

Th17- and Treg-related cytokine and mRNA expression are associated with acute and resolving Kawasaki disease

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Abstract

Background: Kawasaki disease is a vasculitis most commonly afflicting children <5 years of age. Many autoimmune diseases are associated with up-regulation of T helper (Th) 17 cells, and down-regulation Treg cells. Few studies have examined the Th17/Treg expression in Kawasaki disease.

Methods: Blood samples were obtained from 186 children with Kawasaki disease at 24 h before IVIG therapy, followed by 3 days and 21 days after IVIG therapy. Thirty children with an acute febrile infectious disease and 30 healthy children were obtained as control. Plasma levels of Th17- and Treg-related cytokines including IL-6, IL-17A, IL-10, TGF- β , and mRNA expression levels of ROR γ t and Foxp3 were tested.

Results: Patients with Kawasaki disease had higher levels of plasma IL-17A (25.35 ± 3.21 vs 7.78 ± 1.78 pg/ml, $P < 0.001$) and IL-6 (152.29 ± 21.94 vs 38.63 ± 12.40 pg/ml, $P < 0.001$) when compared to the febrile control group. IVIG resulted in a reduction in IL-6 and IL-17A at both 3 and 21 days after IVIG therapy. FoxP3 levels increased significantly 3 days after IVIG therapy (2.28 ± 0.34 vs 0.88 ± 0.14 , $P < 0.001$). IVIG resistance was associated with higher levels of IL-10 and IL-17A.

Conclusion: Kawasaki disease was associated with higher IL-17A and IL-6, a cytokine profile similar to other autoimmune diseases. IVIG therapy resulted in increased expression of Treg-related FoxP3. IVIG resistance was associated with higher levels of IL-10 and IL-17A. Our findings provide further evidence that Kawasaki disease is an autoimmune-like disease.

Kawasaki disease is a common cause of systemic vasculitis in children <5 years old. First described in 1967 by Dr. Tomisaku Kawasaki, diagnosis is dependent on a constellation of clinical symptoms including fever for 5 days and four of the following five symptoms: oral mucosal changes, bilateral conjunctivitis, cervical lymphadenopathy, erythema, or edema of the extremities, and a polymorphous rash (1). Kawasaki disease mainly affects medium- to small-sized vessels, especially the coronary arteries. It is estimated that 25% of patients with Kawasaki disease will develop coronary artery lesions if left untreated, leading to coronary artery aneurysm formation, thrombosis, stenosis, myocardial infarction, or sudden death (2). Standard treatment consists of intravenous immunoglobulin (IVIG) within the first 10 days of fever, which reduces the risk of coronary artery disease to 2–4% (3).

The precise immunopathogenesis of Kawasaki disease remains unclear, although growing evidence indicates that

the development of Kawasaki disease may be an autoimmune-like process. Epidemiological studies have found that the incidence of Kawasaki disease shows seasonal variation, with higher rates of disease from April to June in tropical areas such as Taiwan (4). Furthermore, Kawasaki disease is more common in the Asian population (5) and in children with parents or siblings who were diagnosed with Kawasaki disease in the past (6, 7). It has been speculated that Kawasaki disease may be triggered by an infectious agent, but only causes disease in individuals with a genetic predisposition (8).

Recent studies have found that both T-helper cell type 17 (Th17) and regulatory T cells (Treg) have an important role in the development of autoimmune disease. Th17 cells are associated with tissue damage and autoimmunity, while Treg cells have an antagonistic effect and negatively regulate inflammatory responses. The differentiation of Th17 cells and Treg cells is believed to be reciprocal in nature. Overexpression of

interleukin-17A (IL-17A), one of the major effector cytokines of Th17 cells, has been linked to autoimmune diseases such as rheumatic arthritis and systemic lupus erythematosus (9). Only a handful of studies have examined the expression of IL-17A and other-related cytokines in Kawasaki disease. The aim of our study was to investigate the expression of Th17 and Treg cells in patients with Kawasaki disease and disease outcome.

Materials and methods

Subject recruitment

In total, 186 patients diagnosed with Kawasaki disease were recruited, and all received 2 g/kg of IVIG over a 12-h period at Kaohsiung Chang Gung Memorial Hospital, Taiwan. In addition, 30 children with an acute febrile infectious disease and 30 healthy children were selected as controls. Data including age, gender, clinical symptoms, and duration of fever were collected by chart review. Blood samples were obtained at three time points: within 24 h before IVIG therapy, followed by at 3 and 21 days after IVIG therapy. IVIG resistance was defined as fever persisting for 48 h after IVIG therapy. Two-dimensional echocardiography was performed once at baseline and then at 1 month, and 2 months after IVIG therapy. Coronary artery lesions (CAL) were defined as any interior coronary artery diameter of >3 mm in a patient <5 years old and any lesion >4 mm in patients over 5 years old, or if the internal diameter of a segment was at least 1.5 times that of an adjacent coronary artery as seen on two-dimensional echocardiography. Patients with coronary artery dilatation that resolved within 2 months after the onset of Kawasaki disease were determined to have transient ectasia and were not considered as having a CAL as our previous study (10).

