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**Down-regulation of TLR2, 3, 9 and Signaling Mediators, MyD88 and TRIF,
Gene Transcript Levels in Patients with Kawasaki Disease
Treated with IVIG**

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ABSTRACT

Kawasaki disease (KD) is an acute febrile systemic vasculitis of childhood characterized by elevated levels of inflammatory mediators at the acute stage. High-dose intravenous immunoglobulin (IVIG) is well accepted as a conventional therapy for KD. The aim of the present study was to determine the expression level of Toll like receptors (TLRs) and their corresponding signaling mediators in PBMCs of IVIG-treated KD patients.

TLR2, 3, 9 and signaling mediators, MyD88 and TRIF transcript levels were determined in PBMCs from 31 KD patients, before (acute phase), 2 weeks later (sub-acute phase) and 6 weeks later (convalescent phase) of IVIG therapy using real time PCR. The mean age of the patients was 3.6 years and 65% of subjects were male and 35% were female. 20 age-matched irrelevant febrile patients and 20 healthy subjects were included as control groups.

Elevated levels of TLR2, MyD88, and TRIF gene transcripts were observed in the PBMCs at acute phase of untreated KD patients in comparison with normal subjects. IVIG therapy resulted in significant decrease in TLR2, 3 and 9 (60-90%) as well as MyD88 and TRIF (60-70%) transcripts following 2 and 6 weeks.

With regard to significant up-regulation of MyD88 and TRIF at the acute phase of KD, our findings suggest TLR signaling pathway potential in KD pathogenesis and may also support the assumption of an infectious background in KD. Down-regulation of TLR members and corresponding mediators in IVIG treated patient suggest general TLR pathway suppression as a novel anti-inflammatory mechanism of IVIG.

Keywords: IVIG; Kawasaki disease; MyD88; Toll like receptors; TRIF

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INTRODUCTION

Kawasaki disease (KD) is an acute febrile childhood vasculitis of unknown origin, which is associated with the development of coronary artery abnormalities.¹ KD is clinically characterized by high fever, rashes, cervical lymphadenopathy, conjunctivitis, oral enanthema, erythematous induration of the hands and feet and elevation of serum levels of many proinflammatory mediators.² These symptoms resolve spontaneously within 1-3 weeks after early treatment.^{2,3} The aetiopathogenesis of KD is not well identified but it is generally assumed that occurrence of an undefined infectious trigger in genetically predisposed subjects leads to the disease. Untreated KD could be categorized into three clinical phases. An acute febrile phase for 10-14 days is typically followed by a sub-acute phase of approximately 2 to 4 weeks. A convalescent phase starts 8-10 weeks after the beginning of the illness; when all symptoms resolve and last until all clinocopathological tests return to normal state.^{4,5}

Several clinical features of KD support an infectious trigger including abrupt onset of symptoms, resolution of the symptoms in 1-3 weeks, usually without recurrence, the young aged patients that are most susceptible to ubiquitous microbes, hyper-activation and spontaneous production of inflammatory pathways and cytokines in peripheral immune system cells, the winter-spring predominance of cases in non-tropical climates and the existence of epidemics and clusters of cases.⁴ *Leptospira* spp., *Streptococcus sanguis*, retrovirus, Epstein-Barr virus or cytomegalovirus, toxic shock syndrome toxin 1 and other bacterial toxins, coronavirus NL-63, human bocavirus, and previously unrecognized persistent RNA virus may be involved in the etiology of KD.⁶

Of interest, KD is described by an obvious activation of the innate immune cells with elevation of serum levels of pro-inflammatory cytokines at acute phase.^{7,8} TNF- α is attributed in the vascular injury of KD patients.⁹ Hyper-activation state of nuclear factor kappa B (NF- κ B), master transcription factor for genes encode the inflammatory cytokines of innate immune system, in PBMCs of KD patients may possibly mediate systemic inflammation.¹⁰

