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Inflammasome activation in children with Kawasaki disease and Multisystem Inflammatory Syndrome

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Multisystem inflammatory syndrome in children (MIS-C) is an acute inflammatory response following exposure to SARS-CoV-2, and Kawasaki disease (KD) is a pediatric vasculitis of medium-sized arteries whose etiology is unknown. The clinical presentation and cardiovascular outcomes of MIS-C overlap with those of KD and KD shock syndrome (KDSS).[1] However, MIS-C patients have a greater magnitude of systemic

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Disclosure
None.

Ethics approval and consent to participate
The Human Research Protection Program of the University of California San Diego approved this research protocol, and written informed consent was obtained from the parents of all subjects and adolescent, or child assent was obtained as appropriate.

inflammation. Inflammasomes are intracellular protein complexes of the innate immune system that respond to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern molecules (DAMPs). Canonical inflammasomes, such as NLRP3, activate caspase-1 which then proteolytically cleaves pro-interleukin (IL)-1β and pro-IL-18 and induces pyroptosis, an inflammatory cell death. We previously showed that TIFA [TRAF-interacting protein with a forkhead-associated (FHA) domain], a crucial innate immune mediator, regulates the activation of the NLRP3 inflammasome.[2] Additionally, activation of the non-canonical inflammasome is associated with caspase-4- or caspase-5-dependent pyroptosis, which is known to be induced by intracellular lipopolysaccharide (LPS) from Gram-negative bacteria.[3,4] Interestingly, the activation of caspase-4/5 also induces the canonical NLRP3 inflammasome.

A central enigma is whether MIS-C fits into a spectrum of disease related to typical KD or whether the molecular pattern of activation of inflammation is distinct. To address this question, we first analyzed the whole blood transcription profiles from acute (pre-treatment) and convalescent KD cohorts.[5] Levels of *TIFA*, *NLRP3*, *CASP1*, *CASP4*, *CASP5*, and *IL1B* mRNA were significantly elevated in acute versus convalescent KD patients (Figure [A]). Thus, both the NLRP3/caspase-1-dependent canonical inflammasome and the caspase-4/5-dependent non-canonical inflammasome participate in the pro-inflammatory immune response of KD patients at the transcriptional level. Because MIS-C and KD patients share many clinical features,[1] we compared transcript levels of *TIFA*, *NLRP3*, *CASP1*, *CASP4*, *CASP5*, and *IL1B* in whole blood from recent patients with MIS-C, KDSS, and KD. Blood samples from healthy children with a remote history of KD and normal coronary arteries were used as convalescent controls. The table in Figure [B] describes the demographic and clinical features of these four patient groups. Transcript levels for *TIFA* and markers of the canonical and non-canonical inflammasome were similarly increased in the whole blood of MIS-C, KDSS, and KD, when compared with convalescent controls (Figure [C]). However, plasma levels of IL-1β, as revealed by Meso Scale assay, were low (<1 pg/ml) in most of the patients (Figure [D]), which were unable to correlate with the IL-1β transcript levels in the whole blood.

Because neutrophils are the predominant white blood cell population in MIS-C and KD, we compared the inflammasome activation in granulocytes from acute, pre-treatment MIS-C, KD, and pediatric febrile patients (FC) patients. The clinical features of these individuals are summarized in Figure [E]. Granulocytes from MIS-C, but not from KD and FC patients, showed a significant activation of caspase-4 and caspase-1, demonstrated by the increase in their cleaved forms (Figure [F]). Similarly, TIFA protein expression was induced only in the MIS-C granulocytes. The correlation of TIFA and caspase-4 with MIS-C was strongly supported by data from one MIS-C individual (patient 6) who had blood sampled before treatment on Days 4 and 6. Even on Day 4, the levels of TIFA and caspase-4 in this patient were higher than KD patients having similar plasma levels of CRP and those of FC patients (Figure [E] and [F]). Such TIFA and caspase-4 activation was even more prominent at Day 6. As well, plasma levels of IL-1β were low (Figure [G]).

Altogether, our results demonstrated a robust non-canonical inflammasome induction in granulocytes in MIS-C patients. The small number of patients studied reflects the fact that

this is a rare condition and because these children are very ill at the time of presentation, it is challenging to obtain research samples. Although increased canonical and non-canonical inflammasome-related mRNAs were detected in the whole blood of MIS-C and KD patients, the caspase-4/5-dependent non-canonical inflammasome was unique to MIS-C. The activation of non-canonical inflammasome may lead to the induction of caspase-1-dependent NLRP3 inflammasome, pyroptotic cell death, and release of pro-inflammatory cytokines in MIS-C patients. Because LPS would not be involved in the pathogenesis of MIS-C, molecular patterns other than those from Gram-negative bacteria must be involved in the induction of non-canonical inflammasome in MIS-C. TIFA has been shown to assemble the inflammasome through its own oligomerization[2]. The correlated TIFA induction and caspase-4 activation in MIS-C suggests a role of TIFA in non-canonical inflammasome activation. Currently, intravenous immunoglobulin (IVIG) is the mainstay of therapy for MIS-C and KD. Because our results show specific activation of the non-canonical inflammasome in MIS-C, drugs targeting inflammasomes might be attractive options for the treatment of MIS-C. Although these data point to many similarities between KD and MIS-C, the unique activation of the non-canonical inflammasome in neutrophils may be responsible for the more intense inflammation that is the hallmark of these MIS-C patients.