Measurement of plasma cytokines

Measurement of plasma cytokine levels of IL-10, IL-17A, and IL-6 was performed using Luminex 200 system (Luminex, Austin, TX, USA). Experimental protocol was modified from our previously reported methods (11, 12). Diluted standards or test samples collected were incubated with antibody conjugated beads for 2 h and then at room temperature with detector antibodies. Samples were then incubated with biotin as a reporter for 30 min, followed by fluorescent dye-conjugated streptavidin-phycoerythrin for 30 min. Enzyme-linked immunoassay was used to measure TGF- β (human TGF- β ; R&D Systems, Minneapolis, MN, USA), according to manufactures' instructions.

Analysis of cytokine data

We created standard curves for each cytokine tested on a log-log plot. Plasma concentrations were determined by the corresponding curve-fitting equation for each cytokine (Master Plex QT 1.2 system; Hitachi, San Bruno, CA, USA). Calibration of plasma cytokine concentrations was performed using well-known standard samples according to manufacturer instructions. All samples were tested at least twice, and the average value of each test sample was recorded.

Detection of mRNA expression levels using real-time PCR

Isolation of total RNA from white blood cells was performed using FavorPrep blood/cultured cell total RNA purification kit (Favorgen, cat. No. FABRK001-1; Favorgen Biotech Corp., Ping-Tung, Taiwan). We used high-performance reverse-transcriptase system (EPICENTRE) for reverse transcription of RNA samples. Detection of forkhead box P3 (FoxP3) and retinoic acid-related orphan receptor γ (ROR γ) expression levels was performed using real-time RT-PCR (Green PCR Master Mix and ABI Prism 7500 Sequence Detection System, Applied Biosystems, Waltham, MA, USA). Normalization of FoxP3 and ROR γ to 18S was performed; both were presented as absolute expression levels. Primers used for amplifying 18S mRNA were 5'-GTA ACC CGT TGA ACC CCA TT-3' (forward) and 5'-CCA TCC AAT CGG TAG TAG CG-3' (reverse); FoxP3 mRNA were 5'-CAA GTT CCA CAA CAT GCG AC-3' (forward) and 5'-ATT GAG TGT CCG CTG CTT CT-3' (reverse);(13) and ROR γ mRNA were 5'-TTT TCC GAG GAT GAG ATT GC-3' (forward) and 5'-CTT TCC ACA TGC TGG CTA CA-3' (reverse) (14).

Detection of CD4+IL-17A+ and CD4+ FoxP3+ cells via flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Ficoll-Hypaque density gradient centrifugation. These cells were finally adjusted to a final concentration of 2×10^7 /ml in complete RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from GIBCO, Grand Island, NY, USA) and stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) in the presence of BD GolgiStop protein transport inhibitor for 5 h at 37°C and cultured in a 5% CO₂ incubator. Cells were stained according to the manufacturer's instructions (Human Th17/Treg phenotyping kit, BD Biosciences, San Jose, CA, USA). Then, cells were analyzed on a LSR II flow cytometer (BD Biosciences). Collected data were analyzed using BD FACSDiva Software (BD Biosciences).

Statistical analysis

Statistical analysis was performed using SPSS version 14 software (SPSS Inc., Chicago, IL, USA). Mean and standard error were calculated for all plasma cytokine and mRNA expression levels. Student's *t*-test was used to compare demographic data. Chi-squared test was used to compare categorical variables. Mann-Whitney *U*-test was calculated to compare all cytokine and mRNA expression levels. A *P*-value of <0.05 was considered to be statistically significant.

Results

Demographic data

In all, 186 patients with Kawasaki disease (1.61 ± 0.10 years old, 116 male) were included in this study. Thirty patients with an acute febrile infectious disease (1.91 ± 0.21 years

old, 15 male) and 30 healthy children (2.05 ± 0.25 years old, 15 male) were chosen as control. Causes of acute infection in the control group included the following: upper and lower respiratory tract infection, gastroenteritis, *Moraxella* bacteremia, and urinary tract infection. There was no significant difference in age and gender between patients with Kawasaki disease and those within both control groups. Average duration of fever in patients with Kawasaki disease was 6.75 days. Twenty-three patients (12.3%) were IVIG resistant, and 11 patients (5.91%) were found to have coronary artery lesions 2 months after disease onset (Table 1).