First-line therapy for KD is high-dose intravenous immunoglobulin (IVIG) in combination with high-dose aspirin.¹¹ This therapy inhibits the immune-mediated necrotizing arteritis and attenuates the acute systemic inflammation. However, the precise mechanisms underlying the therapeutic effects of IVIG in KD are unidentified. Several anti-inflammatory mechanisms of action of IVIG are described so far.¹² Aiming innate immune system by IVIG through inhibition of NF- κ B signaling pathway is most recently discovered.¹³ Prevalence of infection history and also hyper-activation of NF- κ B signaling pathway in KD, provide evidences that interaction of microbial components by receptors of innate immune cells may be the key phenomenon of KD initiation and progression.

As the most prominent innate immune receptors, Toll like receptors (TLR) are expressed in several types of the innate immune cells including monocytes, B lymphocytes and granulocytes, as well as epithelial and endothelial cells.^{14,15} TLRs being involved in bacterial infection are the cell-surface receptors TLR2 and TLR4 sensing lipoproteins and Liposaccharides, respectively.¹⁶ Intracellular members of TLR family, e.g. TLR3 and TLR9 recognize viral double stranded RNA and unmethylated CpG, respectively.¹⁴ TLR stimulation primarily activates signaling mediators MyD88 (Myeloid differentiation primary response gene 88, downstream of all TLR members except TLR3) and TRIF (TIR-domain-containing adapter-inducing interferon- β , downstream of TLR3) eventually leads to activation of NF- κ B pathway and secretion of several pro-inflammatory cytokines such as endogenous pyrogens TNF- α , IL-1, IL-6.¹⁷

Moreover, researchers discovered a new mechanism of IVIG in the inflammatory and autoimmune diseases through disturbing TLR activation and disabling NF- κ B pathway.¹³ Taking critical role of TLRs in recognition of microbial antigens and exploiting NF- κ B signaling pathway, it is presupposed that TLR expression and activation may be deregulated in KD patients.

In this study, we analyzed the expression level of TLR2, TLR3, TLR9 gene transcripts and corresponding signaling mediators, MyD88 and TRIF, in the peripheral blood mononuclear cells of KD patients and patients suffering from irrelevant febrile diseases, in comparison

with healthy subjects. We also evaluated TLR gene expressions in the PBMCs of KD patients before (acute phase) and after IVIG therapy (sub-acute and convalescent phases).

MATERIALS AND METHODS

Study Population and Sample Collection

In this study, 31 patients who met the specific diagnostic criteria for KD, based on centers for diseases control and prevention (CDC) criteria (<http://www.cdc.gov/kawasaki/>) and journal of pediatric criteria, were enrolled after obtaining parental informed consent.¹¹ All the patients have been visited and hospitalized in pediatrics ward, Namazi hospital, Shiraz University of Medical Sciences, Iran. All patients received high-dose intravenous immunoglobulin (Fibrogammin, Tokyo, Japan), 1000 mg/kg/day for 2 days. The first venous blood sample was taken from each patient at the first day of diagnosis one day before treatment with IVIG. All samples were obtained from subjects that had suffered from fever at least for 5 days. Samples after IVIG therapy at sub-acute phase were obtained 2 weeks after treatment and samples at the convalescent stage were obtained 6 weeks after treatment. Samples from 20 healthy age-matched volunteers without any sign of fever and systemic inflammation, and 20 age-matched control patients who had been febrile for at least 72 hrs (body temperature >38°C) were also collected. Unrelated febrile patients were included to address the TLR transcript levels in febrile diseases excluding KD patients.

Peripheral Blood Mononuclear Cells

Heparinized peripheral blood was obtained from patients and healthy volunteers. PBMCs were isolated using ficoll density-gradient centrifugation, as previously described.¹⁸ Isolated PBMCs were washed twice with sterile phosphate buffered saline (PBS).

