CXCL10/IP-10 Is a Biomarker and Mediator for Kawasaki Disease

Tai-Ming Ko,* Ho-Chang Kuo,* Jeng-Sheng Chang,* Shih-Ping Chen, Yi-Min Liu, Hui-Wen Chen, Fuu-Jen Tsai, Yi-Ching Lee, Chien-Hsiun Chen, Jer-Yuarn Wu, Yuan-Tsong Chen

Rationale: Kawasaki disease (KD), an acute febrile vasculitis, is the most common cause of acquired heart disease in childhood; however, diagnosing KD can be difficult. Objective: To identify unique proteomic biomarkers that can be used to facilitate earlier diagnosis of KD. Methods and Results: We enrolled 214 children with fever and clinical features suggestive of KD. Of those, only 100 were diagnosed with KD. Their plasma samples were globally analyzed for cytokines, chemokines, and cell adhesion molecules using an unbiased, large-scale, quantitative protein array. This study was conducted in 3 stages: discovery, replication, and blinded validation. During the discovery phase (n [KD]=37; n [control]=20), the expression of interleukin-17F, sCD40L, E-selectin, CCL23 (myeloid progenitor inhibitory factor 1), and CXCL10 (IFN-γ-inducible protein 10 [IP-10]) were upregulated during the acute phase in patients with KD when compared with that in the controls. A notable increase was observed in the IP-10 levels (KD, 3037±226.7 pg/mL; control, 672±130.4 pg/mL; P=4.1×10^-13). Receiver-operating characteristic analysis of the combined discovery and replication data (n [KD]=77; n [control]=77) showed that the IP-10 level had high area under the curve values (0.94 [95% confidence interval, 0.9055–0.9778]; sensitivity, 100%; and specificity, 77%). With 1318 pg/mL as the optimal cutoff, the blinded validation study confirmed that the IP-10 levels were a good predictor of KD. With intravenous immunoglobulin treatment, the IP-10 levels returned to normal. The downstream receptor of IP-10, CXCR3, was activated in the T cells of patients with acute KD. Conclusions: IP-10 may be used as a biomarker to facilitate KD diagnosis, and it may provide clues about the pathogenesis of KD. (Circ Res. 2015;116:876-883. DOI: 10.1161/CIRCRESAHA.116.305834.)

Key Words: biomarkers ■ diagnosis ■ IP-10 ■ mucocutaneous lymph node syndrome ■ vasculitis

Kawasaki disease (KD), a multisystem inflammatory condition observed in younger children, can cause acute vasculitis, most notably affecting the coronary arteries. Without treatment, 20% to 25% of children with KD develop coronary artery abnormalities (CAAs). Intravenous immunoglobulin (IVIG) treatment can reduce the incidence of CAAs to ≤5%, but early detection is necessary.2-4 KD diagnosis is difficult, especially at the early stage. Currently, KD diagnosis is based on clinical symptoms, including fever for ≥5 days, bilateral conjunctival injection without exudate, polymorphous exanthema, changes in the lips and mouth (erythema and cracking of lips, strawberry tongue, and diffuse injection of oral and pharyngeal mucosae), changes in the extremities (erythema and edema of the hands and feet), and cervical lymphadenopathy (≥1.5 cm in diameter).5,6 However, overlapping clinical features and laboratory parameters between KD and other conditions make definitive diagnosis difficult, and no specific laboratory tests are available. Therefore, identification of specific biomarkers to facilitate KD diagnosis by laboratory analysis would be valuable for preventing serious KD sequelae, especially CAAs.7