Kawasaki disease patients had higher Th17-related cytokines

In terms of Th17-related plasma cytokines, patients with Kawasaki disease had significantly higher levels of both IL-17A and IL-6 when compared to both the febrile control group (IL-17A: 25.35 ± 3.21 vs 7.78 ± 1.78 pg/ml, $P < 0.001$, IL-6: 152.29 ± 21.94 vs 38.63 ± 12.40 pg/ml, $P < 0.001$) and the healthy control group (IL-17A: 25.35 ± 3.21 vs 4.24 ± 0.42 pg/ml, $P < 0.001$, IL-6: 152.29 ± 21.94 vs 3.96 ± 1.11 pg/ml, $P < 0.001$) (Fig. 1). Plasma concentrations of IL-6 decreased significantly 3 days after IVIG therapy (14.52 ± 2.11 vs 152.29 ± 21.94 pg/ml, $P < 0.001$). In contrast, IL-17A levels decreased gradually and were significantly lower 21 days after IVIG therapy (7.84 ± 1.43 vs 25.35 ± 3.21 pg/ml, $P < 0.001$).

Treg-cell-related cytokines IL-10 and TGF- β were also tested. IL-10 levels were found to be higher in patients with Kawasaki disease when compared to the febrile control group (109.79 ± 14.81 vs 54.58 ± 16.07 pg/ml, $P = 0.003$) and the healthy control group (109.79 ± 14.81 vs 17.56 ± 7.88 pg/ml, $P < 0.001$). TGF- β levels were higher in patients with Kawasaki disease when compared to the febrile control group (14497.72 ± 1255.36 vs 8202.36 ± 1414.26 pg/ml, $P = 0.029$), but similar to the healthy control group (14497.72 ± 1255.36 vs 15855.83 ± 1907.38 pg/ml, $P = 0.344$). IVIG therapy led to a significant decrease in IL-10 levels 3 days after IVIG therapy (14.67 ± 3.07 vs 109.79 ± 14.81 pg/ml, $P < 0.001$). There was no significant difference in TGF- β levels in patients with Kawasaki disease before or after IVIG therapy (Fig. 1).

Significant increase in Foxp3 mRNA expression after IVIG treatment in Kawasaki disease

Treg and Th17 cells are characterized by the expression of specific transcription factors, FoxP3 and ROR γ t, respectively. Patients with Kawasaki disease did not have a significant difference in FoxP3 or ROR γ t expression levels when compared with either the febrile control group or the healthy control group. Treg-related FoxP3 expression levels increased significantly 3 days after IVIG therapy (2.28 ± 0.34 vs 0.88 ± 0.14 , $P < 0.001$). However, there was no change in Th17-related ROR γ t levels at 3 days or 21 days after IVIG therapy. Of note, while Treg-related FoxP3 mRNA expression decreased to levels similar to the healthy control group by 21 days after IVIG therapy (1.15 ± 0.21 vs 0.72 ± 0.26 , $P = 0.260$), Th17-related ROR γ t expression levels remained higher than the healthy control group at 3 and 21 days after IVIG therapy, although this was not statistically significant (Fig. 2).

Comparison of mRNA and plasma cytokine in patients with IVIG resistance and coronary artery lesions

Patients with Kawasaki disease who were IVIG resistant had higher levels of both IL-10 and IL-17A prior to IVIG therapy when compared with those who were IVIG responsive (IL-10: 356.45 ± 87.46 vs 85.13 ± 11.33 pg/ml, $P < 0.001$; IL-17A: 68.91 ± 23.15 vs 21.00 ± 2.41 pg/ml, $P = 0.001$) (Table 2). Cytokine levels of IL-10, IL-17A, IL-6, TGF- β , and mRNA expression levels of FoxP3 and ROR γ t showed no significant difference in patients with and without coronary artery lesions (Table 3).

Lower percentage of CD4+FoxP3+ cells in patients with Kawasaki disease

Patients with Kawasaki disease had lower percentages of CD4+FoxP3 cells when compared to the febrile control group (4.33 ± 0.20 vs $7.64 \pm 0.54\%$, $P = 0.017$); percentages of CD4+FoxP3+ cells increased 3 days after IVIG therapy, although there was no statistically significant difference when compared with before IVIG treatment. There was no

Table 1 Demographic data

	Healthy control (N = 30)	Fever control (N = 30)	Kawasaki disease (N = 186)	P-value
Age (year)	2.05 ± 0.25	1.91 ± 0.21	1.61 ± 0.10	0.168
Male gender	15 (50%)	15 (50%)	116 (62.4%)	0.237
Clinical data		Upper respiratory tract infection (N = 13)	Average duration of fever: 6.75 days	
		Lower respiratory tract infection (N = 11)	Oral mucosa change: 165 patients (88.7%)	
		Gastroenteritis (N = 4)	Conjunctivitis: 164 patients (88.2%)	
		<i>Moraxella</i> Bacteremia (N = 1)	Lymph node >1.5 cm: 43 patients (23.1%)	
		Urinary tract infection (N = 1)	Limb swelling, peeling: 99 patients (53.2%)	
			Rash: 145 patients (77.9%)	
			IVIG resistance: 23 patients (12.3%)	
			CAL lesions at 2 months: 11 patients (5.91%)	

