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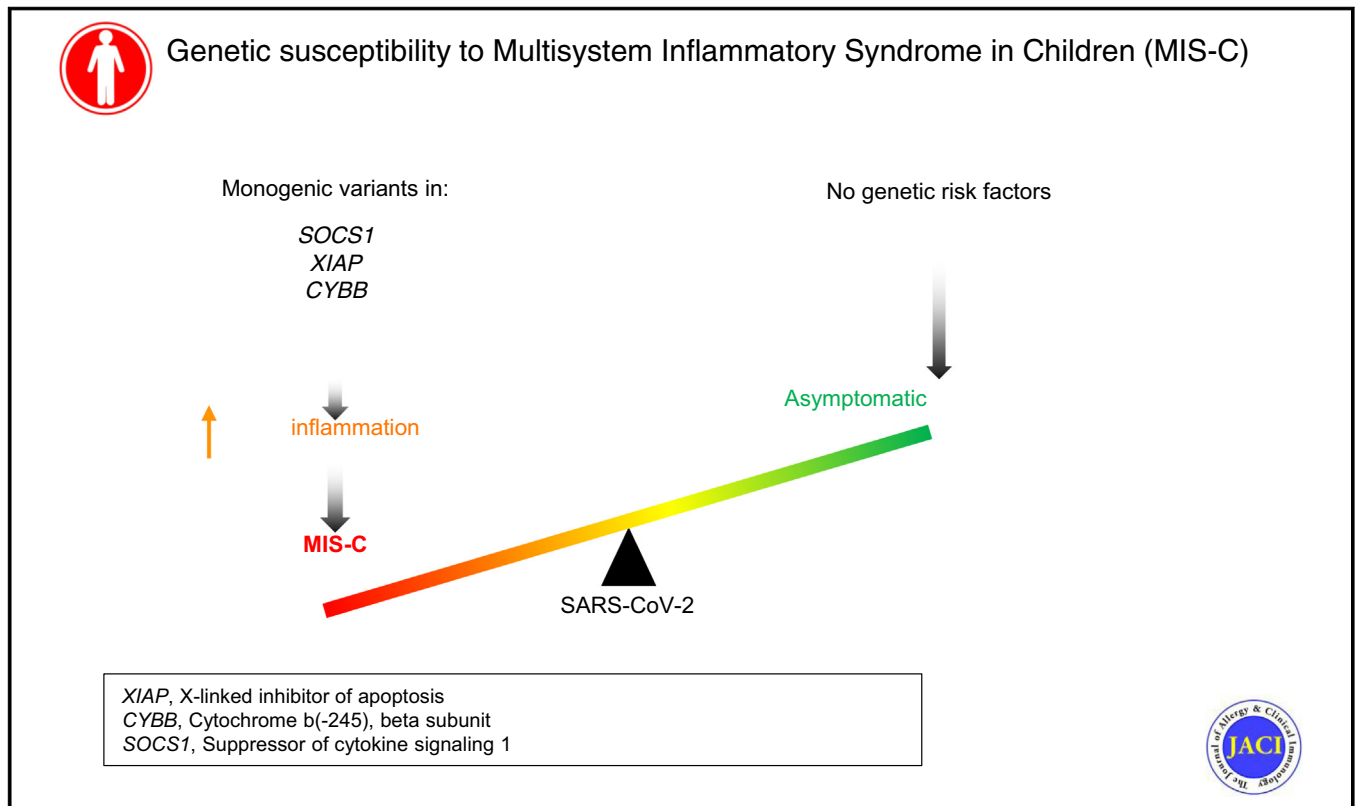
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Mechanisms underlying genetic susceptibility to multisystem inflammatory syndrome in children (MIS-C)



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GRAPHICAL ABSTRACT



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Background: Multisystem inflammatory syndrome in children (MIS-C) is a pediatric complication of severe acute respiratory syndrome coronavirus 2 infection that is characterized by multiorgan inflammation and frequently by cardiovascular dysfunction. It occurs predominantly in otherwise healthy children. We previously reported haploinsufficiency of suppressor of cytokine signaling 1 (SOCS1), a negative regulator of type I and II interferons, as a genetic risk factor for MIS-C.

Objectives: We aimed to identify additional genetic mechanisms underlying susceptibility to severe acute respiratory syndrome coronavirus 2–associated MIS-C.

Methods: In a single-center, prospective cohort study, whole exome sequencing was performed on patients with MIS-C. The impact of candidate variants was tested by using patients' PBMCs obtained at least 7 months after recovery.