RNA Extraction and cDNA Synthesis

Total RNA from 4×10^6 of PBMCs was extracted by Trizol reagent treatment (Invitrogen, USA) in accordance with the manufacturer's instructions. Briefly, the protocol employs guanidine thiocyanate solution,

phenol/chloroform, isopropanol and ethanol for RNA extraction and precipitation. The quality and quantity of the extracted RNA were estimated by spectrometry.¹⁹ Contaminated DNA was removed from RNA by Dnase I treatment (Fermentase, Lithuania) before cDNA synthesis. cDNA was synthesized from 5 µg of total RNA with the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Lithuania) as described previously.²⁰ Briefly, total RNA, Oligo (dT) Primer, Random Hexamer Primer were mixed in a sterile, nuclease-free tube containing DEPC-treated water at total volume of 12 µl and incubated at 70°C for 5 min. The tube was placed back on ice and 5X Reaction Buffer, RiboLock™ RNase Inhibitor (20 u/µl) and 10 mM dNTP Mix were added up to 19 µl and incubated at 37°C for 5 min. In the final step, 1 µl RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 u/µl) (Fermentas, Vilnius, Lithuania) was added and after a brief centrifuge, the mixture was incubated at 42°C for 100 min. The reaction was terminated by heating at 70°C for 5 min.

Semi-Quantitative Real Time PCR

The relative expression level of TLR2, TLR3, TLR9, MyD88 and TRIF gene transcripts before, 2 weeks and 6 weeks after IVIG therapy of KD patients were determined in triplicates for each individual sample by real time PCR using an ABI 7500 system (ABI, Applied Biosystems, USA) with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Expression of 18sRNA housekeeping gene was used as a reference for the level of target gene expression. Specific primers were designed by Primer3 open source software (SourceForge, USA) (Table 1). Thermal cycling for TLR2, 3, 9 and MyD88 genes was 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and elongation at 60°C for 40 sec. Thermal cycling for TRIF genes was 40 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 20 sec and elongation at 60°C for 30 sec. Real time PCR efficiencies of 18s RNA, TLR2, 3, 9, MyD88 and TRIF were determined 92%, 89%, 95%, 96%, 90%, and 94%, respectively. The relative amounts of TLR2, 3, 9, MyD88 and TRIF transcripts were determined from the ΔCt and $2^{(-\Delta Ct)}$ formulas. Target-to-reference gene ratios were calculated with the Pfaffl method.²¹

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Table 1. The sequence of primers

Primer	Sequence
18sRNA Forward	CGAACGTCTGCCCTATCAACTT
18sRNA Reverse	ACCCGTGGTCACCATGGTA
TLR2 Forward	AGTACCTGGGTCTGGACGTG
TLR2 Reverse	CTGGGAACCTCAAGCAGGAAG
TLR3 Forward	CCCACTGAGGAGTCCAACAT
TLR3 Reverse	TTTCTTGCGCTTTCGTTTTT
TLR9 Forward	GCACTTTCCTCTCCGCTAG
TLR9 Reverse	TAAGCTCCATCACTAACAAC
MyD88 Forward	GAGCGTTTCGATGCCTTCAT
MyD88 Reverse	CGGATCATCTCCTGCACAAA
TRIF Forward	AGCGCTTCGACATTCTAGGT
TRIF Reverse	AGAACCATGGCATGCAGGA

Statistical Analysis

Relative expression level of gene transcripts in the PBMCs of KD patients were evaluated to the equivalent values from subjects who suffered from unknown febrile fevers and normal subjects, using nonparametric Mann-Whitney test by SPSS software v. 11.5 (SPSS Inc., Chicago, USA). The correlations among dissimilar values were studied by Pearson tests. In all statistical analysis, $p < 0.05$ was regarded as significant.