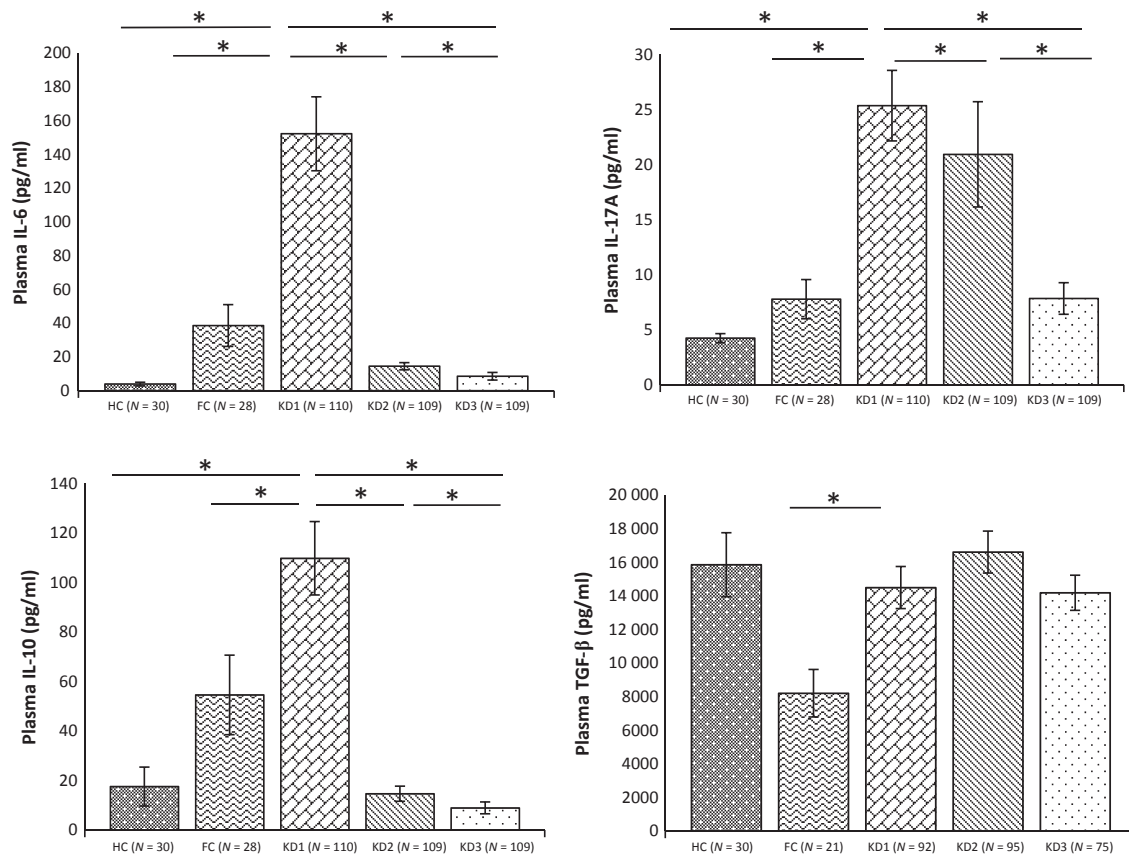


Figure 1 Plasma cytokine data. HC: healthy control group, FC: febrile control group, KD1: patients with Kawasaki disease 24 h prior to IVIG therapy, KD2: patients with Kawasaki disease 3 days after IVIG therapy, KD3: patients with Kawasaki disease 21 days after

IVIG therapy. *A *P*-value of <0.05 between two groups. IL: interleukin, TGF-β: transforming growth factor β, FoxP3: forkhead box P3, and RORγt: retinoic acid-related orphan receptor γt.