Results: We enrolled 18 patients with MIS-C (median age = 8 years; interquartile range = 5–12.25 years), of whom 89% had no conditions other than obesity. In 2 boys with no significant infection history, we identified and validated hemizygous deleterious defects in *XIAP*, encoding X-linked inhibitor of apoptosis, and *CYBB*, encoding cytochrome b-245, beta subunit. Including the previously reported *SOCS1* haploinsufficiency, a genetic diagnosis was identified in 3 of 18 patients (17%). In contrast to patients with mild COVID-19, patients with defects in *SOCS1*, *XIAP*, or *CYBB* exhibit an inflammatory immune cell transcriptome with enrichment of differentially expressed genes in pathways downstream of IL-18, oncostatin M, and nuclear factor κ B, even after recovery.

Conclusions: Although inflammatory disorders are rare in the general population, our cohort of patients with MIS-C was enriched for monogenic susceptibility to inflammation. Our results support the use of next-generation sequencing in previously healthy children who develop MIS-C. (*J Allergy Clin Immunol* 2021;148:732–8.)

Key words: Multisystem inflammatory syndrome in children, MIS-C, COVID-19, SARS-CoV-2, whole exome sequencing

INTRODUCTION

Multisystem inflammatory syndrome (MIS-C) is a life-threatening complication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposure occurring in individuals younger than 21 years of age.^{1,2} As defined by the Centers for Disease Control and Prevention, the diagnostic criteria include fever, elevated inflammatory marker levels, multisystem organ involvement, and SARS-CoV-2 infection or exposure within 4 weeks of symptoms without an alternative diagnosis.³ Because most children with MIS-C have mild or no symptoms at the time of initial infection, MIS-C is thought to be a postinfectious syndrome. Nearly all patients with MIS-C have detectable antibodies to SARS-CoV-2, and many have detectable SARS-CoV-2 virus by RT-PCR testing.^{1,2} The clinical features of MIS-C overlap with those of acute coronavirus disease 2019 (COVID-19) and those of the pediatric vasculitic disease Kawasaki disease.^{1,2} Symptoms include fever, rash, gastrointestinal symptoms, coagulopathy, cardiac dysfunction, and/or shock.^{1,2} Studies of hospitalized adults with severe COVID-19 have identified deleterious genetic

Abbreviations used

CGD:	Chronic granulomatous disease
COVID-19:	Coronavirus disease 2019
CYBB:	Cytochrome b-245, beta subunit
HLH:	Hemophagocytic lymphohistiocytosis
IVIG:	Intravenous immunoglobulin
MIS-C:	Multisystem inflammatory syndrome in children
NOD2:	Nucleotide-binding oligomerization domain-containing 2
SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2
SOCS1:	Suppressor of cytokine signaling 1
TLR:	Toll-like receptor
WES:	Whole exome sequencing
XIAP:	X-linked inhibitor of apoptosis

variants impairing type I interferon signaling in up to 3.5% of patients.^{4,5} In contrast, MIS-C is not associated with preexisting cardiopulmonary, autoimmune and/or immune, or hematologic diseases, and its genetic basis is largely unknown.¹

We previously reported haploinsufficiency of suppressor of cytokine signaling 1 (*SOCS1*), a negative regulator of type I and II interferons, as a genetic risk factor for MIS-C.⁶ Here, we present findings from our prospective cohort sequencing study of children and adolescents with MIS-C.

RESULTS AND DISCUSSION

Whole exome sequencing (WES) was performed on 18 patients with a diagnosis of MIS-C (Table 1). Nearly all of them (89% [n = 16]) had no preexisting medical conditions (other than obesity in 44%). Nine patients required critical care during their hospital stay. This cohort's median age, predominance of Hispanic ethnicity, lack of other comorbidities, and clinical characteristics are concordant with those of previously published cohorts of patients with MIS-C.^{1,2} In addition to *SOCS1* haploinsufficiency,⁶ we identified defects in X-linked inhibitor of apoptosis (*XIAP*) and *CYBB*, amounting to a genetic diagnosis in 3 of 18 patients in our cohort (17%).

Patient 1 is an 11-year-old boy with no history of disease other than obesity and resolved pityriasis lichenoides chronica. He developed fever, vomiting, diarrhea, rash, and conjunctivitis. The result of RT-PCR testing for SARS-CoV-2 was negative on the fourth day of illness but positive on the seventh. The patient had neutrophilia; T-cell lymphopenia; increased levels of the T-cell activation marker soluble CD25; and elevated levels of IL-18, IL-6, IL-10, and C-X-C motif chemokine ligand 9 (CXCL9) indicative of type I and II interferon signaling (Table 2). He developed warm cardiogenic shock in the setting of severely depressed systolic cardiac function and third-degree heart block. He was treated with remdesivir, anakinra, methylprednisolone (2 mg/kg twice daily), intravenous immunoglobulin (IVIG) (1 g/kg), and vasopressors. While taking methylprednisolone, he was found to have EBV viremia (4.3 log₁₀ copies/mL). He had undetectable IgM to capsid and early D antigen, consistent with EBV reactivation. His EBV viremia peaked at 6 log₁₀ copies/mL while he was taking prednisone, and it persisted at 3.9 log₁₀ copies/mL for 9 months until it was cleared by 1 dose of rituximab. After he recovered from MIS-C, his natural killer cytotoxicity function was found to be normal.