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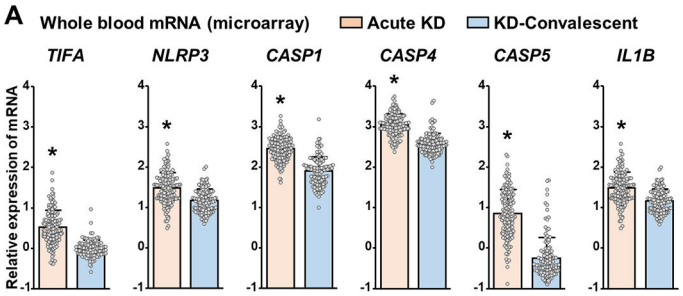
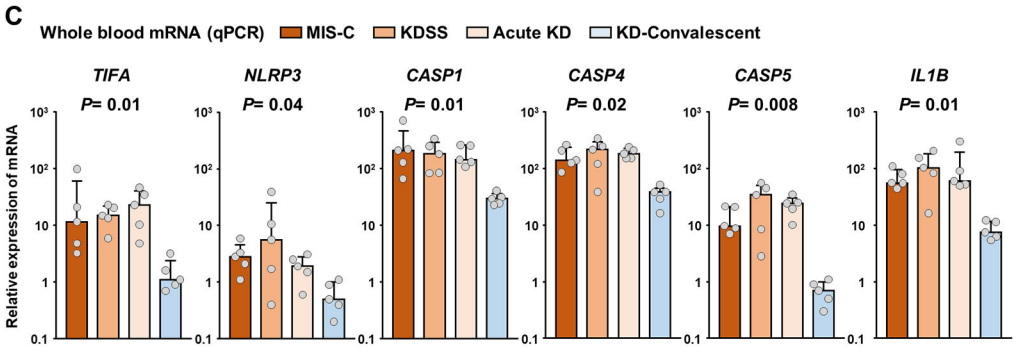


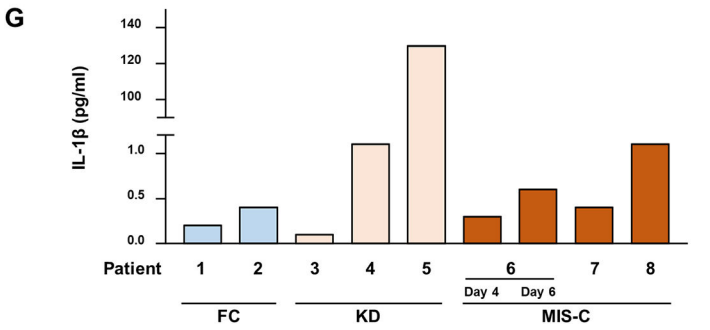
B

	MIS-C (n=5)	KDSS (n=5)	KD (n=5)	KD conv. (n=5)	P
Male, N (%)	3 (60)	1 (20)	3 (60)	1 (20)	NS
Age, years	10.5 (7.5-11.3)	6.9 (6.0-8.3)	9.1 (6.8-9.2)	10.3 (8.7-10.3)	NS
Illness day	3 (3-4)	5 (5-5)	4 (4-5)	484 (429-992)	NS
WBC (10^3/μL)	10.9 (4.9-11.3)	12.5 (12.4-14)	15.9 (14-22.1)	NA	NS
ANC (10^3/μL)	7.3 (4.7-9.0)	11.4 (7.2-11.6)	14.2 (10.6-20.3)	NA	NS
CRP (mg/dL)	20.7 (19.8-24.8)	23.6 (16.6-26.9)	18.8 (14.6-19)	NA	NS
LVEF (%)	58 (57.0-66.0)	51.5 (45.8-56.5)	65.5 (63.0-72.3)	NA	NS
coronary artery aneurysm, N (%) *	2 (40)	1 (20)	2 (40)	1 (20)	NS
SARS-CoV-2 IgG positive or exposure, N	5	0	0	0	NA



E

	FC (n=2)		KD (n=3)			MIS-C (n=3)			
Patient	1	2	3	4	5	6		7	8
Age, years	7.0	1.6	2.8	1.5	3.2	11.2		7.1	13.4
Sex	M	F	F	F	F	F		M	F
Illness day*	8	4	4	7	4	4	6	7	5
WBC (10^3/μL)	9.4	10.6	11.4	22.3	5.4	9.7	11.7	5.7	11.3
ANC (10^3/μL)	5.0	1.1	4.3	11.2	4.1	8.4	9.9	4.6	11.2
ALC (10^3/μL)	3.1	8.8	5.9	8.0	0.9	0.8	1.2	0.8	0
CRP (mg/dL)	<0.5	<0.5	4.3	4.1	6	5.3	27.6	17	30.5
LVEF (%)	NA	NA	61	61	65	NA	67**	44	58
coronary artery aneurysm***	–	–	–	–	–	–	–	+	–
SARS-CoV-2 IgG	–	–	–	–	–	+	+	+****	+