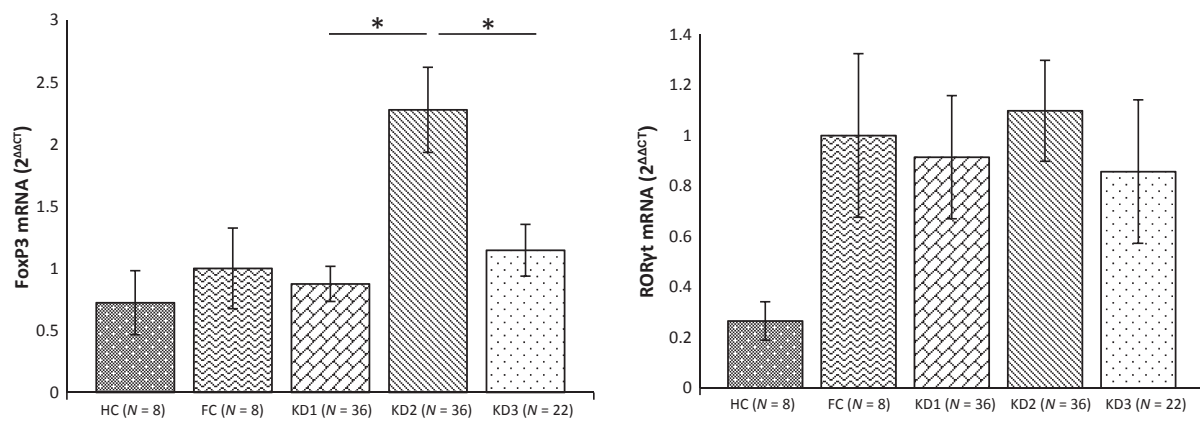


Figure 2 mRNA expression data. HC: healthy control group, FC: febrile control group, KD1: patients with Kawasaki disease 24 h prior to IVIG therapy, KD2: patients with Kawasaki disease 3 days after IVIG therapy, KD3: patients with Kawasaki disease 21 days

after IVIG therapy. *A *P*-value of <0.05 between two groups. IL: interleukin, TGF-β: transforming growth factor β, FoxP3: forkhead box P3, and RORγt: retinoic acid-related orphan receptor γt.

significant difference in terms of CD4+IL-17A+ cell percentages in patients with Kawasaki disease when compared to the febrile control group or after the administration of IVIG therapy (Fig. 3).

Discussion

Kawasaki disease is a pediatric systemic vasculitis resulting from abnormal activation of inflammation in susceptible children. There is an ongoing debate as to how the initial immune activation is brought about, although both animal and human studies suggest an infectious trigger coupled with an abnormal self-directed T-cell response underlies the autoimmune-like response in Kawasaki disease (15). Genetic studies have also found that Kawasaki disease is linked to polymorphisms in CD4, B lymphoid tyrosine kinase (BLK), inositol 1,4,5-trisphosphate 3-kinase C (ITPKC), Fc fragment of immunoglobulin G, low-affinity IIa, receptor (FCGR2A), and caspase 3 (Casp3) genes, highlighting the importance of B and T cells in the pathogenesis of Kawasaki disease. (16–18)

Th17 cells are a newly discovered T-helper cell subset associated with the pro-inflammatory stage of autoimmunity. Their differentiation requires both TGF- β combined with either IL-10 or IL-21 and results in the specific expression transcription factor retinoic acid-related orphan receptor γ t (ROR γ t), and the production of inflammatory cytokines including IL-6 and IL-17A (10). To date, only a few studies have examined the expression of Th17-related ROR γ t and IL-17 in children with Kawasaki disease. Sohn et al. (19) found that IL-17 levels were significantly elevated in children with acute Kawasaki disease. Similarly, in a study of forty-five children by Jia et al. (20), higher levels of ROR γ t and IL-17A were found in children with Kawasaki disease.

In our study of 186 patients, we found that children with Kawasaki disease had higher levels of IL-17A and IL-6 when compared to both the febrile control group and the healthy control group. In comparison with IL-6 which decreased rapidly within 3 days after IVIG therapy, IL-17A levels persisted for a longer period of time, and showed a gradual decline over a 21-day period after IVIG therapy. Although Th17-related ROR γ t expression levels in patients with Kawasaki disease remained higher than the healthy control group at 3 and 21 days after IVIG therapy, this was not statistically significant. Because we did not find a significant difference in ROR γ t mRNA expression between patients with Kawasaki disease and the febrile control group, we could not conclusively prove that the elevation of IL-17A and IL-6 was the result of increased ROR γ t expression. It is possible that the elevation of IL-17A and IL-6 seen in our study is the result of the other inflammatory cells involved. Although IL-17A is primarily secreted by Th17 cells, it can also be produced by CD8 positive T cells, mast cells, and neutrophils (21). Likewise, the expression of IL-6 is not specific to Th17 cells and can also be produced by T cells, B cells, monocytes, and macrophages (22). Overall, our study shows that the acute phase of Kawasaki disease is associated with the elevation of both IL-6 and IL-17A, a cytokine profile which mimics other

autoimmune diseases such as rheumatic arthritis (21, 22). Both IL-17A and ROR γ t expression levels remained elevated 21 days after IVIG therapy, suggesting that either a Th17 or Th17-like immune response is persistent in patients with Kawasaki disease beyond the acute phase and after IVIG therapy.