TABLE I. Summary features of enrolled patients

Feature	MIS-C, no. (%) (N = 18)
Age (y)	
Median	8
Age range	5- 2.25
Sex, no. (%)	
Male	8 (44)
Female	10 (56)
Race and ethnicity, no. (%)	
White, non-Hispanic	2 (11)
Black, non-Hispanic	1 (5.5)
Asian, non-Hispanic	1 (5.5)
Other, non-Hispanic	1 (5.5)
White, Hispanic	5 (28)
Black, Hispanic	1 (5.5)
Other, Hispanic	7 (39)
Prior medical diagnoses, no. (%)	
None (excluding overweight and obesity)*	16 (89)
Body mass index, no. (%)	
Normal (<85th percentile for age and sex)	7 (39)
Overweight (85th-95th percentile for age and sex)	3 (17)
Obesity (>95th percentile for age and sex)	8 (44)
SARS-CoV-2 testing result, no. (%)	
Positive for SARS-CoV-2 RT-PCR	8 (44)
Positive for SARS-CoV-2 serology†	18 (100)
Hospital care required, no. (%)	
Required critical care	9 (50)

*Of the 2 patients with preexisting medical conditions, 1 had sickle cell anemia and the other had Evans syndrome.

†Positive SARS-CoV-2 serology was obtained by using either the Roche Elecsys or Viracor assay.

WES identified a novel hemizygous missense variant in *XIAP* (NP_001158.2: p.Ser421Asn). *XIAP* is a widely expressed protein that contributes to cellular survival, activation, and negative regulation of the NLRP3 inflammasome.⁷ This variant has a minor allelic frequency of 1.1×10^{-5} in the Genome Aggregation Database and was predicted to be benign with a Combined Annotation Dependent Depletion score of 4.9. However, structural modeling predicts that the variant creates a hydrogen bond with alanine 417 within the ubiquitin-associated domain, thereby potentially disrupting the domain's structure and/or capacity for binding ubiquitin (Fig 1, A). The patient exhibited intact *XIAP* protein expression (Fig 1, B). The ubiquitin-associated domain is important for TNF- α secretion following nucleotide-binding oligomerization domain-containing 2 (NOD2) activation.⁷ Compared with the controls, CD14⁺HLA-DR⁺ monocytes from patient 1 secreted less TNF- α in response to NOD2 activation with muramyl dipeptide (Fig 1, C), which is indicative of impaired *XIAP* function. TNF- α secretion after LPS stimulation, which is independent of NOD2 signaling, was intact in the patient (Fig 1, C). Patients with hemizygous loss-of-function variants in *XIAP* are at risk for virally triggered hemophagocytic lymphohistiocytosis (HLH) and cytokine storm syndromes.⁸ This has been attributed to loss of *XIAP*-mediated negative regulation of the NLRP3 inflammasome, which relies on the protein's ubiquitylation function.^{7,9} Mice lacking either *XIAP* or *XIAP*-mediated ubiquitylation secrete increased IL-1 β downstream of Toll-like receptor 3 (TLR3)- or TLR4-mediated activation of the NLRP3 inflammasome.⁹ In contrast, *XIAP* is not required for IL-6 or TNF- α secretion after TLR stimulation.¹⁰ TLR3 binds

to double-stranded viral RNA intermediates, whereas TLR4 binds to SARS-Cov-2 spike protein.^{4,11} Compared with the controls, PBMCs from patient 1 secreted increased IL-1 β after TLR3 and TLR4 stimulation (Fig 1, D). As anticipated, TNF- α secretion after TLR3 or TLR4 stimulation was comparable between patient 1 and the controls (Fig 1, E). These findings show the impaired function of *XIAP*^{Ser421Asn}, leading to inflammatory signaling that likely predisposed this patient to MIS-C.