RESULTS

Demographic Results

Demographic and laboratory findings of 31 KD patients at the time of blood drawing are presented in Table 2. The mean age of the patients was 3.6 ± 1.9 years (ranged 0.5 to 8 years old) and negative control and positive febrile control groups were 4 ± 2.1 years (ranged 1 to 6). 65% of subjects were male and 35% were female. Equality of age and sex frequency of patient and control groups were statistically approved. 100% of patients suffered from fever, inflamed oral mucosa and conjunctivitis. 80% and 65% of patients represented extremity and lymphadenopathy signs at the acute phase of disease, respectively. For minor criteria, 56% of patients had low level (anemic) of Hb and 35% and 70% of patients represented leukocytosis and thrombocytosis, respectively. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were elevated in almost all of

patients (90%) which reflects prevalence of the acute-phase response in KD patients. However, elevated serum level of albumin and ALT were seen only in about 25% of patients. 25% of all patients represented cardiovascular disorder at the time of admission, before IVIG therapy. The incidence of cardiovascular disease folded down to 22% and 19% in sub-acute and convalescent stages following IVIG therapy.

TLR2, 3 and 9 Gene Expressions in PBMCs of KD Patients

At the first set of experiment, real time PCR analysis for TLR2, 3 and 9 gene transcripts levels were normalized by 18sRNA. There were no significant differences in transcript levels of TLR3 and TLR9 in PBMCs of Kawasaki disease and febrile patients with normal subjects. However, TLR2 gene transcript level was significantly elevated in Kawasaki disease in comparison to normal subjects ($p = 0.021$) (Figure 1).

MyD88 and TRIF Gene Expressions in PBMCs of KD Patients

A significant up-regulation in MyD88 transcript levels were observed at acute phase of Kawasaki disease and also unrelated febrile patients compared with normal subjects ($p = 0.03$) (Figure 2). In KD patients, but not unrelated febrile patients, TRIF transcript level was significantly up-regulated in comparison with normal subjects ($p = 0.01$) (Figure 2).

Table 2. Clinical and laboratory findings of 31 KD patients

Patient	Age	Gender	Fever >5 day	Erythematous rash	Extremity involvement	Lymphadenopathy >1.5cm	Conjunctivitis	Oral mucosa abnormality	WBC >15×10 ³	ESR >40	PLT >450×10 ³	CRP ≥3mg	Alb <3g/dl	ALT	U/A WBC>10	Cardiovascular symptom
1	0.5	M	+	+	+	-	+	+	-	+	+	+	-	-	-	-
2	6	M	+	+	+	-	+	+	+	+	+	+	-	-	-	-
3	7	M	+	+	-	+	+	+	+	+	-	+	-	-	-	-
4	2.5	M	+	+	+	-	+	+	-	+	+	+	-	-	-	+
5	5	F	+	+	+	-	+	+	+	-	+	+	-	-	-	-
6	8	M	+	+	+	-	+	+	+	-	+	+	-	+	-	-
7	3	M	+	-	+	+	+	+	-	+	+	+	+	-	-	+
8	2.6	M	+	+	-	+	+	+	-	+	+	+	+	ND	-	-
9	ND	F	+	+	+	-	+	+	-	+	+	+	+	+	-	-
10	5	F	+	-	+	+	+	+	-	+	+	-	-	+	-	-
11	2.2	F	+	+	+	-	+	+	-	+	+	+	-	+	-	-
12	2.4	M	+	+	+	-	+	+	-	+	+	+	ND	-	-	-
13	3	F	+	+	-	+	+	+	+	+	+	+	-	-	+	-
14	ND	F	+	+	+	-	+	+	-	+	+	+	-	-	-	-
15	4	F	+	-	+	+	+	+	-	+	+	+	-	-	-	-
16	ND	M	+	+	+	+	+	+	+	+	-	+	-	ND	-	-
17	1.9	M	+	+	+	+	+	+	-	+	-	+	-	+	+	-
18	3	M	+	+	+	+	+	+	-	+	+	+	+	ND	+	+
19	1.6	F	+	+	+	-	+	+	+	+	+	-	-	-	-	-
20	2.6	F	+	+	+	+	+	+	+	+	+	+	+	-	-	+
21	1.3	M	+	+	+	+	+	+	-	+	+	+	+	-	-	-
Patient	Age	Gender	Fever >5 day	Erythematous rash	Extremity involvement	Lymphadenopathy >1.5cm	Conjunctivitis	Oral mucosa	WBC >15×10 ³	ESR >40	PLT >450×10 ³	CRP >3mg	Alb <3g/dl	ALT	U/A WBC>10	Cardiovascular Before IVIG
22	4.6	M	+	+	+	+	+	+	-	+	+	+	-	-	-	-
23	4	F	+	+	+	+	+	+	-	+	-	+	-	-	-	+
24	4	M	+	+	+	+	+	+	-	-	-	-	-	+	-	-
25	2.5	M	+	+	+	+	+	+	-	+	-	+	-	-	-	+
26	4.6	F	+	+	+	+	+	+	+	+	+	+	-	+	-	-
27	3	M	+	+	+	+	+	+	+	+	-	+	-	-	-	-
28	6.6	M	+	+	+	+	+	+	-	+	-	+	-	+	+	+
29	7	M	+	-	+	+	+	+	+	+	-	+	-	-	+	-
30	2	M	+	-	-	+	+	+	-	+	+	-	+	-	+	+
31	5	M	+	+	+	-	+	+	+	-	-	+	-	-	-	-