Previous studies have suggested that KD is an immunemediated disease. This notion is also supported by our
Recent findings and those of a Japanese study on immune-related genes (ie, FCGR2A, IGHV, BLK, and CD40), which were identified to be responsible for KD susceptibility. Furthermore, examination of the gene expression profile in the peripheral blood mononuclear cells and urine proteomics supported the hypothesis that cytokine regulators and inflammatory molecules may be associated with KD pathogenesis. Several studies have identified that cytokines and immunoregulatory molecules are associated with KD. The average level of serum interleukin-6 was found to be elevated in patients with acute KD, and the genetic polymorphism in IL-18 has been shown to increase the KD risk. Furthermore, the increase in the meprin A and filamin C levels in the urine of patients with acute-phase KD may be potential markers that could aid in KD diagnosis. Although numerous studies have shown that the expression of multiple genes can be upregulated in acute KD, the differentiation of patients with KD from those with non-KD-related fever in a simple and reliable way remains a challenge. The difficulty in KD diagnosis could be related to the shared inflammatory pathways between a KD and non-KD fever. Nonetheless, there may be unidentified plasma proteins that can act as sufficiently sensitive and specific diagnostic biomarkers for clinical use in diagnosing KD among febrile subjects.

To identify KD-specific effector molecules, we globally analyzed the profiles of cytokines, chemokines, and cell adhesion molecules (CAMs) in patients with KD. This study aimed to identify a protein biomarker that can be used to facilitate the early diagnosis of KD.

### Table 1. Demographic and Clinical Characteristics of Enrolled Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>KD (N=100)</th>
<th>FC (N=114)</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>1.7±1.6</td>
<td>3.6±2.9</td>
</tr>
<tr>
<td>Sex (men %)</td>
<td>66</td>
<td>62</td>
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<tr>
<td>White blood cells per μL</td>
<td>13829.7±4802.7</td>
<td>10733.6±5227.5</td>
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<tr>
<td>Glutamate oxaloacetate transaminase, U/L</td>
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<td>35.8±12.6</td>
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<tr>
<td>Glutamate-pyruvate transaminase, U/L</td>
<td>90.2±100.7</td>
<td>20.3±11.8</td>
</tr>
<tr>
<td>Number of principal clinical features</td>
<td>4±1</td>
<td>2±1</td>
</tr>
<tr>
<td>Duration of fever, d</td>
<td>5±2</td>
<td>5±2</td>
</tr>
<tr>
<td>Left main coronary artery, mm</td>
<td>2.19±0.43</td>
<td>N.A.</td>
</tr>
<tr>
<td>Right coronary artery, mm</td>
<td>1.94±0.45</td>
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</tr>
</tbody>
</table>

All variable data are expressed as mean±SD. FC indicates febrile control; and KD, Kawasaki disease.

### Methods

#### Ethical Statement

The study was approved by the Institutional Review Board and the Ethics Committee of the Institution Review Board of the China Medical University Hospital, Kaohsiung Chang Gung Memorial Hospital, and Academia Sinica in Taiwan. Written informed consent was obtained from the subjects or their parents.

#### Patients

We enrolled 214 Han Chinese children with a fever and clinical features suggestive of KD. Of those, only 100 were eventually diagnosed with KD. The demographic and clinical characteristics of these children are shown in Table 1, and final diagnoses of the 114 children with non-KD are shown in Online Table 1.

The children participating in the study were recruited in Taiwan from medical centers in different geographical areas—the Chang Gung Memorial Hospital Systems including 4 hospitals in the southern and northern part of Taiwan and the China Medical University Hospital Medical Center, including 3 regional hospitals in the central part of Taiwan. KD was diagnosed using known clinical diagnostic criteria. Of the 100 patients with KD, 37 were included in the study’s discovery phase, 40 in the replication phase, and 23 in the blinded validation phase, which included 3 patients with incomplete presentation of KD (defined as the presence of ≤4 principal symptoms of the Japanese criteria).