Patient 2 is a 16-year-old male whose clinical course has been recently described.¹² He was healthy until 3 weeks before admission, when he developed hematochezia. He had neutrophilia, CD4⁺ and CD8⁺ T-cell lymphopenia, procalcitonin and C-reactive protein levels exceeding the upper normal limits by 100- and 30-fold, a mild coagulopathy, and a positive result of IgG testing for SARS-CoV-2 (Table II). Gastrointestinal biopsies revealed duodenitis, patchy colitis, and crypt abscesses consistent with Crohn disease; notably, he also had duodenal submucosal vasculitis atypical for inflammatory bowel disease. Despite treatment with broad-spectrum antibiotics, methylprednisolone (0.3 mg/kg twice daily), vitamin K, and bowel rest, he had persistent episodes of fever, elevated inflammatory marker levels, and worsening hematochezia. After 7 days of hospitalization, he became febrile to 40°C and severely hypotensive. He had persistent T-cell lymphopenia with a predominance of naive CD4⁺ and CD8⁺ T cells and an elevated soluble CD25 level (Table II). No secondary infections were identified. As his duodenal vasculitis, coagulopathy, and compensated shock were more consistent with MIS-C than with inflammatory bowel disease, he was given methylprednisolone (increased to 0.5 mg/kg twice daily) and high-dose IVIG. He had rapid improvement of his inflammatory marker levels, hematochezia, and diarrhea. He is currently taking infliximab and is clinically well.

In patient 2, WES identified a novel hemizygous missense variant in *CYBB* (p. Arg229Thr), which encodes the p91^{phox} subunit of the NADPH oxidase that is essential for the phagocytic oxidative burst. Deleterious variants in *CYBB* cause chronic granulomatous disease (CGD). Although the crystal structure of the extracellular domain harboring this variant has not yet been identified (Fig 1, F), this variant is predicted to be pathogenic, with a Combined Annotation Dependent Depletion score of 31, and it has a minor allelic frequency of 3.9×10^{-4} in the Genome Aggregation Database. The patient's neutrophil oxidative burst was impaired but not absent (Fig 1, G). This contrasts with the minimal neutrophil oxidative burst typical of *CYBB* variants that causes classical CGD, thereby indicating the variant's hypomorphic effect on p91^{phox} function. Unlike patients with typical inflammatory bowel disease, the majority of patients with CGD experience inflammatory sequelae, including infection-associated cytokine storm syndromes and granulomatous lesions.¹³ Loss of NADPH oxidase function impairs the generation of reactive oxidant species that inhibit type I interferon signaling, resulting in a proinflammatory macrophage phenotype.¹³⁻¹⁵

We previously showed that *SOCS1* haploinsufficiency in a patient with a history of MIS-C leads to increased tonic type I and/or II interferon signaling in unstimulated PBMCs on account of reduced inhibition of the Janus-activated kinases. Transcriptomic analysis of unstimulated PBMCs obtained at least 7 months after recovery from SARS-CoV-2 infection identified 712 genes with more than a 1.5-fold difference between the 3 patients in this cohort with likely genetic susceptibility to MIS-C and 4 otherwise healthy children with a history of mild COVID-19. Differentially

TABLE II. Results of immunologic evaluation of the patients

Indicator	Patient 1	Patient 2		Reference value
	Day 1	Day 1	Day 11	
Hemogram results				
White blood cells (10 ³ cells/ μ L)	18.0	21.7	29.4	5.52-9.29
Neutrophils (10 ³ cells/ μ L)	15.4	25.6	24.3	3.04-6.06
Lymphocytes (10 ³ cells/ μ L)	1.27	0.69	1.12	1.17-2.30
Monocytes (10 ³ cells/ μ L)	0.60	0.24	1.55	0.19-0.72
Platelets (10 ³ cells/ μ L)	173	395	621	189-342
Inflammatory markers				
C-reactive protein (mg/dL)	21.9	12.8	10.8	\leq 0.5
Fibrinogen (mg/dL)	551	528	623	200-400
Ferritin (ng/mL)	1138	231	1116	10-80
D-dimer (μ g/mL)	3.1	9.1	4.2	\leq 0.5
Soluble CD25 (pg/mL)	14,800	nd	1550	\leq 1033
Lymphocyte subsets				
CD3 ⁺ (cells/ μ L)	516	475	883	1000-2600
CD3 ⁺ CD4 ⁺ (cells/ μ L)	357	262	443	530-1500
Naive (% CD4 ⁺)	64.8	nd	62.6	21-61.4
Central memory (% CD4 ⁺)	16.7	nd	20.6	26.8-62.1
Effector memory (% CD4 ⁺)	13.1	nd	16.1	7.6-25.1
TEMRA (% CD4 ⁺)	5.4	nd	0.8	0.1-4.0
CD3 ⁺ CD8 ⁺ (cells/ μ L)	145	196	196	330-1100
Naive (% CD8 ⁺)	60.3	nd	79.6	11.4-66.5
Central memory (% CD8 ⁺)	16.7	nd	2.4	3.7-23.2
Effector memory (% CD8 ⁺)	13.1	nd	13.6	16.8-54.6
TEMRA (% CD8 ⁺)	18.6	nd	4.4	5.6-43.9
CD19 ⁺ (cells/ μ L)	421	197	387	110-570
Naive (% CD19 ⁺)	65.6	nd	72.1	48.4-79.7
Unswitched memory (% CD19 ⁺)	8.10	nd	8.8	7.0-23.80
Switched memory (% CD19 ⁺)	21.1	nd	14.3	8.30-27.8
Plasmablast (% CD19 ⁺)	9.7	nd	2.7	0.1-2.4
CD3 ⁻ CD56 ⁺ (cells/ μ L)	73	60	81	70-480
Immunoglobulin levels				
IgG (mg/dL)	1147	1423	1522	639-1344
IgM (mg/dL)	320	90	148	40-240
IgA (mg/dL)	169	nd	97	70-312
Positive titers to pneumococcal subtypes (out of 23 subtypes)	17	nd	8	$>$ 14
Tetanus (IU/mL)	0.1	nd	3.62	$>$ 0.15
Cytokines (pg/mL)				
IL-2	7	nd	$<$ 5	\leq 12
IL-12	$<$ 5	nd	$<$ 5	\leq 6
IL-10	33	nd	11	\leq 18
IL-6	44	nd	8	\leq 5
IL-18	1427	nd	nd	89-540
CXCL9 (induced by type I and type II interferons)	1575	nd	nd	$<$ 121