ND: not diagnosed

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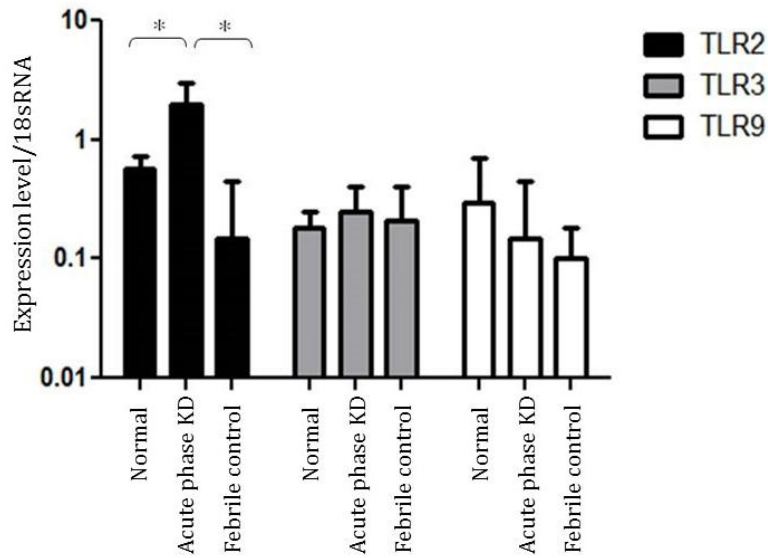


Figure 1. Relative gene expressions of TLR2, 3 and 9 in PBMCs of the normal subjects, acute phase of KD and unrelated febrile patients, normalized by 18sRNA, as internal control. The data are presented as the mean \pm standard error. * represent p value < 0.05 (compared with normal subjects).

