Analysis of bio-markers serum levels in IVIG and infliximab refractory Kawasaki Disease patients

Running title: Infliximab therapy for Kawasaki disease

Keywords

Kawasaki disease; infliximab; granulocyte-colony-stimulating factor; interferon-gamma inducible protein 10; soluble tumor necrosis factor-alpha receptor

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Abstract

Introduction: Infliximab (IFX) is effective for treatment of refractory Kawasaki disease (KD). However, the precise mechanisms and biomarkers for IFX efficacy are unknown. We tried to evaluate the effect and response to IFX therapy by measuring serum cytokine levels.

Methods: Twenty-nine children with KD who had been resistant to 2 courses of high-dose intravenous immunoglobulin were enrolled and treated with IFX. Plasma samples were analyzed for cytokines before and after IFX administration.

Results: Serum levels of interleukin-6, granulocyte-colony-stimulating factor (G-CSF), interferon-gamma-induced monokine, interferon-gamma inducible protein 10 (IP-10), monocyte chemotactic protein 1, and soluble tumor necrosis factor-alpha receptor (sTNFR) 1 and 2 were significantly elevated before IFX treatment, but promptly decreased after the administration. The pre-treatment G-CSF and sTNFR1 levels in non-responders to IFX were significantly higher than in responders, who were defined as patients who defervesce (<37.5°C). After IFX administration, elevated cytokines declined to normal ranges in responders, but in non-responsive group, G-CSF and sTNFR1 remained elevated without failing to normal levels.

Conclusions: IFX treatment significantly reduced the levels of serum cytokines, chemokines, and sTNFRs in refractory KD. G-CSF and sTNFR1 may be indicators predictive of poor response to IFX.

1. INTRODUCTION

Kawasaki disease (KD) is an acute systemic inflammatory vasculitis in infants and young children [1], preferentially affecting the coronary arteries that is the leading cause of acquired heart disease. The therapeutic methods for KD have progressed drastically over the last half century. High-dose intravenous immunoglobulin (IVIG) is effective in suppressing inflammation and reducing the prevalence of coronary artery abnormalities when administered in the early stages of KD [2]. However, 15-20% of affected individuals do not respond to the initial IVIG treatment and harbor greater risk of coronary artery aneurysm formation [3, 4]. Recently, anti-tumor necrosis factor (TNF)-α therapy has emerged as a rescue treatment for patients with refractory KD. As anti-TNF-α monoclonal antibody, infliximab (IFX) has been reported as safe and effective for reducing fever duration [5-10]. Nevertheless, the precise mechanisms and prognostic biomarkers for IFX therapy in KD remain poorly understood.

Previous studies have suggested KD to be an immune-mediated disease in which cytokines might be responsible for disease development and progression. Several investigations have identified elevated serum levels of multiple pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1β, IL-6, IL-8, IL-18 and interferon (IFN)-γ, in acute phase KD [11-13]. Increased expression of monocyte chemotactic protein (MCP)-1 has also been observed in KD, whose expression declines after IVIG [14]. High levels of soluble tumor necrosis factor-alpha receptor (sTNFR) 1 were detected in the serum during acute stage of KD, and a positive correlation was seen for TNF-α and sTNFR [15]. Lastly, granulocyte colony-stimulating factor (G-CSF) is elevated in most of KD patients, and elevated G-CSF can predict IVIG treatment failure in KD [16].

Ko et al. most recently identified serum interferon-gamma inducible protein-10 (IP-10) to be specifically increased in KD in comparison with other febrile diseases and thereby excellent biomarker for differentiating KD cases [17]. IP-10 is secreted by several cells, including T lymphocytes, neutrophils, monocytes, and endothelial cells, under the influence of multiple cytokines, such as IFN-γ, IFN-α, and TNF-α, depending on the cell type [18, 19]. IP-10 promotes the migration of activated Th1 lymphocytes [20] and functions, not only as a chemotactic factor, but also as a potent inhibitor of angiogenesis [21].