Bolded values are outside the normal range. At the time of this blood draw, patient 1 had not received any immunomodulatory medications, whereas patient 2 had received methylprednisolone (0.3 mg/kg) treatment for 6 days. IL-12, IFN- γ , IL-4, IL-5, IL-13, IL-17, IL-1 β , IL-8, and TNF- α levels were normal in both patients. Neither patient had received IVIG before testing. Naive T cells, CD45RA⁺CCR7⁺, Central memory T cells, CD45RA⁻CCR7⁺, effector memory T cells, CD45RA⁻CCR7⁻, TEMRA, CD45RA⁺CCR7⁻. Naive B cells, CD27⁺IgD⁺, unswitched memory B cells, CD27⁺IgD⁺, switched memory B cells, CD27⁺IgD⁻, plasmablasts CD24^{low}CD38^{high}. nd, Not detected; TEMRA, terminally differentiated effector cell.

expressed genes were enriched in signaling pathways downstream of the inflammatory response, nuclear factor κ B, IL-18, oncostatin M, and type I interferon signaling (Fig 2, A). Increased level of IL-18, a marker of inflammasome activation, is a known finding in individuals with defects in XIAP, CYBB, or SOCS1.^{14,16,17} Oncostatin M, a member of the IL-6 family of cytokines, induces type I interferon-stimulated genes.¹⁸ Type I interferon signaling triggers activation of the NLRP3 inflammasome in tissues such as the gastrointestinal and respiratory epithelium and coronary artery endothelium, as well as in myeloid cells.^{19,20} However, recovered patients lacking a genetic

diagnosis for MIS-C did not exhibit significantly increased interferon or inflammatory gene expression in their unstimulated PBMCs (Fig 2, A). This contrasts with the robust immune cell activation characteristic of active MIS-C highlighted here by single-cell RNA sequencing of PBMCs from a patient with ongoing MIS-C despite the lack of any identifiable genetic risk factors. Compared with the control cells, the cells of the patient with active MIS-C exhibited enrichment of differentially expressed genes promoting interferon signaling in CD4⁺ T cells, CD8⁺ T cells, CD14⁺CD16^{low} classical monocytes, and natural killer cells (Fig 2, B). Thus, the pathways upregulated in PBMCs