#### Multiplex Analysis and Quantification of Cytokines, Chemokines, and CAMs

Fresh heparinized blood samples that were obtained from the study subjects were centrifuged at 2000g for 10 minutes. Then the plasma samples were extracted from the aliquots and were stored at −80°C for further analysis. Samples were run in duplicate using the Bio-Plex Pro Human Th-17 Cytokine Panel 15-Plex (Bio-Rad, Hercules, CA). The complete list of cytokines (IL-1β), IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L, and tumor necrosis factor-α was quantified in these cohorts, and their detection limits and reproducibility were provided in the product manual. Fifteen distinct sets of fluorescently dyed beads loaded with capture monoclonal antibodies specific for each cytokine were used. The signal was measured and quantified using the Bio-Plex Protein Array System (Bio-Rad). Assays were performed using Bio-Plex Protein Array System integrated with Bio-Plex Manager Software, version 3.0 (Bio-Rad). Reporter conjugate emission wavelengths were adjusted using the Bio-Plex Calibration Kit (Bio-Rad). Fluidics performance, consistent optical alignment, doublet discrimination, and identification of individual bead signatures were validated using the Bio-Plex Validation Kit, version 3.0 (Bio-Rad). For the initial screening, plasma from 6 patients with KD was examined using human protein array (AAH-CYT-G8-8; Raybiotech Inc, Norcross, GA), which assesses 54 chemokines and CAMs to identify proteins showing an upregulated expression in KD. The complete chemokine/CAM names are...
available at http://www.raybiotech.com. The identified upregulated genes, namely, IL-9, IFN-γ-inducible protein 10 (IP-10), E-selectin, and myeloid progenitor inhibitory factor 1 (MPIF-1), were further quantified in the remaining patients with KD using ELISA. The limits of detection for the E-selectin, MPIF-1, and IP-10 ELISA were 30, 7, and 8 pg/mL, respectively. The reproducibility (intra-assay: coefficient of variance<10%; interassay: coefficient of variance<12%) and specificity of IP-10 were validated; this ELISA kit shows no cross-reactivity with any of the cytokines tested. Dilution ranged from 1:2 to 1:20 according to the manufacturer’s instructions (RayBiotech Inc).

Flow Cytometry

The peripheral blood mononuclear cells were isolated from the heparinized blood by Ficoll-Isopaque density gradient separation (Pharmacia Fine Chemicals, Uppsala, Sweden). Immunophenotypic analyses were performed using distinct fluorochrome-conjugated monoclonal antibodies that recognize human CD3 (UCHT1; BD Biosciences, San Jose, CA) or CXCR3 (1C6/CXCR3; BD Biosciences). After the peripheral blood mononuclear cells, cells were incubated with dilute antibody (1:200) for 1 hour at room temperature, they were examined by multicolor flow cytometry using a FACS Calibur device (BD Biosciences). Data were obtained using CellQuest acquisition software (BD Biosciences), and 0.5 to 2.0×10⁶ events were recorded for analysis in each experiment.

Statistical Analysis

Statistical significance was assessed using unpaired Student t test and the Prism4 software (GraphPad, San Diego, CA). Receiver-operating characteristic curve analysis was performed using SAS software, version 9.3 (SAS Institute Inc, Cary, NC). The receiver-operating characteristic curve plots sensitivity and 1–specificity and provides a summary of sensitivity and specificity across a range of cutoff points for a continuous predictor. Between-group differences were determined using ANOVA and logistic regression analysis. The optimal cutoff value of each candidate biomarker was determined as the sum of its maximum sensitivity and specificity.

Results

Plasma Profile: The Discovery Study

Using the cytokine multiplex system and protein array, 69 inflammatory cytokines were analyzed in total. In the initial screening, the plasma levels of 15 cytokines in 20 non-KD febrile controls and 37 patients with KD were determined. The levels of IL-17F and sCD40L were significantly higher in the patients with KD than in the febrile controls (Figure 1O). Only 1 cytokine, IL-33, was found to be downregulated (Figure 1).

![Figure 1. Plasma cytokine, chemokine, and cell adhesion molecule levels during the acute phase of Kawasaki disease (KD).](image)