In the present study, we investigated the cytokine profiles of patients with IVIG-resistant KD before and after IFX therapy to identify biomarkers that could estimate the effect of IFX among responders, and non-responders.

2. PATIENTS AND METHODS

2.1. Ethical Statement

This investigation on IFX therapy for refractory KD and its analysis of cytokine profiles for KD were approved by the institutional review board of Shinshu University (No. 993 and 2781). Written informed consent was obtained from legal guardians.

2.2. Patients

We enrolled 29 children (12 girls and 17 boys; mean age: 37 months) with 2nd IVIG resistant KD transferred to Shinshu University Hospital from July 2007 through April 2015. All subject were of Japanese ethnicity and fulfilled the criteria outlined by Diagnostic Guidelines for Kawasaki Disease (5th revision) published by the Kawasaki Disease Research Committee in Japan [22], with the exception of 1 case of incomplete type of KD. The patients had received initial treatments at institutions other than our hospital of 2.0 g/kg IVIG for 1-2 days twice, alone with either 30 mg/kg/day oral aspirin or 3-5 mg/kg/day flurbiprofen due to the liver dysfunction at the onset of KD. Refractory KD to IVIG was defined as axillary temperature of over 37.5°C persisting more than 24 hours after the 2nd IVIG.

IFX treatment was begun within 8 days of disease onset in all patients. Intravenous 5 mg/kg IFX in 100 ml saline over 2 hours was administered in an open-label manner. No anaphylactic reactions were observed. Patients were re-assessed 24 hours after IFX infusion. Clinical data, including age, sex, duration of illness, and the results of routine laboratory tests, such as white blood cell (WBC) count, neutrophil count, C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), were obtained from medical records. Strong inflammatory activity was evident in all KD patients such as elevated WBC count and serum CRP levels before IFX administration (Table 1).

We defined patients who responded to IFX (responder group, n=13) as those who had defervesced (<37.5°C) within 24 hours after the completion of IFX treatment with no subsequent recurrent fever. Those patients who exhibited persistent fever (>37.5°C) lasting more than 24 hours or recrudescent fever associated with KD symptoms after a 24-hour afebrile period were placed in the non-responder group (n=16). The pre-treatment clinical characteristics and laboratory data of the overall cohort and patient subgroups are summarized in Table 1. Fever lessened slightly in 12 patients in non-responder group, but remained over 37.5°C at 24 hours following IFX administration. No significant differences were apparent between the responder and non-responder groups regarding age at KD onset, principal KD symptoms, WBC

count, neutrophil count, or serum levels of CRP, AST, ALT, or LDH before IFX infusion (Table 1). Eleven of 16 non-responders required additional therapy: 8 received additional IVIG therapy and 3 were further treated with plasma exchange therapy. In contrast, no patient needed supplemental KD treatment in responders.

2.3. Measurement of serum cytokines

Blood samples were collected just prior to IFX treatment and at 24-96 hours after completion. There was no significant difference in the timing of blood sample collection after IFX between the groups (median time after IFX administration; responders, 38.3 hours; non-responders; 39.5 hours, p=0.57). Collected sera were immediately frozen and stored at -40°C until measurement of cytokines. Serum values of IL-1β, IL-6, IL-8, IL-10, IL-17A, IFN-γ, TNF-α, G-CSF, IP-10, interferon gamma induced monokine (MIG), and MCP-1 were determined by BDTM Cytometric Bead Arrays (BD Biosciences, Piscataway, USA) according to the manufacturer's instructions. Data were analyzed using the BD Cytometric Bead Array software (version 3.1). sTNFR1 and sTNFR2 were quantified by ELISA (R&D Systems, Minneapolis, USA), and IL-18 was quantified by ELISA (MBL, Nagoya, Japan) according to manufacturer's directions.

2.4. Statistical analysis

Group comparisons for laboratory data, and cytokine and chemokine levels were performed using the Kruskal-Wallis test. Changes in each parameter were assessed using the Friedman test. Statistical differences were considered to be significant when the p value upon two-tailed t-testing was less than 0.05. All statistical analyses were performed using JMP9 statistical software (JMP Statistical Discovery, North Carolina, USA).

