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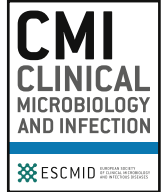
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Research Note

Cross-protective humoral immunity to coronaviruses from SARS coronavirus 2—naïve sera of children with Kawasaki disease

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ABSTRACT

Objectives: SARS coronavirus 2 (SARS-CoV-2)—associated multi-system inflammatory syndrome in children indicates that viruses can trigger a Kawasaki disease (KD)-like hyperinflammation. A plausible hypothesis was that coronavirus-specific ‘holes’ in humoral immunity could cause both diseases.

Methods: To determine whether SARS-CoV-2—naïve patients with KD have inferior humoral immunity for the novel coronavirus, sera of children with KD and control children from year 2015 to 2021 were subjected to ELISA, microwestern, and neutralization assays to evaluate the capabilities in recognizing the receptor-binding domain of SARS-CoV-2, spotting spike proteins of three respiratory syndromic coronaviruses, and blocking SARS-CoV-2 from binding to angiotensin-converting enzyme 2 receptors *in vitro*, respectively.

Results: 29 patients with KD before 2019, 74 patients with KD in 2019 or 2020, 54 non-febrile controls, and 24 febrile controls were included in the study. SARS-CoV-2 was recognized on ELISA for both patients with KD in 2016 and those with KD in 2020. Microwestern demonstrated cross-reactive IgG in an all-or-none manner towards three spike proteins of syndromic coronaviruses regardless of sample year or KD status. The ratio between the sera that recognized all spike proteins and those that recognized none (51 vs. 47) was significantly higher from patients with KD than from non-febrile controls (17 vs. 32; *p* 0.047) but not from febrile controls (13 vs. 11; *p* 0.85). Most positive sera (12 of 17 controls, 5 of 8 patients with KD before 2019, and 28 of 33 patients with KD in 2019 or 2020) offered protection comparable to low-titre sera from the WHO reference panel.

Discussion: Humoral immunity of SARS-CoV-2—naïve children with KD was not inferior to that of controls in offering cross-protection against the novel coronavirus. **Tzu-Yi Lin, Clin Microbiol Infect 2022;■:1**

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Introduction

Kawasaki disease (KD) is the most common form of paediatric systemic vasculitis [1]. Intravenous immunoglobulin is now the standard treatment for KD; however, coronary arterial lesions (CALs) still occur in 5% to 20% of patients during the acute stage [2,3]. With the global outbreak of coronavirus disease 2019 (COVID-19), clinicians in Italy first reported an unusual 30-fold increase in

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the incidence of KD-like diseases [4]. Whittaker et al. [5] also reported clinical courses of 58 children with multi-system inflammatory syndrome (MIS-C), of whom 45 had evidence of recent COVID-19. Given the clinical similarities between MIS-C and KD, the role of coronaviruses in KD pathogenesis is intriguing.

Genome-wide association studies have linked *FCGR2* [6], *CD40* [7,8], and *BLK* [7] to KD, implying that humoral immunity could carry recognisable features among patients with KD. In Taiwan, strict public-health control measures prevented major outbreaks of COVID-19 before the Omicron variant. No cases of MIS-C were reported before 2022. This context offers an opportunity to test whether patients with KD have coronavirus-specific 'holes' in humoral immunity. We used SARS coronavirus 2 (SARS-CoV-2) as a novel coronavirus to address the hypothesis. Sera collected from patients with KD before 2019 and in 2019–2020 were tested for humoral reactivity against SARS-CoV-2 and two other coronaviruses that cause SARS.

Methods

Patients and samples

Patients and controls were recruited from National Taiwan University Hospital with approval from the Institutional Review Board [202101080RINB]. We enrolled 103 patients with KD, of whom 29 (male-to-female ratio, 13:16; median age, 1.6 years) were diagnosed before 2019 and 74 (male-to-female ratio, 58:16; median age, 1.8 years) were diagnosed in 2019 or 2020. Diagnoses were made using the clinical criteria set by the American Heart Association [9]. Among the 54 non-febrile controls (male-to-female ratio, 34:20; median age, 5.3 years), 19 were healthy volunteers and 35 were patients without KD who visited the paediatric cardiology clinic; 13 of the 54 non-febrile controls were recruited before 2019. Among the febrile controls, eight of the 24 (male-to-female ratio, 16:8; median age, 4.6 years) were recruited before 2019. Diagnoses and ages of the febrile controls are tabulated in Table S1. Sera from patients with KD were collected before intravenous immunoglobulin therapy. The WHO reference panel was bought from the National Institute for Biological Standards and Control [10].

