Original Article
The plasma level of soluble IL-2 receptor (sIL-2R) is elevated in children with acute Kawasaki disease

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Abstract: Purpose: Kawasaki disease (KD) is a systemic vasculitis syndrome caused by immune dysfunction involving regulatory T cells (Tregs). Circulating soluble interleukin 2 receptor (sIL-2R) has been shown to regulate T-lymphocyte activation in various immunological disorders. To investigate the effect of plasma sIL-2R on Tregs, we analyzed the relationship between sIL-2R, Tregs and the IL-2/STAT5 signaling pathway in children with KD. Methods: Blood samples were collected from 33 children with KD before and after intravenous immunoglobulin therapy, as well as from 14 age-matched healthy controls. Circulating levels of CD4+CD25+Foxp3+ Tregs, levels of phosphorylated-STAT5 (pSTAT5), and IL-2R in CD4+CD25+Foxp3+ Tregs, and the levels of sIL-2R and IL-2 family cytokines in plasma were measured by flow cytometry, cytometric bead array and Real-time PCR, respectively. Results: The proportion of Tregs, mRNA levels of associated factors (Foxp3, GITR, CTLA-4 IL-2Rα and IL-2Rβ), and protein levels of pSTAT5 in Tregs were significantly reduced (P < 0.01), while plasma sIL-2R concentrations were significantly increased in acute KD (P < 0.01). Moreover, circulating sIL-2R levels were higher in KD patients with coronary artery lesions (KD-CAL+) than those without coronary artery lesions (KD-CAL-) (P < 0.01). Plasma sIL-2R levels were negatively correlated with expression of IL-2Rβ, Foxp3 mRNA and pSTAT5 protein (P < 0.01). Moreover, pSTAT5 protein expression was found to positively correlate with Foxp3 mRNA expression (P < 0.01). Intravenous immunoglobulin (IVIG) treatment effectively eliminated these differences between patients and controls. Conclusion: Aberrant signaling of the IL-2/STAT5 pathway may be mediated by increased sIL-2R and may contribute to downregulation of Tregs in KD patients.

Keywords: sIL-2R, IL-2, STAT5, Kawasaki disease, Tregs

Introduction
Kawasaki disease (KD) is an acute systemic vasculitis syndrome in which the medium-sized blood vessels become inflamed, and most frequently affects infants and children under five years of age. Even though the etiology of KD is unknown, epidemiological data suggests that infectious agents can trigger onset of KD. Previous studies have shown that aberrant autoimmune responses are involved in the pathogenesis of KD, however the precise mechanisms remain unclear [1-3]. CD4+CD25+Foxp3+ regulatory T cells (Tregs) are critical for the maintenance of autoimmune tolerance and immune homeostasis. The transcription factor Foxp3, which is required for Treg development and function, is considered the “master regulator” of Tregs [4-6]. Several studies have reported reduced levels and defective function of Tregs during the acute phase of KD, which could be partially recovered in response to intravenous immunoglobulin (IVIG) therapy.

While Treg dysfunction has been implicated in the immune dysfunction in KD, the cause remains unclear [7, 8]. The IL-2/STAT5 signaling pathway has been reported to play a role in regulation of Treg differentiation [9-11], however, whether it is associated with the Treg abnormalities in acute KD remains to be determined. In this study, we investigated expression of IL-2/STAT5 signaling pathway associated molecules in KD patients to further investigate the mechanisms responsible for Treg downregulation in KD.

Materials and methods

Subjects
Thirty-three children in the acute febrile stage of KD (24 males and 9 females; median age: 12
months; age range: 3-54 months) were enrolled in this study (Table 1). These participants were recruited from Shenzhen Children's Hospital between November 2012 and May 2013. KD was diagnosed according to the clinical criteria published by the Kawasaki Disease Research Committee of Japan. Fever onset was recorded as the first day of an acute KD phase. Blood samples were obtained at the acute stage (range: 5-10 days; median: 7 days) before IVIG therapy. Post-therapy samples were also obtained after IVIG therapy (range: 7-13 days; median: 8 days). Fourteen age-matched children (8 males and 6 females; median age: 16 months; age range: 4-38 months) who were physically healthy, without any clinical signs of infection or inflammation, were enrolled as healthy controls (Ctrl).