A–O, Plasma cytokine levels are measured in non-KD febrile controls (n=20) and patients with KD (n=37) using the Bio-Plex system. P–R, The levels of plasma E-selectin, myeloid progenitor inhibitory factor 1 (MPIF-1), and IFN-γ-inducible protein 10 (IP-10) identified from the protein array are determined by ELISA in the febrile controls (n=20) and KD cases (n=37). The P values of interleukin (IL)-17F, IL-33, sCD40L, E-selectin, MPIF-1, and IP-10 were 1.5×10⁻², 4.7×10⁻¹, 2.8×10⁻⁴, 8.6×10⁻³, 2.3×10⁻⁴, and 4.1×10⁻⁴, respectively. Each dot represents the average of 3 analyses with variation <5% SD from a single individual. *P<0.05, **P<0.01, ***P<0.001, unpaired Student t test. TNF indicates tumor necrosis factor.
For the remaining 54 inflammatory chemokines and CAMs, a proteomics approach was used to identify candidate biomarkers in a set of plasma samples obtained during the acute phase in 6 patients with KD randomly selected from the discovery phase. These data were compared with those of the controls with a non-KD fever and skin rash. The average expression levels of 10 cytokines or CAMs were ≥1.3-fold higher in the patients with KD than in the controls (Online Table II). Among these 10 proteins, IL-9, IP-10, E-selectin, and MPIF-1 showed an increase in the average expression of ≥2-fold in patients with KD, and this result was found in all 6 patients tested. Furthermore, the PDGF-AA, IL-2R-α, CD14, IGF-II, and Siglec-5 genes were downregulated in the patients with acute-phase KD, showing a ≥1.8-fold decrease (<60%, data not shown) compared with the controls. ELISA was then conducted with a larger sample size (20 non-KD febrile controls and 37 patients with KD) to quantify candidate biomarkers (IL-9, IP-10, E-selectin, and MPIF-1). Consistent with the protein array data for the patients with acute-phase KD, there were significant increases in the IP-10, MPIF-1, and E-selectin levels (Figure 1P–1R). However, the increase in the IL-9 levels became insignificant when the sample size increased (data not shown). Among the 6 candidate KD biomarkers (IL-17F, IL-33, sCD40L, E-selectin, MPIF-1, and IP-10), IP-10 showed the most significant increase in patients with KD (3037±226.7 pg/mL) compared with the controls (672±130.4 pg/mL; values in patients with KD versus values in non-KD febrile controls; P=4.1×10^{-11}; Figure 1R).

**IP-10 Levels: The Replication Study and Combined Studies**

To validate the role of IP-10 further, a replication study involving an additional 40 patients with KD and 57 non-KD febrile controls was performed. As shown in Figure 2A, this study also showed a significant increase in the IP-10 levels in patients with KD when compared with those in the febrile controls. When the data from the replication study were combined with those of the discovery study (combined studies), the IP-10 level was significantly elevated in 77 patients with KD (3587±210.2 pg/mL) compared with the 77 non-KD febrile controls (921±106.2 pg/mL; values in patients with KD versus values in non-KD febrile controls; P=2.8×10^{-20}; Figure 2B).

To confirm the role of IP-10 as a biomarker in KD diagnosis further, receiver-operating characteristic curve analyses were performed using values of IP-10 from the combined studies. IP-10 showed an extremely high area under the curve values...
of 0.94 (95% confidence interval, 0.9055–0.9778; Figure 2C) when non-KD febrile patients were used as the controls. With a plasma IP-10 level of 1318 pg/mL as the optimal cutoff value, as defined by the sum of maximum sensitivity and specificity, IP-10 showed a high sensitivity (100%) and specificity (77%) compared with the non-KD febrile controls (Figure 2C).

**Blinded Validation Study**

The final study phase was conducted using plasma samples from 60 children who were suspected with KD. The plasma IP-10 levels were measured in samples labeled in a blinded fashion, and the results were unblinded and analyzed. Using a cutoff value of 1318 pg/mL, 29 samples were IP-10 positive and 31 were IP-10 negative. KD was successfully diagnosed in 22 of the 29 IP-10 positive samples (including 2 cases of incomplete presentation of KD); the remaining 7 samples were diagnosed with a non-KD fever (Figure 3). Of the 31 IP-10 negative samples, 30 were from non-KD febrile controls and 1 was from an incomplete presentation of KD patient. Overall, the IP-10 cutoff value of 1318 pg/mL showed good ability to distinguish between 23 patients with KD and 37 non-KD febrile controls (sensitivity, 96% [22/23]; specificity, 81% [30/37]).

