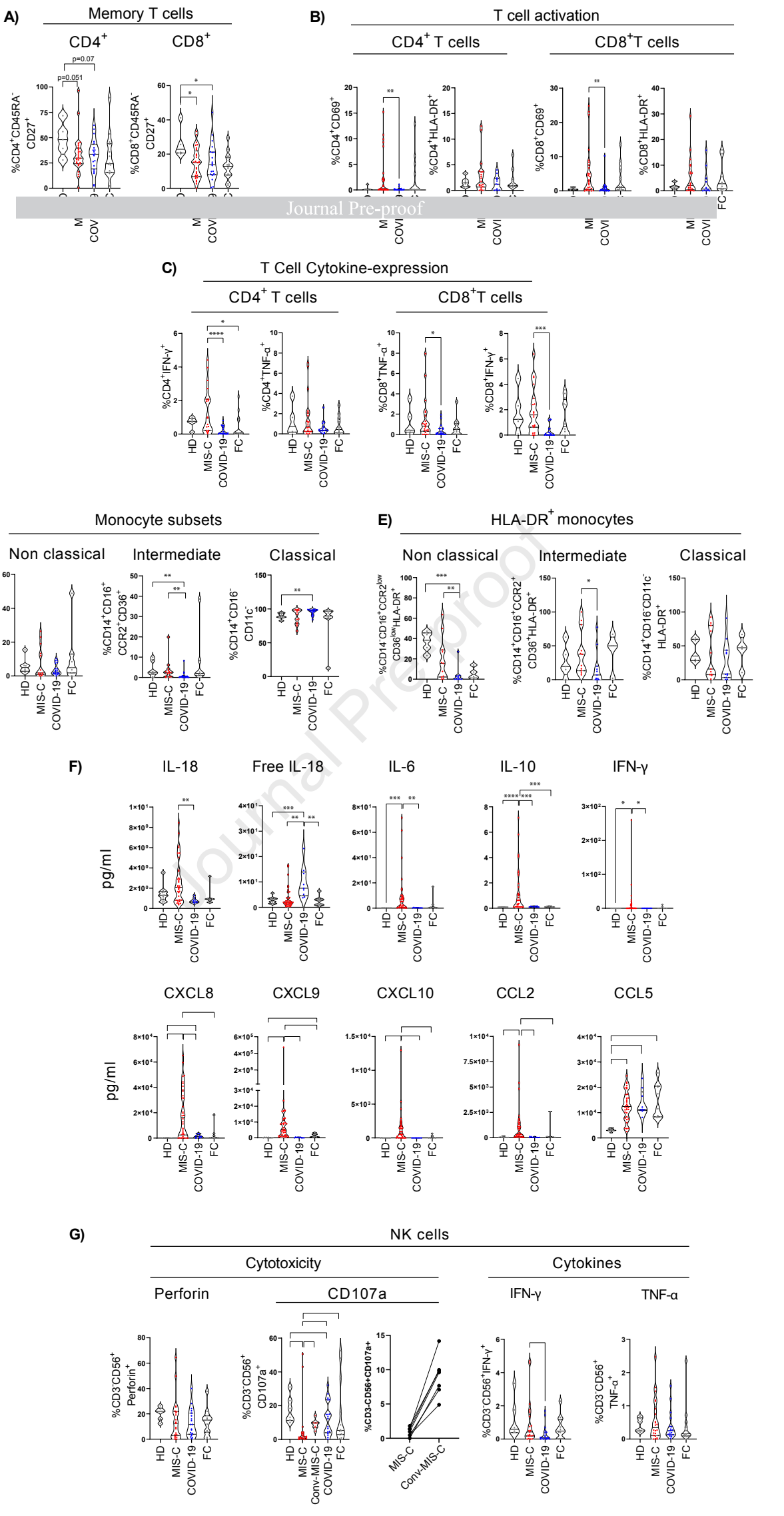
656 Figure 5. **Cellular immune response in PBMCs from convalescent COVID-19 and MIS-C patients**. (A)
657 $CD4^+$ and $CD8^+$ memory T cells subsets of healthy donors (HD), convalescent COVID-19 and MIS-C
658 patients upon stimulation with SARS-CoV-2 Spike protein normalized by unstimulated PBMCs. (B)
659 IFN-γ^+ $CD4^+$ and $CD8^+$ memory T cells subsets of convalescent COVID-19 and MIS-C patients upon
660 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 children controls sampled at the beginning of pandemic (unvaccinated no COVID contact documented) 13 convalescent MIS-C and 12 convalescent COVID-19 concatenated samples (E) Neutralizing antibodies titration of convalescent COVID-19 n=20 patients (F) Neutralizing antibodies titration of convalescent MIS-C n=18 patients. G) Neutralizing antibodies 1/IC50 comparison between convalescent MIS-C and COVID patients. Abbreviation as follows: MIS-C-Conv: Convalescent MIS-C, sampled after 6 months of disease onset. COVID-19-Conv: Convalescent COVID-19 sampled after 6 months of disease onset Mann-Whitney comparisons, *$p<0.05$