3. RESULTS

3.1. Serum cytokine, chemokine, and sTNFR levels

We first assessed the concentrations of serum cytokines, chemokines, and sTNFRs before IFX administration in our cohort. As reported elsewhere in KD patients at disease onset [14-16, 23-24], serum IL-6, IL-18, G-CSF, IP-10, MIG, MCP-1, and sTNFR1 and 2 were significantly elevated before IFX treatment in comparison with healthy controls (Figure 1). IFN-γ and IL-1β were elevated in some patients only (Figure 1). We did not detect any marked differences in

serum IL-8, IL-10, IL-17A, or TNF-α as compared with controls (Figure 1). At 24-96 hours after the completion of IFX infusion, IL-6, G-CSF, IP-10, MIG, MCP-1, sTNFR1, and sTNFR2 levels were significantly decreased (all $p<0.0001$) (Figure 2). In contrast, IL-18 had not decreased appreciably ($p=0.66$) (Figure 2). Elevated IFN-γ and IL-1β existing in the same patient decreased to healthy control levels after therapy (data not shown).

3.2. Serum cytokines, chemokines, and sTNFRs between responders and non-responders

We next compared the serum levels of cytokines, chemokines, and sTNFRs between responders and non-responders to IFX therapy. Serum G-CSF and sTNFR1 were significantly elevated among non-responders compared with responders before IFX administration ($p=0.002$, $p=0.0129$, respectively) (Figure 3). Pre-treatment IL-6, IL-18, IP-10, MIG, MCP-1 and sTNFR2 between the two groups were not significantly different (Figure 3). At 24-96 hours after the completion of IFX infusion, serum values of IL-6, IP-10, MIG, MCP-1, and sTNFR2 in both responders and non-responders had declined to those of healthy controls (Figure 3). Interestingly, serum G-CSF and sTNFR1 in non-responders decreased greatly, but still maintained higher levels in comparison with responders ($p= 0.01$, $p= 0.0069$, respectively) (Figure 3) and healthy controls ($p=0.0011$, $p=0.0024$, respectively).

4. DISCUSSION

The present study revealed that serum IL-6, IL-18, G-CSF, MIG, MCP-1, IP-10, sTNFR1 and sTNFR2, were markedly up-regulated in KD patients who failed to respond to initial and 2nd IVIG therapy. IFX treatment significantly reduced all the cytokine levels, apart from IL-18. Notably, G-CSF and sTNFR1 among non-responders before and after IFX therapy were significantly higher than those of responders, indicating that they may represent biomarkers predicting a poor response to IFX.

The observed elevations in serum cytokines, chemokines, and sTNFRs before IFX therapy were consistent with those of previous reports on KD patients before IVIG treatment [14-16, 23, 24]. We did not detect IL-10 or TNF-α in most of our cohort in our system, despite significant increases having been reported elsewhere in acute phase KD [13, 24]. In agreement with earlier studies [9, 25], significant reductions in serum IL-6 and sTNFR1 by IFX in KD were noted in this series. Given that IFX suppressed all elevated recording apart from IL-18, our findings indicated that serum levels of those molecules might represent a useful tool to estimate the

therapy efficacy of IFX in IVIG-resistant KD patients. Serum IL-18 decreases gradually in response to treatment in systemic juvenile idiopathic arthritis [26]. Thus, it may have declined in a delayed fashion after IFX administration in our cohort.

We witnessed that the serum levels of G-CSF and sTNFR1 were higher in IFX non-responders before and after IFX infusion, but decreased significantly. Such findings were not evident other proinflammatory cytokines, including IL-6, IL-18, IP-10, MIG, MCP-1, and sTNFR2. G-CSF is an inflammatory cytokine associated with the proliferation, differentiation, and survival of progenitor cells committed to a neutrophilic lineage. Accordingly, neutrophil amounts were significantly higher in non-responders than in responders after IFX therapy (median, [range]: responders 3.7, [5.1-2.3] x 10^3 /μl; non-responders 8.9, [10.7-7.6] x 10^3 /μl; p=0.0056). These results suggest that insufficient improvement of neutrophilic inflammation may be associated with an unresponsiveness to IFX in IVIG-resistant KD patients.