Regulatory T cells (Treg) regulate immune responses and suppress autoimmune reactions. Treg cells require TGF- β for differentiation, specifically express the transcription factor FoxP3, and secrete inhibitory cytokines including IL-10 and TGF- β (14). Most studies to date have shown that IVIG therapy seems to increase FoxP3 expression (23–25), but may not affect TGF- β levels (26, 27). Although IL-10 is considered an anti-inflammatory cytokine, prior studies have shown that IL-10 is actually elevated in the acute phase of Kawasaki disease and then significantly subsides after IVIG treatment (28–30). Taken together, IL-10 may play an inflammatory rather than anti-inflammatory role in Kawasaki disease.

In our study, we found that patients with Kawasaki disease have a slightly lower level of FoxP3 mRNA expression at baseline, with a statistically significant increase in FoxP3 mRNA expression after IVIG therapy. This was also reflected in our flow cytometry data which showed that patients with Kawasaki disease had lower percentages of CD4+FoxP3+ cells when compared to the febrile control group, which increased after IVIG therapy. However, this increase in FoxP3 mRNA expression was not mirrored by concordant changes in plasma IL-10 or TGF- β concentrations. Recent studies have found that IVIG can both modulate Treg cell function and increase Treg cell expansion. Proposed mechanisms include direct binding of Treg cells and induction of expansion, neutralization of inflammatory cytokines, and altered cytokine production by antigen-presenting cells that induce Treg cell proliferation (31). The results of our study provide further evidence that IVIG therapy increases Treg cell expansion and are associated with the resolution of Kawasaki disease. Because IL-10 and TGF- β did not increase after IVIG therapy, it is possible that Treg cells mediate immune suppression through other cytokine pathways or through direct cell interaction.

Even though IVIG therapy is considered the standard treatment for Kawasaki disease, IVIG resistance may occur in 10–20% of patients and is associated with higher rates of coronary artery formation (32). Retreatment with a second dose of IVIG is often recommended, although no prospective trials exist to evaluate its effect in comparison with other treatment options (33, 34). In our study, we found that patients with IVIG resistance had higher levels of IL-10 and IL-17A before IVIG therapy, which may be used as potential biomarkers of IVIG resistance in Kawasaki disease. In addition, we found that IL-17A levels tended to persist longer after IVIG therapy when compared to IL-10 or IL-6. Given that IL-17A levels persist for a longer period of time after IVIG therapy when compared to other cytokines, targeted suppression of IL-17A may prove to be an effective alternative treatment in patients who are IVIG resistant.

Table 2 Plasma levels of Th17/Treg cytokines and mRNA expression in Kawasaki disease in IVIG resistance and IVIG responsive patients

	KD 1			KD 2			KD 3		
	IVIG sensitive, mean $\pm$ SE (N)	IVIG resistant, mean $\pm$ SE (N)	P-value	IVIG sensitive, mean $\pm$ SE (N)	IVIG resistant, mean $\pm$ SE (N)	P-value	IVIG sensitive, mean $\pm$ SE (N)	IVIG resistant, mean $\pm$ SE (N)	P-value
IL-10 (pg/ml)	85.13 $\pm$ 11.33 (100)	356.45 $\pm$ 87.46 (10)	<0.001*	14.88 $\pm$ 3.35 (99)	12.56 $\pm$ 4.62 (10)	0.813	9.38 $\pm$ 2.53 (100)	4.20 $\pm$ 2.35 (9)	0.369
IL-17A (pg/ml)	21.00 $\pm$ 2.41 (100)	68.91 $\pm$ 23.15 (10)	0.001*	18.49 $\pm$ 4.21 (99)	45.02 $\pm$ 31.79 (10)	0.208	6.84 $\pm$ 0.97 (100)	19.00 $\pm$ 13.77 (9)	0.908
IL-6 (pg/ml)	142.94 $\pm$ 22.37 (100)	245.85 $\pm$ 89.71 (10)	0.154	14.84 $\pm$ 2.29 (99)	11.34 $\pm$ 3.87 (10)	0.690	8.96 $\pm$ 2.43 (100)	4.64 $\pm$ 2.30 (9)	0.739
TGF- β (pg/ml)	15204.83 $\pm$ 1368.54 (81)	9290.87 $\pm$ 2555.97 (11)	0.108	17119.65 $\pm$ 1379.53 (82)	1341.61 $\pm$ 2664.78 (13)	0.407	14065.15 $\pm$ 1064.78 (70)	15919.11 $\pm$ 5471.06 (5)	0.755
FoxP3	0.82 $\pm$ 0.12 (33)	1.54 $\pm$ 1.15 (3)	0.841	2.29 $\pm$ 0.37 (33)	2.09 $\pm$ 0.91 (3)	0.977			
ROR γ t	0.94 $\pm$ 0.26 (33)	0.61 $\pm$ 0.51 (3)	0.841	1.14 $\pm$ 0.21 (33)	0.63 $\pm$ 0.59 (3)	0.290			

KD1: patients with Kawasaki disease 24 h prior to IVIG therapy, KD2: patients with Kawasaki disease 3 days after IVIG therapy, KD3: patients with Kawasaki disease 21 days after IVIG therapy, IL, interleukin; TGF- β , transforming growth factor β ; FoxP3, forkhead box P3; ROR γ t, retinoic acid-related orphan receptor γ t.