Informed consent forms were obtained from the parents of all participants as a condition of study enrollment, and the study was approved by the local Medical Ethics Committee. Blood samples were immediately analyzed without stimulation of mitogens or culture in vitro, except where indicated. All patients with KD received 2 g/Kg IVIG and were administered aspirin orally. All KD patients responded well to IVIG therapy except one patient in which fever persisted for 72 h after IVIG. This patient therefore received a second dose of IVIG (Table 2). All KD patients received their first two-dimensional echocardiographic examination within 10 days. Coronary artery lesion (CAL) was defined by an arterial internal diameter > 3 mm (< 5 years) or > 4.0 mm (≥ 5 years), or by the presence of coronary artery aneurysms. Coronary artery aneurysms were considered “present” if the maximum internal lumen diameter was enlarged by at least 1.5-fold. Patients with KD were divided into the KD-CAL+ group and KD-CAL- group according to the echocardiographic examination results.

Blood samples

Venous blood (5 mL) was collected from KD patients and healthy controls in anti-coagulant tubes containing EDTA-Na₂. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation for subsequent flow cytometry analysis. Plasma was obtained after centrifugation and stored at -80°C for analysis by cytometric bead array (CBA). CD4⁺CD25⁺ T cells were immediately isolated from the patients’ peripheral blood samples using microbeads (11363D, Dynal; Invitrogen, USA) according to the manufacturer’s instructions. Cell populations have at least 97% purity as assayed by flow cytometry. Viability was assessed by trypan blue exclusion assay, and samples in which over 95% of cells were viable were used for experiments.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from CD4⁺CD25⁺ T cells according to the manufacturer’s instructions using the miRNeasy Mini Kit (Qiagen, Germany). The integrity of the total isolated RNA was confirmed by an average ODₐ₅₀/OĐ₉₀ absorption ratio of 1.98. cDNA was synthesized with oligo-dT primers and RevertAid™ H Minus reverse transcriptase (Fermentas, Lithuania). Negative control samples (no first-strand synthesis) were prepared by performing reverse transcription in the absence of reverse transcriptase.

LightCycler real-time PCR

The cDNA levels of Foxp3, GITR, CTLA4, IL-2Rα, IL-2Rβ and IL-2Rγ were quantitated by real-time
PCR using the Quantitect™ SYBR green PCR Kit (Takara, Japan) and a LightCycler® 2.0 (Roche Molecular Biochemicals, Switzerland). The primers used for the real-time PCR assays are listed in Table 3. The second derivative maximum method was performed for CP determination using LightCycler software V3.5.30 (Roche Molecular Biochemicals). After normalization with Relative Quantification Software V1.0 (Roche Molecular Biochemicals), the target gene levels were expressed relative to GAPDH.

Flow cytometry analysis of Tregs

Whole blood samples were incubated with anti-human CD4-eFluor 450 and anti-human CD25-PerCP-Cy5.5 (eBioscience, USA) at 4°C for 30 min. The cells were then fixed and permeabilized (eBioscience, USA) according to the manufacturer's instructions and incubated with anti-human Foxp3-APC (eBioscience), and anti-mouse STAT5-Alexa Fluor 488 (BD Pharmingen, USA), or isotype controls (BD Pharmingen, USA). The proportion of CD4^+CD25^+ T cells and fluorescence intensity (MFI) of phosphorylated (p)-STAT5 protein in CD4^+CD25^+ T cells were analyzed using a FACSCanto II cytometer with FACS Diva software (version 6.1.3, Beckman Coulter, USA).

CBA detection of plasma sIL-2R and IL-2 family cytokines

Plasma levels of sIL-2R and IL-2 family cytokines were measured using a CBA kit (eBioscience) according to the manufacturer's instructions. All samples were measured in duplicate.