ELISA for SARS-CoV-2, micro western blotting, neutralisation assay

Please see supplementary material for description of the experiments.

Statistical analysis

Chi-square tests were adopted to evaluate the differences in microwestern staining patterns or ELISA results among the groups. A t-test was used to address the differences in neutralizing power among patients with KD with CAL z scores of <2.0 or >2.0; a p value of <0.05 was considered significant.

Results

Presence of SARS-CoV-2 recognizing IgG among children with KD

Previously, Chang et al. [11] reported no humoral cross-reactivity to SARS-CoV-2 proteins in children with KD before the COVID-19 pandemic. We conducted a similar IgG ELISA using samples obtained from patients with KD in 2020. However, SARS-CoV-2 receptor-binding domain (RBD) was recognized in 12 of 32 children, even though widespread COVID-19 community transmission had not occurred in Taiwan at that time (Fig. S1a). The same assay was repeated with the KD sera from year 2016. Four of

20 samples still yielded positive results (Fig. S1b), indicating the presence of cross-reactivity to SARS-CoV-2 in the naïve KD sera. No significant differences existed between the 2016 and 2020 results (p 0.18).

Humoral reactivity to spike proteins of three coronaviruses causing acute respiratory syndrome

To evaluate the spectrum of IgG cross-reactivity, we used microwestern blotted with the spike proteins of three coronaviruses that cause acute respiratory syndrome [12]. Samples included sera from patients with KD before 2019 ($n = 29$), patients with KD in 2019 or 2020 ($n = 74$), and controls without KD ($n = 78$). Six blotting patterns were identified (Fig. 1a), ranging from three positive (Fig. 1a, pattern 1) to three negative (Fig. 1a, pattern 6). A polarized behaviour was demonstrated in 171 samples that recognised spike proteins in an all-or-none manner. Ten samples recognized only one or two proteins (Fig. 1a, patterns 2 to 5). Both control and KD samples exhibited this polarized behaviour. By regarding pattern 1 as the positive category versus pattern 6 as the negative category, chi-square statistics revealed a significant difference for non-febrile controls versus patients with KD (p 0.047) but insignificance for pre-2019 versus 2019–2020 patients with KD (p 0.11) or febrile controls versus patients with KD (p 0.85) (Fig. 1a). IgG reactivity was not related to SARS-CoV-2 infection; only nine of 85 samples from patterns 1, 2, and 3 blotted positively for IgM against the SARS-CoV-2 nucleocapsid protein. No significant differences in IgM reactivity were found among different groups (Fig. 1b).

Neutralizing capabilities of spike protein recognizing serum

We further evaluated the neutralizing power of samples recognizing the SARS-CoV-2 spike protein ($n = 41$ for patients with KD and $n = 17$ for controls) *in vitro*. Sera that competitively blocked the binding between the RBD of the SARS-CoV-2 spike protein and the angiotensin-converting enzyme 2 receptor to below 70% were designated as neutralizing (Fig. 2a, red dotted line). WHO standards [10] (20/150, high titre; 20/148, mid titre; 20/144 and 20/140, low titre; 20/142, negative) were simultaneously assayed as reference controls. Almost all study samples (12 of 17 controls, five of eight patients with KD before 2019, and 28 of 33 patients with KD in 2019 or 2020) had neutralizing capabilities categorized into the WHO low-titre groups. However, there were no significant differences among the controls, patients with KD before 2019, and patients with KD in 2019 or 2020. There were also no differences (p 0.50) among patients with KD with CAL z scores of <2.0 or >2.0 (Fig. 2b).