**Association of Plasma IP-10 Levels With Fever Duration and Intravenous Immunoglobulin Treatment**

To determine whether increased IP-10 levels could be detected during the early stage of KD, 37 KD samples obtained within 4 days of onset of fever (mean, 3.4±0.90 days; range, 1–4 days) were examined, and the results were compared with those of 46 samples obtained at a later stage of the disease (mean, 6.0±1.05 days of the onset of fever; range, 5–8 days). IP-10 levels were increased significantly in the early disease stage (3054±331.0 pg/mL; Figure 4A). Using 1318 pg/mL as the optimal cutoff value, 81% (30) of the 37 patients with KD were identified as being in the early stage (<4 days), whereas 96% (44) of the 46 patients with KD were in the acute stage (>5 days).

IP-10 levels were also examined in 45 patients before and 1 week after the initiation of IVIG treatment. High IP-10 levels before treatment returned to normal with IVIG treatment (before treatment, 3323±224.9 pg/mL; after treatment, 348±64.8 pg/mL; Figure 4B), except in 1 patient with KD who was resistant to the first round of IVIG treatment and required a second course of therapy.

**Figure 3.** Plasma levels of IFN-γ-inducible protein 10 (IP-10) in the blinded validation study. A, Plasma IP-10 levels in the validation study (patients aged <6 years), which included febrile controls (F/hF, n=37), incomplete patients with Kawasaki disease (KD) (iK, n=3), and patients with KD (K, n=20), are determined using ELISA. Error bars indicate the SD from triplicate values.

**Figure 4.** Plasma IFN-γ-inducible protein 10 (IP-10) levels in Kawasaki disease (KD) in relation to the duration of fever (A) and intravenous immunoglobulin (IVIG) treatment. A, Dot plot of IP-10 plasma levels in patients with KD with blood obtained <4 days (mean, 3.4±0.90 days; range, 1–4 days) from the onset of fever or >5 days (mean, 6.0±1.05 days; range, 5–8 days) from the onset of fever. B, Plasma IP-10 levels before and 1 week after intravenous immunoglobulin (IVIG) treatment in 45 patients with KD. Each dot represents the average of 3 analyses with variation <5% (SD/average) from a single individual.
Cell Surface Chemokine Receptor CXCR3 in T Cells

IP-10 downregulates the cell surface chemokine receptor CXCR3 in T cells. To the cell surface expression of CXCR3 in T cells of 6 patients with KD was analyzed. The mean fluorescence intensity of CD3+ T cells was measured, and there was a 3.3-fold decrease in mean fluorescence intensities in patients with acute stage KD when compared with the mean fluorescence intensities of 3 healthy donors (Figure 5). In the recovery stage, the expression levels of CXCR3 were restored to normal (data not shown).

Discussion

Prompt diagnosis and IVIG treatment of KD is important because delays can increase the incidence of CAAs and other devastating cardiac complications. Several previous studies reported increased levels of certain cytokines or cytokine regulators in association with KD,13,18–21 and a haptoglobin phenotype that may help in diagnosis at a late stage has been identified.25 Although we confirmed the elevation of IL-6 levels compared with healthy controls, this marker was not as specific or sensitive of a predictor of KD when compared with IP-10 because of lack of discrimination in the suspected cases with fevers (Figure 1). Although IL-1β was critical in the development of coronary lesions in a mouse model of KD26, the plasma levels of IL-1β were not significantly elevated in the patients with acute KD in the present study (Figure 1). Increase in meprin A and filamin C levels in the urine of patients with acute-phase KD might be helpful for KD diagnosis using urine samples11; however, their use in the blood is not clear. In the present study, using protein array, we found that a novel KD-specific potential biomarker IP-10 had the strongest association with KD among 69 key immune-associated molecules studied. With 1318 pg/mL as the optimal cutoff, IP-10 was identified as an excellent biomarker for differentiating KD cases from non-KD cases, including subjects who were highly suspected of KD, such as febrile cases with scarlet fever (Online Table 1). When compared with the previously reported biomarkers in the blood, IP-10 seems to be the most significant biomarker that can be used as a predictor for KD diagnosis.