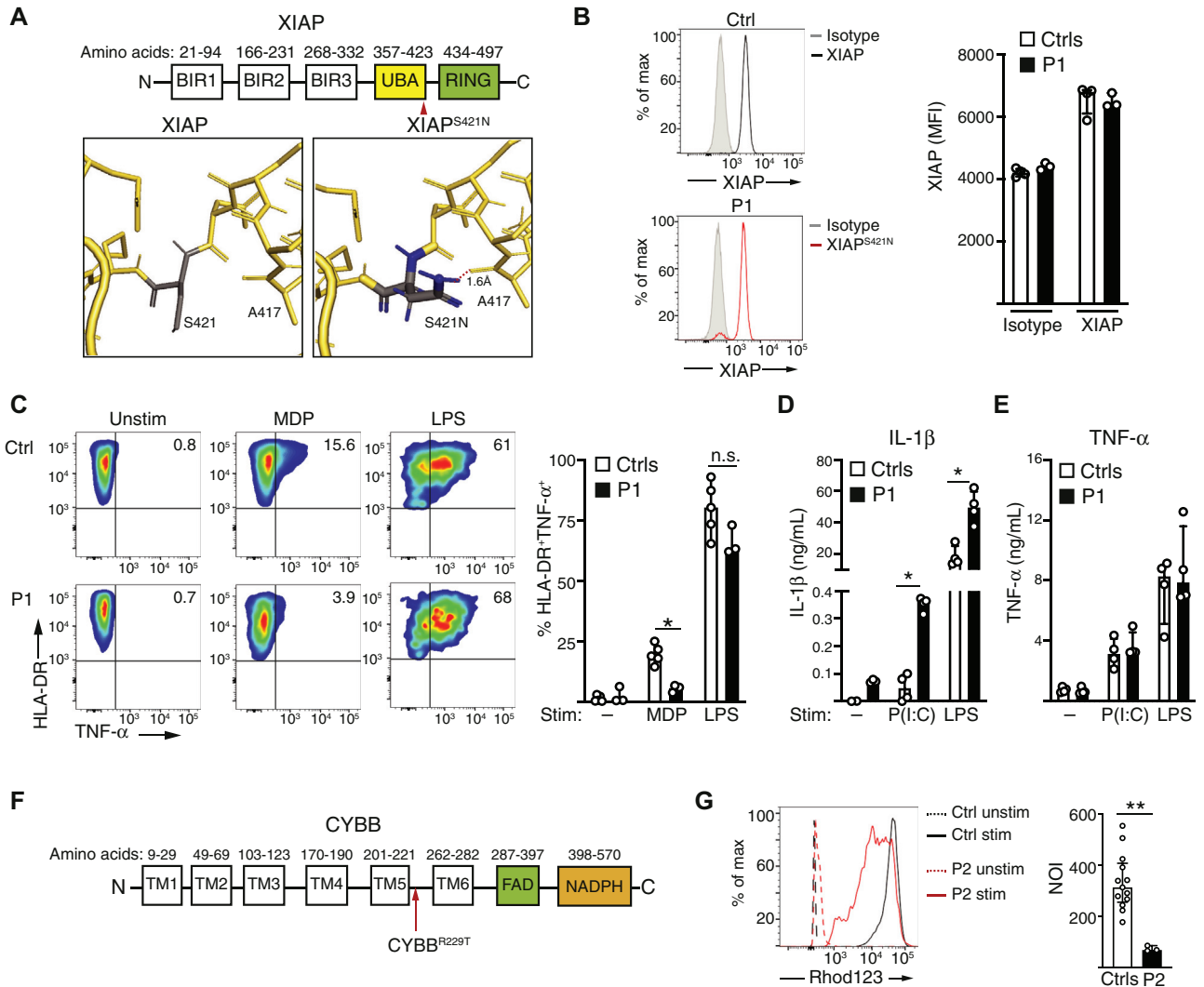


FIG 1. Genetic risk factors for MIS-C. **A**, Schematic of XIAP with structural modeling of the ubiquitin-associated (UBA) domain identifies a new hydrogen bond (indicated by the dotted red line) formed between the S421N mutant found in patient 1 and alanine 417. **B**, Flow cytometric quantification of XIAP protein expression in CD14⁺ monocytes from a control (Ctrl) and the patient from 2 experiments with 3 controls. **C**, Quantification of HLA-DR⁺TNF- α ⁺ monocytes, gated on CD14⁺ cells, after stimulation with 200 ng/mL of muramyl dipeptide or LPS for 2.5 hours in 2 experiments with 5 controls. **D**, IL-1 β secretion after stimulation of PBMCs from 4 controls and patient 1 with indicated stimuli, pooled from 2 experiments. **E**, TNF- α secretion after stimulation of PBMCs from 4 controls and patient 1, pooled from 2 experiments. **F**, Schematic of CYBB. **G**, Quantification of the neutrophil oxidative burst in the presence and absence of stimulation with phorbol 12-myristate 13-acetate, pooled from 3 independent experiments with 13 controls. *max*, Maximum; *MFI*, mean fluorescence intensity; *n.s.*, not significant; *NOI*, neutrophil oxidative index; *stim*, stimulated; *unstim*, unstimulated.

from recovered, genetically susceptible patients converge with those also increased during active MIS-C.

Patients previously known to have primary immunodeficiencies or autoinflammatory disorders are at increased risk of development of life-threatening COVID-19 rather than MIS-C.²¹ The cohort that we have presented is unique from those of prior studies in that our patients have hypomorphic variants with mild to no immunologic sequelae before SARS-CoV-2 infection. Therefore, varying degrees of immunologic impairment may result in clinical outcomes distinct from those of SARS-CoV-2 infection. Our report thus provides proof of principle for recent perspectives proposing

that genetic risk factors for MIS-C may be incompletely penetrant.²² Future studies with larger cohorts are needed to determine additional genetic risk factors for MIS-C, because the risk of MIS-C may vary among different genetic causes of autoinflammation.