Abe et al. identified that G-CSF was a good biomarker for predicting the response to IVIG in patients with KD based on higher serum levels of G-CSF in IVIG non-responders than in responders [16]. Thus, it is possible that G-CSF may be used as a prediction marker of not only IVIG therapy, but also IFX therapy in KD patients. Meanwhile, sTNFR1 is ubiquitously expressed on human cells [27] and released into the circulation via shedding or alternative splicing in response to various stimuli, such as TNF-α [28]. A positive correlation between TNF-α and sTNFR1 has been reported in KD patients [15]. Although we could not detect serum TNF-α in most cases in our system of the low sensitivity, sTNFR1 may be an indicator of anti-TNF-α therapy response.

Kobayashi and Egami scoring systems have been used to predict IVIG resistance in Japan [29, 30]. Recently, Tang et al. identified a new scoring showing a relatively better performance than Kobayashi and Egami scoring systems in east China population [31]. Because there was no significant deference of most of the variables included in those systems before IFX administration between our IFX resistant and responded group (data not shown), we did not identify their variables as risk factors for IFX resistance. Therefore, the scores calculated by Tang, Kobayashi and Egami systems in IFX resistant group did not differ those in responded group. These results suggest that their scoring models may not have useful performance as the predictor of IFX resistance in the IVIG resistant KD patients.

KD is a pediatric vasculitis with coronary artery aneurysm as its main complication, and preventing the development of coronary artery aneurysm is the most important in treatment.

Although associations between coronary artery aneurysm formations and IP-10 were not evaluated in this study, it is reported that patients with thoracic aortic aneurysms have shown significantly elevated serum IP-10 levels as compared with controls [29]. The internal luminal diameter of coronary artery was assessed using two-dimensional echocardiography following IFX therapy at 2 weeks after disease onset (data not shown). However, we could not evaluate the association between coronary artery aneurysm formation and serum cytokine levels because of a small number of patients having coronary artery dilatation.

In conclusion, IFX administration significantly reduced the elevated serum cytokines, chemokines, and sTNFRs in IVIG-resistant KD patients, indicating a strong suppression of inflammation. The cytokines, chemokines, and sTNFRs mentioned represent useful biomarkers for evaluating the efficacy of IFX therapy, while elevated G-CSF and sTNFR1 levels may indicate poor response to IFX. Further large-scale studies on IFX treatment for refractory KD and analyses of relevant biomarkers for IFX efficacy are warranted.

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Informed consent

Informed consent was obtained from all individual participants included in the study.

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Conflict of interest

Authors declare no conflicts of interest.

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Table 1. Clinical characteristics and laboratory data of 29 KD patients prior to IFX administration

	Total	Responders	Non-responders	P value analysis**
Donors(n)	29	13	16	
Age(months)*	34, 12-85	38.0, 13-65	32.5, 12-85	
Sex(male; female)	17; 12	7; 6	10; 6	
Diagnosis (day)*	4, 3-8	4, 3-8	4, 3-7	
Infliximab infusion (day)*	8, 7-12	9, 7-12	8, 7-12	
Body temperature(℃) *	38.8, 39.5-38.5	38.8, 39.4-38.3	39.1, 39.8-38.5	0.88
Clinical manifestations of KD without fever before IFX (n)				
Conjunctivitis	26	12	14	
Mucositis	29	13	16	
Rash	18	8	10	
Extremity changes	20	8	12	
Lymphadenopathy	27	13	14	
Additional treatment after IFX (n)				
None (n)	5	2	3	
3rd IVIG (n)	8	0	8	
Steroids (n)	0	0	0	
Plasma exchange (n)	3	0	3	
Urinastatin (n)	22	11	11	
WBC (x 10^3/μl) *	13.6, 15.9-12.2	13.9, 15.9-12.5	13.2, 15.9-12.1	0.78
Neutrophils (x 10^3/μl) *	9.0, 11.5-7.3	8.6, 11.5-6.6	9.6, 11.7-7.8	0.57
CRP (mg/dl) *	12.5, 16.1-5.7	10.9, 13.0-4.2	14.0, 17.9-9.4	0.10
Plt (x 10^4/μl) *	41.7, 50.0-30.6	32.2, 42.7-30.3	46.4, 52.7-32.4	0.18
AST (IU/L) *	31, 39-26	28, 38-25	31, 41-28	0.91
ALT (IU/L) *	31, 44-24	24, 32-23	40, 66-29	0.86
LDH (IU/L) *	258, 292-210	243, 292-203	268, 295-224	1.0