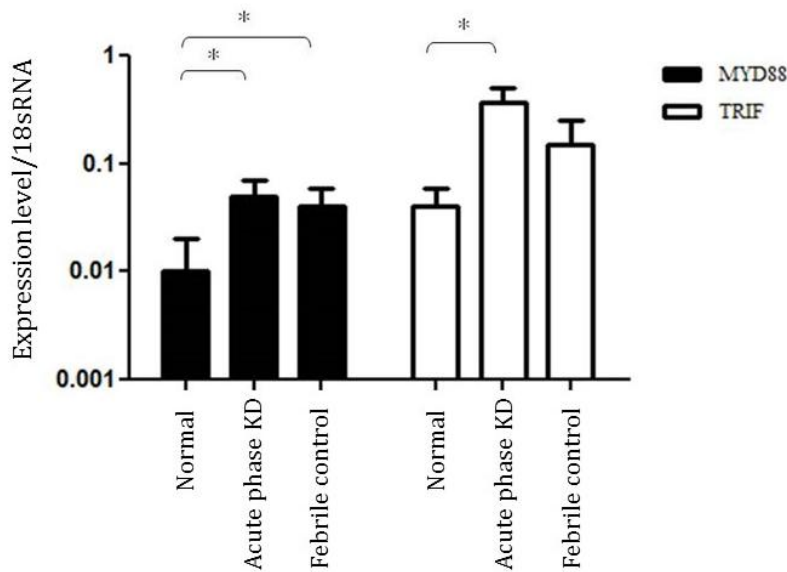


Figure 2. Relative gene expressions of MyD88 and TRIF in PBMCs of the normal subjects, acute case of KD and unrelated febrile patients, normalized by 18sRNA, as internal control. The data are presented as the mean \pm standard error. * represent p value < 0.05 (compared with normal subjects).

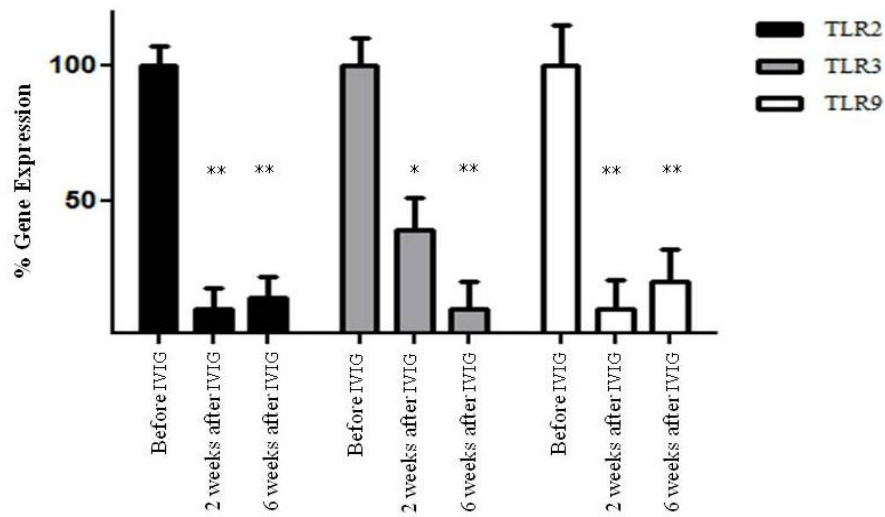


Figure 3. Down-regulated transcript levels of TLR2, 3 and 9 in IVIG treated KD. Real time PCR analysis for TLR transcripts were done following 2 and 6 weeks of IVIG therapy of 31 patients with KD. * and ** represent p value <0.05 and <0.001 , respectively (compared with before IVIG treatment).

Down-Regulation of TLRs and Signaling Mediators after IVIG Therapy

Figure 3 and 4 represent the real time PCR results of PBMCs obtained from KD patients before and after IVIG treatment. Among them, all the genes showed expression to be less than one-third to -tenth following IVIG therapy. Data showed that TLR2 transcript level has been significantly down-regulated, about 10 fold 2 weeks (sub-acute phase) and 6 weeks (convalescent phase) following

IVIG therapy ($p=0.003$ and $p<0.001$) (Figure3). The expression of both TLR3 and TLR9 were significantly down-regulated following 2 weeks (sub-acute phase) and 6 weeks (convalescent phase) of IVIG therapy (60 to 90% down-regulation, p value >0.05 , figure 3). As shown in figure 4, MyD88 and TRIF transcripts levels were also significantly down-regulated by mean of 65%, 2 and 6 weeks after IVIG therapy (Figure 4).