*A P-value of <0.05 between two groups.

Table 3 Th17/Treg cytokine and mRNA expression in Kawasaki disease patients with and without coronary artery lesions

	KD1			KD2			KD3		
	No CAL, mean $\pm$ SE (N)	CAL at 2 months, mean $\pm$ SE (N)	P-value	No CAL, mean $\pm$ SE (N)	CAL at 2 months, mean $\pm$ SE (N)	P-value	No CAL, mean $\pm$ SE (N)	CAL at 2 months, mean $\pm$ SE (N)	P-value
IL-10 (pg/ml)	111.31 $\pm$ 15.21 (106)	69.49 $\pm$ 63.22 (4)	0.162	15.04 $\pm$ 3.18 (105)	5.10 $\pm$ 3.85 (4)	0.321	9.16 $\pm$ 2.41 (105)	3.49 $\pm$ 3.49 (4)	0.178
IL-17A (pg/ml)	25.88 $\pm$ 3.35 (106)	11.37 $\pm$ 8.44 (4)	0.086	21.00 $\pm$ 4.93 (105)	18.90 $\pm$ 16.59 (4)	0.380	7.85 $\pm$ 1.47 (105)	7.68 $\pm$ 6.43 (4)	0.449
IL-6 (pg/ml)	153.41 $\pm$ 22.69 (106)	122.78 $\pm$ 58.39 (4)	0.898	14.81 $\pm$ 2.18 (105)	6.76 $\pm$ 4.12 (4)	0.611	8.81 $\pm$ 2.32 (105)	3.31 $\pm$ 3.30 (4)	0.258
TGF- β (pg/ml)	14483.33 $\pm$ 1360.37 (84)	14648.84 $\pm$ 2280.09 (8)	0.506	16559.25 $\pm$ 3066.17 (87)	17190.01 $\pm$ 3066.17 (8)	0.511	13928.20 $\pm$ 1124.25 (67)	16370.87 $\pm$ 2862.86 (8)	0.311
FoxP3	0.89 $\pm$ 0.16 (32)	0.89 $\pm$ 0.13 (3)	0.331	2.04 $\pm$ 0.24 (32)	4.69 $\pm$ 3.47 (3)	0.680	1.14 $\pm$ 0.23 (20)	1.22 $\pm$ 0.37 (2)	0.568
ROR γ t	0.81 $\pm$ 0.21 (32)	2.26 $\pm$ 2.03 (3)	0.906	1.10 $\pm$ 0.22 (32)	1.15 $\pm$ 0.62 (3)	0.768	0.75 $\pm$ 0.27 (20)	1.92 $\pm$ 1.86 (2)	0.568

CAL: Coronary artery lesion, KD1: patients with Kawasaki disease 24 h prior to IVIG therapy, KD2: patients with Kawasaki disease 3 days after IVIG therapy, KD3: patients with Kawasaki disease 21 days after IVIG therapy, IL, interleukin; TGF- β , transforming growth factor β ; FoxP3, forkhead box P3; ROR γ t, retinoic acid-related orphan receptor γ t.