Discussion

We investigated whether coronavirus-specific humoral 'holes' existed among patients with KD by challenging coronaviral antigens to naïve sera. In addition to positive ELISA results (Fig. S1), micro western analyses demonstrated cross-reactivity in a polarized manner for sera from both controls and patients with KD (Fig. 1a). Most samples blotted positively against either all three spike proteins of coronaviruses causing acute respiratory syndrome or none. Although SARS coronavirus 1 caused a short outbreak in Taiwan in year 2003, neither Middle East respiratory syndrome coronavirus nor SARS-CoV-2 caused major outbreaks in Taiwan before the emergence of the SARS-CoV-2 Omicron variant. The observed cross-reactivity was most likely induced by other common coronaviruses, such as human coronavirus 229E, OC43, or NL63, already in the community. Functionally, the cross-reactivity to SARS-CoV-2 provided neutralizing powers corresponding to

A

Pattern	1			2			3			4			5			6				
	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-			
d	SARS nCoV MERS			SARS nCoV MERS			SARS nCoV MERS			SARS nCoV MERS			SARS nCoV MERS			SARS nCoV MERS				
His Tag																				
a	Kawasaki Disease 2019-2020 (n=74)			41(55.4%)	-	1(1.4%)	-	1(1.4%)	-	1(1.4%)	31(41.9%)									
	Kawasaki Disease Before 2019 (n=29)			10(34.5%)	-	1(3.4%)	2(6.9%)	-	16(55.2%)											
	Non-Febrile Controls (n=54)			17(31.5%)	2(3.7%)	-	-	3(5.6%)	32(59.3%)											
	Febrile Controls (n=24)			13(54.2%)	-	-	-	-	11(45.8%)											

- a KD before 2019 vs. after 2019: p value 0.11 (>0.05) by Chi-Square (Pattern 1 vs. 6)
 b KD vs. Non-Febrile Controls: p value 0.047 (<0.05) by Chi-Square (Pattern 1 vs. 6)
 c KD vs. Febrile Controls: p value 0.85 (>0.05) by Chi-Square (Pattern 1 vs. 6)
 d SARS, nCoV, and MERS denote SARS-CoV-1, SARS-CoV-2, and MERS-CoV, respectively

B

SARS-CoV-2 Nucleocapsid Protein	His Tag	
a	Kawasaki Disease 2019-2020 (n=42)	5(11.9%) 37(88.1%)
	Kawasaki Disease Before 2019 (n=11)	1(9.1%) 10(90.9%)
b	Non-Febrile Controls (n=19)	1(5.3%) 18(94.7%)
	Febrile Controls (n=13)	2(15.4%) 11(84.6%)

- a KD before 2019 vs. after 2019: p value 0.79 (>0.05) by Chi-Square
 b KD vs. Non-Febrile Controls: p value 0.44 (>0.05) by Chi-Square
 c KD vs. Febrile Controls: p value 0.69 (>0.05) by Chi-Square

Fig. 1. Micro western analyses. (a) Spike proteins of three coronaviruses that cause acute respiratory syndrome were blotted with KD sera from before 2019 ($n = 29$), 2019–2020 ($n = 74$), and controls without KD ($n = 78$). Six staining patterns are delineated; however, most samples reacted in a polarized fashion such that either all three proteins (pattern 1) or no proteins (pattern 6) were recognised. Chi-square tests were performed for pattern 1 versus pattern 6. There were no differences between pre-2019 and 2019–2020 KD samples (p 0.11) or between KD and febrile controls (p 0.85); however, KD samples did have a significantly higher percentage positive staining against all three spike proteins (p 0.047; $p < 0.05$) compared with non-febrile controls. (b) Reactivity was not related to SARS-CoV-2 infections. Only nine of 85 samples with IgG reactivity patterns 1, 2, and 3 blotted positively for serum IgM versus SARS-CoV-2 nucleocapsid protein, with no significant differences between groups. KD, Kawasaki disease; MERS, Middle East respiratory syndrome coronavirus; nCoV, novel coronavirus; SARS-CoV-1, SARS coronavirus 1; SARS-CoV-2, SARS coronavirus 2.