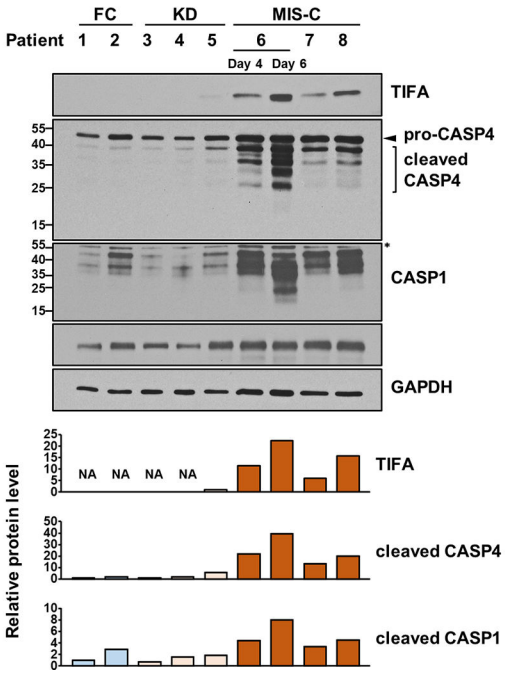


Figure.

(A) mRNA transcripts in whole blood from patients with acute (n=146) and paired convalescent (n=131) KD were measured by microarray.[5] Transcript levels of *NLRP3*, *CASP1*, *CASP4*, *CASP5*, *IL1B*, and *TIFA* were compared. Results were expressed as mean ± SD, and the statistical significance was determined by 2-tailed Student *t* test.

(B) Demographics of MIS-C, KDSS, acute KD patients and KD convalescent healthy controls. Patients with MIS-C were diagnosed between May-June 2020 based on the CDC case definition (4/5 patients were SARS-CoV-2 nucleocapsid IgG antibody positive by

the Abbott Architect assay). The 5th patient whose IgG was negative had a parent who was an essential worker. The patients with KD were diagnosed according to AHA criteria between April 2012 to February 2019 before SARS-CoV-2. The patients with KDSS were diagnosed between October 2004 and March 2020 (the single KDSS patient from 2020 was SARS-CoV-2 IgG antibody negative). KD, KDSS and controls were matched 1:1 with MIS-C patients based on age (± 6 years), illness day (± 3 days), and when possible, acute, pre-treatment CRP level. Control subjects were healthy children with a remote history of KD and normal echocardiograms (429–992 days after the onset of KD). Four of five control subjects were paired to acute KD and KDSS. *P* values were determined by the Kruskal-Wallis test for continuous variables and Chi-square test or Fisher exact test for categorical variables. Numbers are median ± IQR. NS: not significant, NA: not applicable. WBC: white blood cell count, ANC: absolute neutrophil count, CRP: C-reactive protein, LVEF: left ventricular ejection fraction. * Z-Worst: [the highest Z score (internal diameter of the right and left anterior descending coronary arteries normalized for body surface area) during the first six weeks after fever onset.] ≥ 2.5. (**C**) Whole blood mRNA from patients with MIS-C (n=5), KDSS (n=5), acute KD (n=5), and KD convalescent controls (n=5) were measured by RT-qPCR using SYBR green I or TaqMan assays. Transcript levels of *TIFA*, *CASP1*, *CASP4*, *CASP5*, *NLRP3*, and *IL1B*, were normalized to *TAF1B* and compared. Results were expressed as median ± IQR, and the statistical significance was determined by Kruskal-Wallis test between disease groups and KD convalescent group. (D) Plasma IL-1β levels of MIS-C, KDSS, acute KD patients and KD convalescent healthy controls listed in (B) were detected with Meso Scale Discovery electrochemiluminescence assay whenever possible. **(E)** Demographics of MIS-C patients, acute KD, and pediatric febrile controls (FC). *Illness day 1: first calendar day of fever. **: LVEF on illness day 8 was 47%. ***: Z-Worst during the first six weeks after fever onset ≥ 2.5. ****: SARS-CoV-2 PCR-positive on illness day 7 and IgG positive on illness day 20. ALC: absolute lymphocyte count, M: male, F: female, −: negative, +: positive. **(F)** Crude granulocyte preparations were isolated from whole blood of MIS-C, acute KD, and FC patients listed in (E) with the use of Histopaque-1077 and Histopaque-1119 gradients. Cell lysates were analyzed by Western blot with anti-caspase-4, anti-caspase-1 or anti-TIFA with GAPDH as loading controls. Levels of TIFA, cleaved caspase-4, and cleaved caspase-1 were quantified by ImageJ and normalized to that of GAPDH. NA: not applicable. *$P<0.05$, **$P<0.01$ compared with KD convalescent control group. (G) Plasma IL-1β levels of MIS-C, acute KD, and FC patients listed in (E) were detected with Meso Scale Discovery electrochemiluminescence assay.