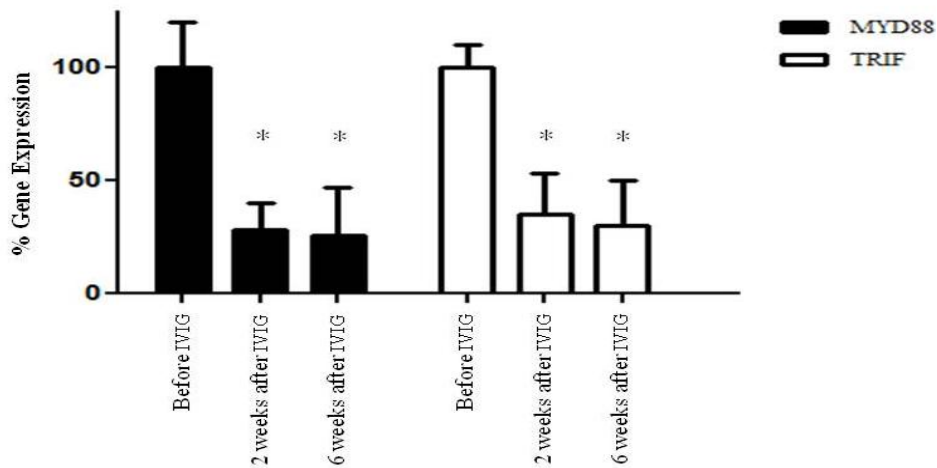


Figure 4. Down-regulated transcript levels of MyD88 and TRIF in IVIG treated PBMCs of KD. Real time PCR analysis for TLR gene transcripts were done following 2 and 6 weeks of IVIG therapy of 31 patients with KD. * represents p value <0.05 (compared with before IVIG treatment).

DISCUSSION

KD is known as an inflammatory cytokine-based disease.^{7,22} Hyperactive state of peripheral cells of the innate immune system in KD may be reflecting a chronic interaction of patient's immune system with one or more infectious agents.⁶ Comprehensive data from expression pattern of TLR pathways as key elements of innate immune system may introduce TLRs as a new culprit in the Kawasaki disease aetiopathogenesis and promising targets for KD treatment.

In this study, to further illuminate potential role of TLRs in the pathophysiology of KD, we analyzed the expression levels of TLR2, TLR3, TLR9 and corresponding signaling mediators, MyD88 and TRIF, in PBMCs of 31 patients with KD disease and 20 unrelated febrile patients in comparison with 20 normal age-matched subjects using semi quantitative real time PCR. We also sought potential of IVIG on the regulation of TLR transcript levels in KD patients. In comparison to normal subjects, we didn't observe any significant differences in transcript levels of TLR3 and TLR9 in KD patients during acute phase and also in febrile patients (Figure 1). However, TLR2 transcript level of KD patients were significantly up-regulated in comparison with febrile and normal controls. Our data on TLR2 over-expression is in agreement with the recent report by Rosenkranz et al that TLR2 and MyD88 contribute to *Lactobacillus casei* extract induced focal coronary arteritis in a mouse model of KD.²³ They demonstrated that *Lactobacillus casei* extract induces release of inflammatory cytokines through TLR2/NF- κ B (but not TLR4) fashion using both murine and human cells. TLR2-deficient mice as well as MyD88-deficient mice were resistant to the induction of KD by *Lactobacillus casei* extract compared with wild-type mice. However, no significant correlation between TLR2 expression level and cardiovascular disorder in KD patients in our study was found (data not shown).

Expression level and phosphorylation state of MyD88 reflect activation of all members of TLR family, but TLR3. TRIF, however, renders a similar role in the TLR3 signaling pathway.^{14,24} In our study, significant up-regulation of MyD88 and TRIF were seen in acute phase of KD compared to normal subjects.