Statistical analysis

SPSS software for Windows version 13.0 was used for statistical analysis (SPSS Inc., USA). The data are expressed as mean ± standard deviation. Student t-test was used to compare the difference between two groups while the significance among three groups was determined using one-way ANOVA. Pearson correlation was applied to detect correlations between different study parameters. P-values < 0.05 were considered indicative of statistical significance.

Results

Acute KD patients have fewer CD4^+CD25^+Foxp3^+ Treg cells

The percentage of Tregs in patient whole blood was quantified by flow cytometry (Figure 1). KD patients' had a significantly lower proportion of peripheral Tregs than healthy subjects (1.25±0.78% vs. 3.96±0.29%, P < 0.01), but the proportion of peripheral Tregs gradually increased following IVIG therapy (3.59±0.96%, P < 0.01) (Figure 1A, 1B). We performed real-time PCR to
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A

CD25 PerCP-Cy5.5-A

FOXP3 APC-A

B

Treg proportion

Ctrl KD KD^{V/IG}

C

Target gene (CASP8)

Ctrl KD KD^{V/IG}

Ctrl KD KD^{V/IG}

Target gene (GITR)

Ctrl KD KD^{V/IG}

Target gene (CD30L)

Ctrl KD KD^{V/IG}
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Figure 1. The expression of CD4+CD25+Foxp3+ Treg and Treg-related factors from patients with KD. 33 KD patients and 14 control subjects were enrolled in this study. All KD patients received IVIG treatment. Ten patients presented with coronary artery lesions (KD-CAL+), as measured by echocardiography. A. Typical flow cytometry dotplots showing the gating strategy for CD4+CD25+Foxp3+ Tregs. CD4+ lymphocytes were gated by forward and side scatter and CD4 positive. Gated cells were further analysed with CD25 and Foxp3 in healthy controls, patients with KD and KD patients after IVIG treatment (KDIVIG). B. The proportions of CD4+CD25+Foxp3+ Tregs. C. The expressions of Treg-related factors. Relative expressions were determined by real-time PCR using GAPDH as an endogenous reference gene. Normalized values are derived from the ratios of target gene/GAPDH mRNA expression. Data are shown as mean ± SD. vs. KD group, *P < 0.01.

Figure 2. The expression of pSTAT5 in CD4+CD25+ T cells from KD patients. A. Flow cytometry histogram for pSTAT5. B. The MFI for pSTAT5 in CD4+CD25+ T cells. Data are shown as mean ± SD. Characteristics of the Ctrl and KDIVIG populations were compared with KD using one-way ANOVA. vs. KD group, *P < 0.01.

Table 4. Concentrations of cytokines in plasma from patients with acute KD and healthy subjects (X ± s, pg/ml)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>sIL-2R</th>
<th>IL-2</th>
<th>IL-7</th>
<th>IL-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>14</td>
<td>5312.36±1671.43</td>
<td>76.40±9.52</td>
<td>46.91±11.92</td>
<td>2.44±0.82</td>
</tr>
<tr>
<td>KD</td>
<td>33</td>
<td>25941.48±14812.34</td>
<td>77.91±16.53</td>
<td>42.55±15.88</td>
<td>2.59±0.85</td>
</tr>
<tr>
<td>KDIVIG</td>
<td>33</td>
<td>14956.73±8757.46</td>
<td>81.03±12.26</td>
<td>46.87±20.51</td>
<td>2.54±0.70</td>
</tr>
</tbody>
</table>

Characteristics of the Ctrl and KDIVIG populations were compared with KD using one-way ANOVA. vs. Ctrl group, *P < 0.01; vs. KD group, **P < 0.01.

The expression levels of IL-2R in acute KD patients

CD4+CD25+ T cells were isolated from patients' whole blood, and intracellular pSTAT5 was quantified by flow cytometry (Figure 2). The pSTAT5 expression of Tregs was significantly lower in the KD group than the control group (MFI: 33.48±15.39 vs. 56.29±11.66, P < 0.01). IVIG therapy partially rescued pSTAT5 expression to levels not significantly different from control group levels (52.73±18.43) (Figure 2B).