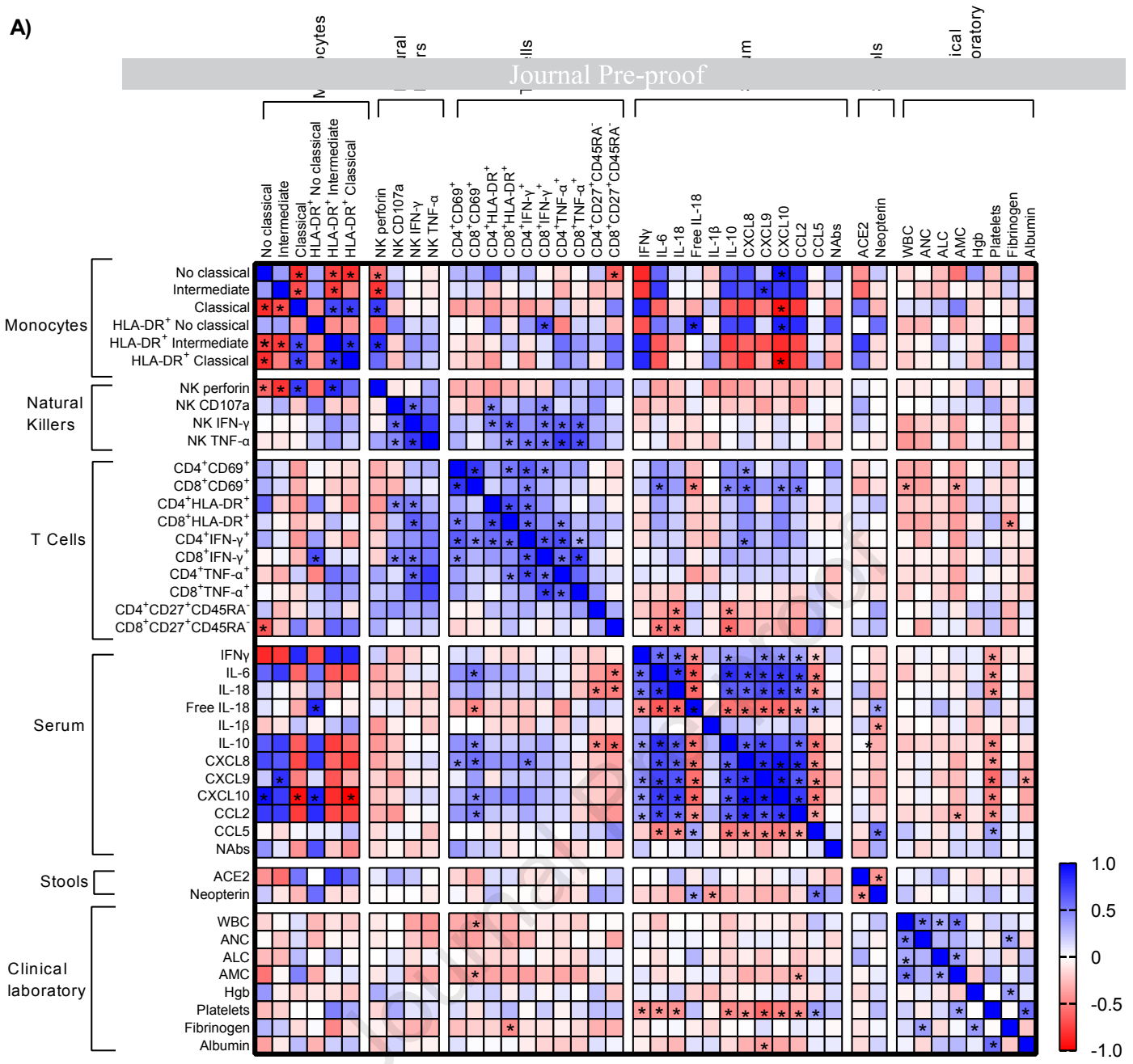
676







A)



B)