In identifying a genetic variant that affects immunity in 17% of patients, our study suggests that MIS-C may indicate an underlying disorder of immune dysregulation. As clinical-grade WES has become increasingly accessible as a diagnostic tool, this study has clinically relevant implications for the use of WES in identifying inborn errors of immunity in patients with MIS-C.

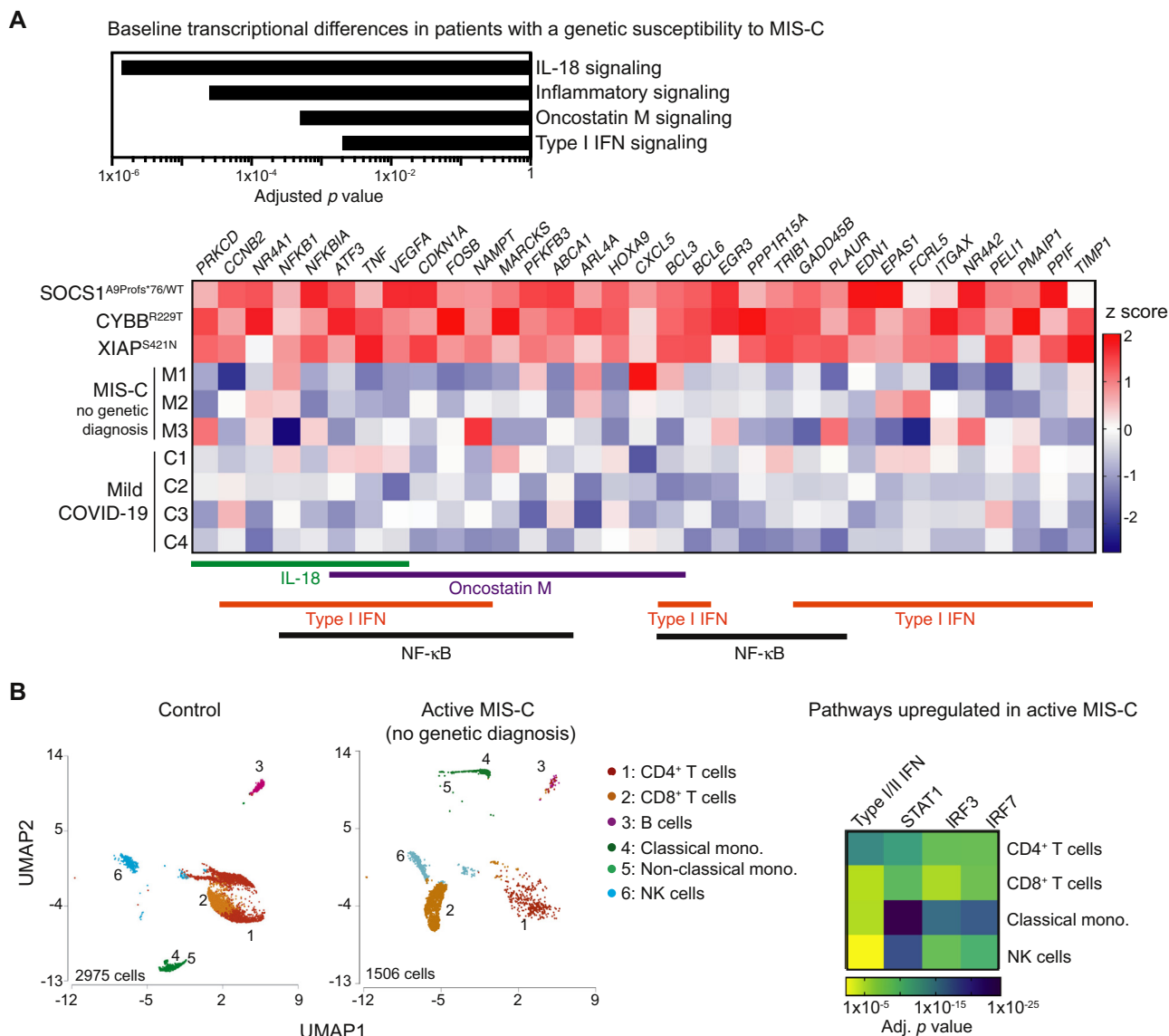


FIG 2. A, Transcriptome analysis of unstimulated PBMCs from patients 1 and 2, as well as those from the patient with *SOCS1* haploinsufficiency whom we previously described,⁶ compared with PBMCs from 4 otherwise healthy individuals who had previously had mild COVID-19. PBMCs were collected at least 7 months after recovery, at which time the individuals had returned to their baseline state of health. * $P < .05$; ** $P < .01$ by the Mann-Whitney test. **B**, Uniform Manifold Approximation and Projection (UMAP) plots depict transcriptional clusters generated by single-cell RNA sequencing of PBMCs from a control and a patient with active MIS-C but no identifiable genetic diagnosis. This research sample was taken early in the patient's course; she had received 1 dose of IVIG but subsequently required additional doses of IVIG and methylprednisolone before clinical improvement occurred. Ingenuity Pathway Analysis of differentially expressed genes indicates upregulation of signaling pathways downstream of type I and/or II interferons, *STAT1*, *IRF3*, and *IRF7*. *mono*, Monocyte; *NF-κB*, nuclear factor κB; *NK*, natural killer.

For detailed methods, please see the [Methods](#) section of this article's Online Repository materials (available at www.jacionline.org).

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Key messages

- In this prospective study of 18 patients with MIS-C, 17% of patients were found to have a genetic variant impairing negative regulation of interferon and inflammatory signaling.
- A history of MIS-C should prompt consideration of WES for the identification of variants affecting host immunity in affected children.

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METHODS

Study design

This prospective observational study was approved by the Boston Children's Hospital Institutional Review Board. Informed assent and/or consent was obtained from participants and/or their legal guardians.

WES

WES was performed by GeneDx using IDT xGen probes with an average coverage across the WES of 100× and more than 95% of targets covered at 20×, as previously described.^{E1} Variant calling and candidate variant analysis were completed by utilizing the Boston Children's Hospital Genomic Learning System, as previously described.^{E1} Minor allelic frequencies for the specified variants were identified by using the Genome Aggregation Database.^{E2}

Single-cell RNA sequencing