The expression of Foxp3, GITR and CTLA-4 in patients with acute KD

evaluate the level of Foxp3, GITR and CTLA-4 expression in CD4+CD25+ T cells from patients with KD. As shown in Figure 1C, Foxp3, GITR and CTLA-4 mRNA levels were substantially lower in the KD group than the Control group (Foxp3: 1.32±0.62×10−4 vs. 3.39±0.97×10−4, P < 0.01; GITR: 3.36±1.04×10−4 vs. 42.4±19.6× 10−4, P < 0.01; CTLA-4: 4.88±1.25×10−2 vs. 12.9±2.36×10−2, P < 0.01). However, expression of these genes was significantly increased after IVIG therapy (Foxp3: 3.13±0.93×10−1, P < 0.05; GITR: 43.7±8.37×10−4, P < 0.05; CTLA-4: 10.1±2.89×10−2, P < 0.01).

Tregs from KD patients express lower levels of pSTAT5

The concentration of plasma sIL-2R and IL-2 family cytokines was detected by cytometric bead array. As show in Table 4, the concentration of plasma sIL-2R was significantly higher in KD samples than control group samples (P < 0.01). IVIG therapy partially rescued sIL-2R levels, but not to control levels (P < 0.01). Moreover, the concentration of plasma sIL-2R in KD patients with coronary artery lesions (KD-CAL+) was much higher than in those without coronary artery lesions (KD-CAL−) (45005±9664 vs. 17652±6626 pg/mL, P < 0.01) (Figure 3). The concentrations of IL-2, IL-7 and IL-15 in plasma did not differ significantly between KD, Control and KDIVIG groups (P > 0.05). The concentrations of plasma IL-2Rα was positively correlated with level of CRP (r=0.63, P < 0.01).

The expression levels of IL-2Rα in acute KD patients

Expression of IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (CD132) mRNA in CD4+CD25+ cells was detected by real-time PCR. As show in...
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Figure 3. The concentrations of plasma sIL-2R in acute KD. Data are shown as mean ± SD. Characteristics of the Ctrl and KD populations were compared with KD using one-way ANOVA vs. KD-CAL+ group. *P < 0.01. Ctrl, healthy control; KD-CAL+, KD patients with coronary artery lesion; KD-CAL−, KD patients without coronary artery lesion.

Figure 4, expression of IL-2Rα and IL-2Rβ was significantly decreased during acute KD (P < 0.01), and IVIG therapy dramatically elevated IL-2Rα and IL-2Rβ expression (P < 0.01). However, expression of IL-2Rγ did not differ significantly between KD patients and healthy controls (P > 0.05). Regression analysis indicated that the concentration of sIL-2R in plasma was negatively correlated with mRNA expression of IL-2Rβ and Foxp3, and with protein expression of pSTAT5. Moreover, Treg pSTAT5 content was positively correlated with the mRNA levels of Foxp3 (Figure 4).

Discussion

Kawasaki disease is a systemic vasculitis syndrome related to immune dysfunction. The immunopathogenesis of KD remains unknown and requires further study [1-3] Tregs constitute a developmentally and functionally distinct T cell subpopulation, and are required for sustained immunological self-tolerance and homeostasis. The transcription factor Foxp3 is specifically expressed in Tregs and is a key regulator of their differentiation and immunosuppression [4-6]. Several studies have reported that the number and activity of Tregs is reduced in acute KD, but are somehow recovered after IVIG therapy [7-8]. Consistent with previous findings, our data demonstrates that Treg numbers and the expression of Treg-related factors (Foxp3, GITR, CTLA-4) were significantly lower in KD patients than healthy controls. IVIG therapy partially recovered Treg number and gene expression, however the mechanism remains unclear.