KD, Kawasaki disease; IFX, infliximab; WBC, white blood cells; CRP C-reactive protein; Plt; platelets;

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

*Data are expressed as median, range. ** derived from comparison of responders and non-responders.

Figure 1.
Concentrations of serum cytokines, chemokines, sTNFR before IFX administration in the KD patients and healthy controls.
Serum IFN-γ, IL-17A, IL-8, IL-1β, IL-10, TNF-α, MCP-1, G-CSF, IP-10, MIG, IL-6, sTNFR1, sTNFR2, and IL-18 were measured in patients in KD before administration and healthy controls. Bars indicate the mean for each group. p values are derived from comparisons of KD patients with healthy controls.

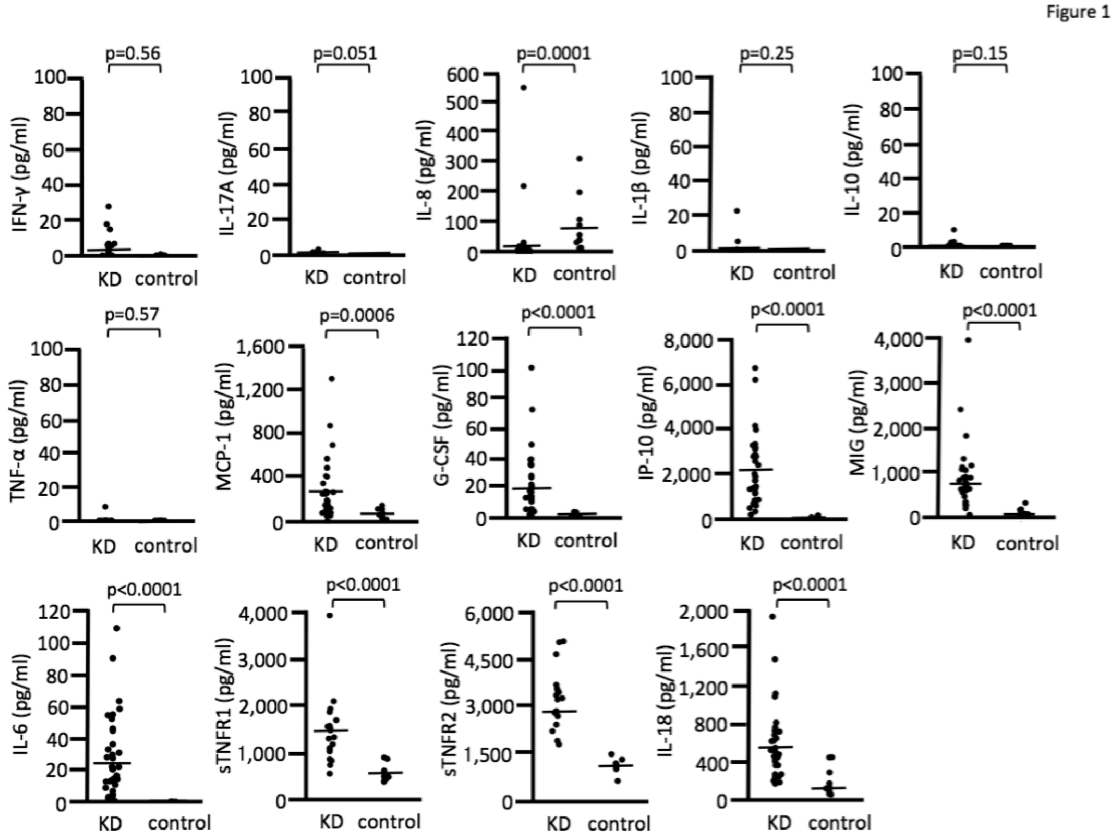

Figure 2.
Alterations in serum cytokines by IFX in KD patients.
Serum levels of IL-6, G-CSF, IP-10, MIG, sTNFR1, sTNFR2, IL-18, and MCP-1 were indicated before and after IFX therapy in KD patients. p values indicate changes before and after treatment.

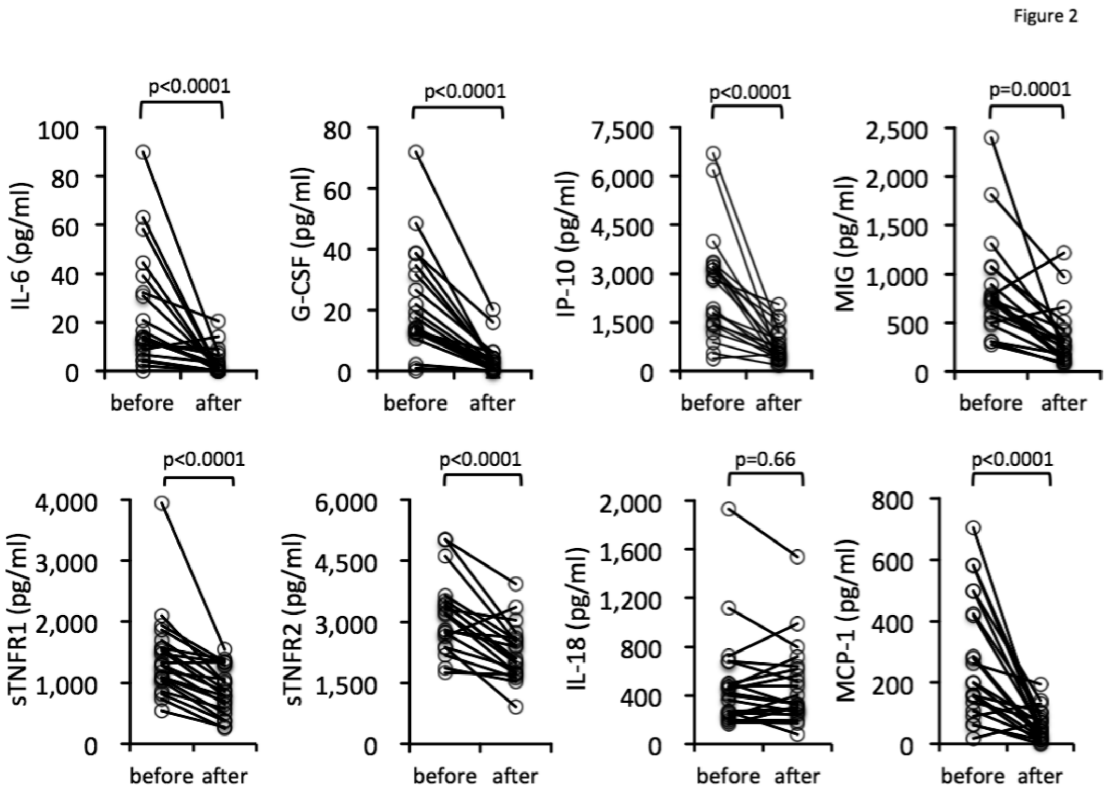

Figure 3.
Alterations in serum cytokine levels by administration of IFX in responders and non-responders.
Serum IL-6, G-CSF, IP-10, MIG, sTNFR2, sTNFR2, IL-18, and MCP-1 were indicated in responders and non-responders before and after IFX. p values are derived from comparisons of responders and non-responders before and after IFX administration.