For each sample, approximately 17,000 PBMCs at a concentration of 1000 cells/μL were input into a 10× Genomics Chromium Controller. Chromium Next GEM Single Cell 3' kits (version 3.1) were used to generate single-cell gene expression libraries, which we subsequently sequenced by using an Illumina NextSeq 500 system with 150-bp paired-end sequencing. Libraries were processed by using Cell Ranger version 3.1 (10× Genomics, Pleasanton, Calif) and GrCh38 as the reference. Partek Flow was used to analyze the data output from Cell Ranger (data filtering, log normalization, integration, scaling, dimensionality reduction, and cluster identification). We excluded genes expressed in fewer than 5 cells, as well as those encoding ribosomal structural proteins and noncoding ribosomal RNA.^{E3} Low-quality cells with more than a 10% mitochondrial gene content or fewer than 200 features were also excluded. Principal component analysis was performed by using the elbow heuristics method to determine the 15 top principal components for subsequent clustering analysis using the Louvain clustering algorithm, followed by Uniform Manifold Approximation and Projection visualization. Pathway analysis of differentially expressed genes within cell types was performed by using Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, Calif).

Flow cytometry

Protocols for staining of XIAP were performed as previously described.^{E4} For quantification of intracellular TNF-α, PBMCs were rested overnight in a 48-well plate and nonadherent cells were removed the following day. Adherent PBMCs, consisting primarily of monocytes, were stimulated with lipidated muramyl dipeptide (200 ng/mL, InvivoGen, San Diego) or LPS (200 ng/mL, InvivoGen) for 2.5 hours with brefeldin A, followed by flow cytometric staining for HLA-DR⁺CD14⁺TNF-α⁺ monocytes as previously

described.^{E5} IL-1β secretion downstream was measured by using cytometric bead array (BD Biosciences, Piscataway, NJ) per the manufacturer's protocol after PBMCs were stimulated with Poly(I:C) (10 μg/mL; InvivoGen, San Diego, Calif) or LPS (100 ng/mL; InvivoGen) for 24 hours.

Transcriptome analysis

mRNA was isolated from PBMCs by using the RNeasy Mini Kit (Qiagen), followed by cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific). The Ion AmpliSeq Transcriptome Human Gene Expression Kit was used to prepare bar-coded libraries and sequenced by using an Ion S5 next-generation sequencer. The AmpliSeqRNA plug-in (ThermoFisher Scientific) was used to calculate differential gene expression analysis. Pathway analysis was performed by using Ingenuity Pathway Analysis and Gene Set Enrichment Analysis (Broad Institute and University of California San Diego)^{E6,E7} on genes with at least a 1.5-fold difference between the controls and patients ($P < .05$). Final analyses were performed by using adjusted P values calculated with the Benjamini-Hochberg procedure.

Statistical analysis

All tests were 2 sided, and P values less than .05 were considered significant when using the indicated statistical tests. Statistical analyses were performed by using Prism 8.0 software (GraphPad Software, San Diego, Calif).

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