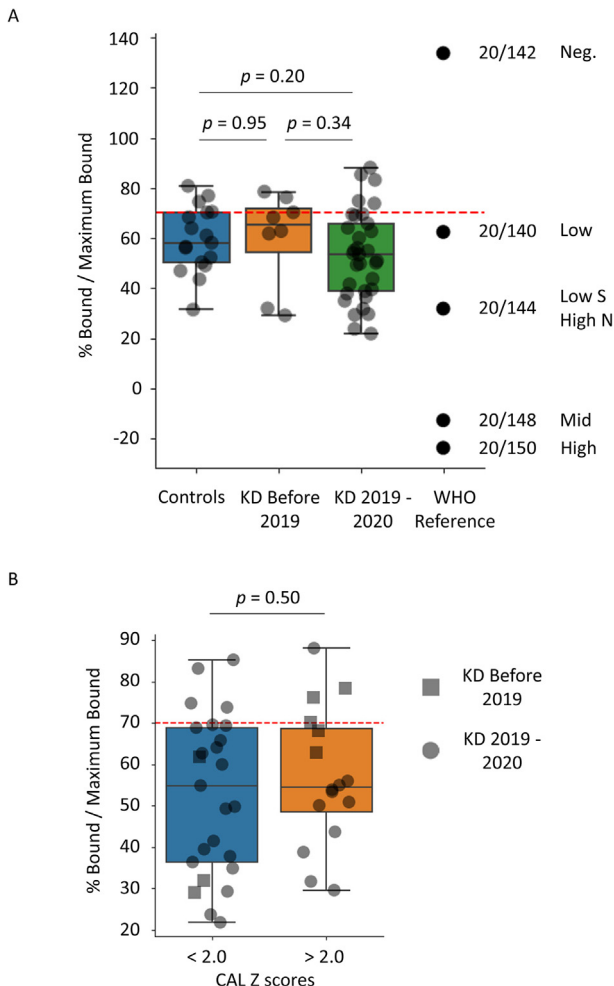


Fig. 2. Neutralization assay. (a) Competitive inhibition of SARS coronavirus 2 receptor-binding domain binding to angiotensin-converting enzyme 2 receptor by KD and control sera was evaluated *in vitro*. Samples that lowered binding below 70% maximum (red dotted line) were designated as neutralizing. As reference controls, the WHO standard panel was also subjected to the neutralization assay (five black dots on the rightmost column). Study samples comprised those recognizing the SARS coronavirus 2 spike protein ($n = 41$ for patients with KD and $n = 17$ for controls). Nearly all sera from patients with KD (five of eight before 2019 and 28 of 33 in 2019 or 2020) and controls (12 of 17) were neutralizing to an extent comparable to WHO low-titre standards (20/144 and 20/140). (b) There were no differences between patients with KD with CAL z scores of < 2.0 and > 2.0 in terms of neutralizing power ($p = 0.50$; $p > 0.05$, t-test). Squares marked samples before 2019, and circles denoted those diagnosed in 2019 or 2020. CAL, coronary arterial lesion; KD, Kawasaki disease; Neg, negative.

those offered by low-titre sera of the WHO standard panel (Fig. 2a). These results indicate that community-acquired humoral immunity is capable of containing SARS-CoV-2 infection to some extent for patients with KD, contradicting our initial hypothesis.

Chang et al. [11] reported no cross-reactivity to SARS-CoV-2 proteins from children with KD; however, a follow-up study [13] confirmed the presence of cross-reactivity to the S2 region from pre-pandemic samples in the New York area. Our results were based on the S1 RBD of SARS-CoV-2. This RBD-blocking capability is distinct from that in an earlier study conducted in Europe, which showed that pre-existing humoral immunity in children mainly targets the S2 subunit [14]. Geographic variations in terms of humoral cross-protection could be a factor contributing to children's disease phenotypes differing between regions.

Although we found no coronavirus-specific 'holes' in humoral immunity from patients with KD, it is still possible that humoral

reactivities do differ for yet unidentified collateral antigens. Alternatively, coronavirus-specific humoral immunity could be inappropriately amplified under some circumstances. A conditional strategy to stratify patients with KD by viruses and/or other personalized factors may be required to decipher KD aetiology.

Author contributions

TYL conducted the experiments. YLL, KLW, MCY, CNH, CMF, and MTL collected the samples and helped with the experiments. LMH, LYC, and HHL contributed to the study design. MTL and HHL supervised the study. MTL and HHL contributed equally to this work and share last authorship.

Transparency declaration

The authors declare that they have no conflicts of interest. This work was supported by grants 107-2321-B-002-026, 108-2321-B-002-016, 108-2314-B-400-029, and 109-2321-B-002-045 from the Ministry of Science and Technology (now National Science and Technology Council), Taiwan.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2022.09.018>.

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