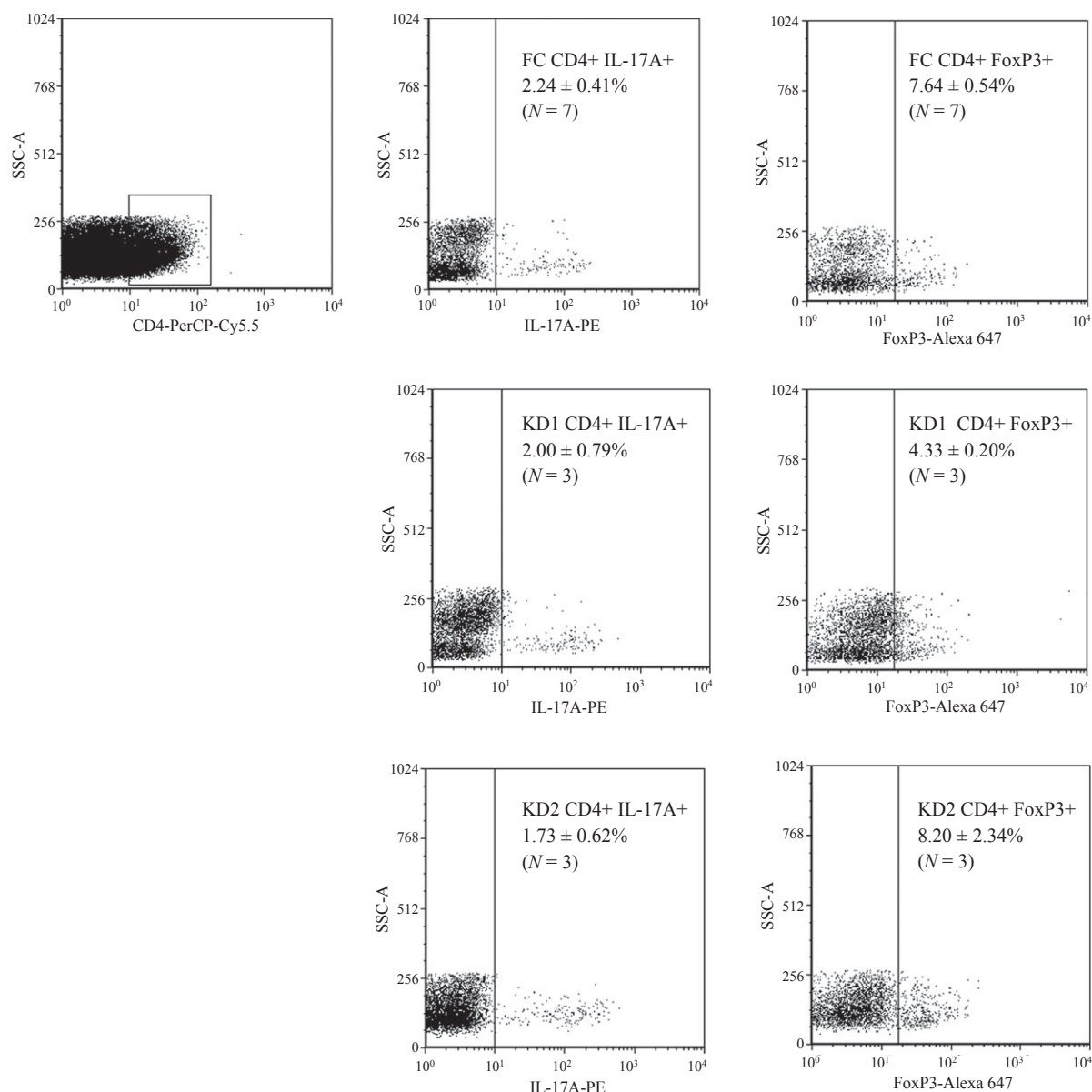


Figure 3 Proportions of CD4+ IL-17A+ and CD4+ FoxP3+ cells. FC: febrile control group, KD1: patients with Kawasaki disease 24 h prior to IVIG therapy, KD2: patients with Kawasaki disease 3 days

after IVIG therapy. IL: interleukin, and FoxP3: forkhead box P3. All values are expressed as mean ± standard error.

To our knowledge, our study is the largest study to date that examines Th17- and Treg-related mRNA and cytokine expression in patients with Kawasaki disease. Three time points of data were collected: at baseline, 3 days, and 21 days after IVIG therapy, which allowed us to examine the expression of Th17 and Treg in acute stage of Kawasaki disease, immediately after IVIG therapy and after the resolution of fever. Of note, baseline blood draws were on average collected after 5.5 days of fever prior to IVIG therapy; mean duration of fever was 6.75 days in our study which is shorter than other studies that report on average 9–10 days of fever.

(20, 23) This may be due to earlier diagnosis and treatment, although rate of IVIG resistance and coronary artery lesions was 12.3% and 5.91%, respectively, which is similar to prior reports (32, 35).

In conclusion, this study shows that patients with Kawasaki disease have elevated IL-17A and IL-6 at baseline, a cytokine profile that is similar to the inflammatory phase of other autoimmune diseases. In addition, higher IL-17A levels were associated with IVIG resistance and persisted for a longer period of time when compared to other cytokines. IVIG therapy resulted in increased expression of Treg-related FoxP3

mRNA, which suggests that IVIG therapy mediates its immunosuppressive effects through the upregulation of Treg cells. We believe that our findings provide further evidence that the immune response in Kawasaki disease is similar to other autoimmune diseases. Suppression of IL-17A may be an alternative therapeutic target in patients with Kawasaki disease, especially those who are IVIG resistant.

Author contributions

Mindy Ming-Huey Guo acquired and analyzed data, drafted the article, revised for intellectual content, and provided final approval of the version to be published. Wan-Ning Tseng and Hui-Mei Pan acquired data, helped in reviewing, and provided approval of version to be published. Chia-Hung Ko acquired data, helped in reviewing, and provided approval of version to be published. Kai-Sheng Hsieh acquired data, helped in review, and provided approval of version to be

published. Ho-Chang Kuo carried out conception and design, acquired data, revised for intellectual content, and provided approval of version to be published.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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