Signal transducers and activators of transcription (Stats) represent a small but critical family of transcription factors that play important roles in transmitting cytokine signals. Consequently, Stats are critical for immunoregulation and the development of immune cells [10]. Activation of STAT5 plays an essential and direct positive role in regulating Foxp3 and Tregs [10-12]. Peripheral Treg counts were significantly lower in STAT5 knockout mice [12], and Foxp3 expression is lower in human T cells with STAT5 mutations [13]. In this study, we observed that the pSTAT5 content of acute KD patient Tregs was significantly lower than that of healthy control Tregs. After IVIG therapy both Foxp3+ and pSTAT5 expression increased, suggesting that STAT5 signaling pathway abnormalities may contribute to depletion of Tregs in patients with acute KD.

We further investigated the mechanisms by which pSTAT5 is downregulated in acute phase KD. Previous studies have shown that IL-2 signals through a receptor complex consisting of three subunits, namely the IL-2Rα, IL-2Rβ and common γ-chain. IL-2 can bind IL-2R, IL-15 can bind IL-2Rβ-γc, and IL-7 can bind IL-2Rγ, inducing STAT5 phosphorylation and upregulation of Foxp3 in Tregs [10, 11, 14]. To better understand the effect of IL-2 family cytokines (IL-2, -7, and -15) on STAT5 activation, we examined the plasma concentration of IL-2 family cytokines in acute KD, and found no difference between acute KD and normal children, indicating that these IL-2 family cytokines may not be the major drivers of downregulation of pSTAT5 during acute KD.

The role of sIL-2R expression in autoimmune disease has been extensively studied in systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis [15-17]. Serum sIL-2R is a useful parameter for evaluating disease stage and monitoring disease progression during post-therapy follow-up, though it is not an organ-specific parameter [18]. We found increased levels of plasma sIL-2R in acute KD patients, and plasma sIL-2R levels positively correlated with serum CRP levels. Moreover, patients with coronary artery lesions (KD-CAL+) had much higher plasma sIL-2R levels than those without coronary artery lesions (KD-CAL−),
indicating that elevated sIL-2R may be involved in development or worsening of KD.

The soluble IL-2 receptor (sIL-2R) is cleaved from surface IL-2R and released into the circulation [19]. High circulating levels of sIL-2R could indicate excessive T cell activation and an inflammatory state [18-20]. Here we explored not only plasma levels of sIL-2R in patients with acute KD, but also the relationship between sIL-2R, Tregs and the IL-2/STAT5 signaling pathway. In this study, the levels of
expression IL-2Rα, and IL-2Rβ expression were significantly reduced during acute KD. Correlation analysis indicated that plasma sIL-2R concentration was negatively correlated with mRNA levels of IL-2Rβ, Foxp3 and cellular pSTAT5 content, which were positively correlated with the levels of Foxp3 mRNA. Therefore, we speculate that aberrant IL-2/IL-2Rβ/STAT5 signaling, resulting from increased plasma sIL-2R, may contribute to down-regulation of Foxp3+ Tregs in acute KD.

IVIG is an increasingly popular therapy for autoimmune and systemic inflammatory diseases due to its immunomodulatory and anti-inflammatory potential [21]. IVIG can protect against some autoimmune diseases by increasing the number of peripheral Tregs, however the mechanisms responsible for the therapeutic effects of IVIG remain to be defined [22]. In our study, the increased levels of plasma sIL-2R were reversed after IVIG therapy, and both IL-2/IL-2Rβ/STAT5 signaling and the proportion of circulating Tregs were restored. Although it is unclear how IVIG reduced the plasma levels of sIL-2R, and restores IL-2/IL-2Rβ/STAT5 signaling to increase circulating levels of Foxp3+ Tregs, our results still provide new insights into the immunoregulatory mechanism of IVIG.

Our data demonstrates that the down-regulation of Tregs may be associated with increased sIL-2R levels, leading to aberrant IL-2/IL-2Rβ/STAT5 signaling and the proportion of circulating Tregs were restored. Although it is unclear how IVIG reduced the plasma levels of sIL-2R, and restores IL-2/IL-2Rβ/STAT5 signaling to increase circulating levels of Foxp3+ Tregs, our results still provide new insights into the immunoregulatory mechanism of IVIG.

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Disclosure of conflict of interest

None.

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