Comparison of febrile patients and normal subjects pointed out MyD88, but not TRIF over-expression. This part of our data is similar to a recent study in which over-expression of MyD88 and TRIF in KD patients was proven.²⁵ Up-regulation of TLR4 and MyD88 during acute phase of KD suggests that aberrant activation of TLR4 via bacterial lipopolysaccharides might be one of the initiating factors of immune deregulation in KD.²⁵ Altogether, up-regulation of TLR2, MyD88 and TRIF during acute phase of KD in the present study suggested that dysregulation of TLRs might be one of the key factors of inflammatory state in KD.

Mainstay of treatment for KD is high-dose IVIG and immune suppressive drugs.^{1,26} IVIG arrests the immune-mediated necrotizing arteritis associated with the disease and alleviates the acute systemic symptoms of inflammation. These observations point out that the IVIG therapy in KD is largely mediated by robust inhibition of activated immune system cells in the peripheral circulation. Recently, it has been shown that IVIG suppressed NF- κ B activation in mononuclear cells.²⁷ However, which NF- κ B upstream inflammatory pathways are actually affected by IVIG remains to be answered.

Real time PCR data showed that TLR2, 3 and 9 gene transcripts have been significantly down-regulated 2 and 6 weeks after IVIG therapy. Following IVIG therapy, TLR2, 3 and 9 gene transcripts were profoundly down-regulated (ranged 60-90%, $p > 0.05$) in KD patients 2 weeks after IVIG therapy. Interestingly, the effect was persistent over 6 weeks after IVIG treatment. Over 2 and 6 weeks after IVIG therapy, MyD88 and TRIF transcripts were also persistently down-regulated about 60-70% in KD patients comparable to the level of normal subjects. This part of experiments indicates that IVIG may attenuate TLR signaling not only by targeting cell surface receptor expression but also via disturbing signaling cascade mediators. Present data may provide more evidences for a new IVIG mechanism of action. Molecular mechanisms by which IVIG inhibits of TLR expression and signaling meet more demands to response.

It has recently been identified in a high throughput analysis that the therapeutic effects of IVIG in KD may be mediated by suppression of an array of immune

activation and inflammatory genes in peripheral blood immune cells.²⁸ Interestingly, IVIG down-regulates TLR4 and TLR1 in PBMCs of KD.²⁷ IVIG also inhibits CpG induced TLR9 activation in B cells.¹³

CpG/TLR9 interaction may trigger the production of ANCA, contributing to the development of relapses in antibody-associated vasculitis (AAV) and KD.^{29,30} Increased frequency of IgA-secreting cells following TLR9 engagement was observed in patients with KD.³¹ In support, it is recently shown that IVIG could attenuate TLR9 activation and signaling in SLE patients.¹³ Similar to MyD88 inhibitors, IVIG also inhibits TLR-induced production of the inflammatory cytokines, but not anti-inflammatory IL-10 by B cells. Moreover, IVIG induces CD22, a B cell inhibitory receptor, which is involved in IVIG mediated inhibition of TLR9 signaling.^{13,32}

Correlation and association between TLR expression and clinical and laboratory findings in KD were analyzed to see if TLRs could be considered as potential predisposition biomarkers for clinical features of KD. There were no significant differences of TLR expression levels in male and females. No significant associations between TLR expression levels and laboratory findings such as ESR, CRP, WBC, Hb, and ALT were observed (data not shown).

Overall, the data should be of interest for future studies focused on KD pathogenesis, diagnosis, and therapy. It is important to further elucidate the precise molecular mechanisms of IVIG in TLR pathways to develop a new therapeutic target for KD patients. However, to consider TLR as a definite causative factor in KD, in vivo studies using mouse model of Kawasaki treated with different agonistic and antagonistic pharmacological agents for TLR should be designed. Genetic polymorphism analysis in order to see if TLRs and TLR signaling mediators are associated with KD could provide invaluable data in the world of KD.

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