Elevated chemokine levels, including IP-10, have been reported in KD when compared with patients with Henoch–Schönlein purpura.27 Elevated IP-10 levels have also been observed in other inflammatory diseases, such as infectious diseases and some autoimmune disorders.28 In addition, IP-10 has been recognized as a potential biomarker for predicting the severity of some diseases, such as hepatitis C virus and rhinovirus infections. However, the levels of IP-10 in these conditions are relatively lower (<500 pg/mL)29,30 than those observed in the acute stage of KD in the present study (3587±210 pg/mL). Furthermore, these diseases can easily be clinically differentiated from KD.

IP-10 is secreted by several cell types, including monocytes, endothelial cells, and fibroblasts, and can be induced by Th17–associated cytokines.31 It shows potent lymphocyte chemoattractant activity and binds to a common receptor, CXCR3, whose expression is unregulated on tissue-infiltrating T cells. We found that CXCR3 was downregulated in T cells during the acute stage of KD. The observation that CXCR3 needs IFN-γ for the expression and is localized to sites of inflammation indicates that the IP-10–CXCR3 axis may play an important role in effector lymphocyte recruitment to inflammatory tissue. Previous studies using CXCR3-deficient or IP-10–deficient mice found reduced levels of tissue-infiltrated T cells in several disease models, including inflammation and transplantation.32,33 In addition, in vitro studies have shown that CXCR3 ligands can promote the adhesion of lymphoblasts to human endothelial cells. IL-6–triggered STAT3 phosphorylation is an important upstream signal for IP-10 production by macrophages.34 Therefore, IP-10 further amplifies autocrine IL-6 production by activating B cells to sustain STAT3 signals, which may explain the high levels of IL-6 observed in the plasma samples of patients with acute-phase KD. STAT3 phosphorylation is an indispensable downstream signaling event for the differentiation of macrophages and B cells to immunoglobulin A–secreting plasma cells, which may infiltrate the inflamed tissues of patients with KD.35 Moreover, the increase in plasma levels of CXCL9, another ligand of CXCR3, in patients with KD (data not shown) supports the notion that activation of the CXCR3 pathway may be important for the development of KD.

In summary, the present study found a significant elevation in the plasma IP-10 levels in patients with acute stage KD. A...
limitation of this study was that all the enrolled subjects were Han Chinese; therefore, we were unable to determine whether the optimal IP-10 cutoff value (1,318 pg/mL) was applicable to other races. Because IP-10 functions as a chemotactant, this increase provides a critical indicator for further investigating the pathogenesis of KD. Because of the high sensitivity and specificity of IP-10 as a potential biomarker for KD, this molecule may be useful for diagnosing KD and monitoring patients' treatment responses.

Acknowledgments

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References

The results identify a practicable biomarker and a clear cutoff for early KD diagnosis. Plasma IFN-γ-inducible protein 10 (IP-10) levels were significantly elevated, and the IP-10 downstream pathway was activated in patients with acute stage KD. Thus, IP-10 could be used as a biomarker for KD, and it may also provide a novel signal pathway for further investigating KD pathogenesis.

Novelty and Significance

What Is Known?

- Kawasaki disease is the most common cause of acquired heart disease in young children.
- Delay of diagnosis can increase the incidence of coronary artery abnormalities and other devastating cardiac complications.
- Diagnosing Kawasaki disease (KD) can be difficult because of its varied clinical manifestations and lack of specific laboratory tests.

What New Information Does This Article Contribute?

- We examined the global profile of cytokines, chemokines, and cell adhesion molecules in plasma samples from a large cohort of patients with clinical features suggestive of KD.
- Plasma IFN-γ-inducible protein 10 (IP-10) levels were significantly elevated, and the IP-10 downstream pathway was activated in patients with acute stage KD.
- The results identify a practicable biomarker and a clear cutoff for early KD diagnosis.
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Online Table I. Final Diagnoses of 214 Pediatric Patients.

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Online Table II. Signaling Intensities of the 10 Selected Candidate Genes Encoding Chemokines and CAMs from the Plasma of Acute-Phase Kawasaki Disease (KD) Patients.

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<td>26,188</td>
<td>27,011</td>
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All tests were performed in duplicate. Internal negative controls were used to determine the cut-off rate for a positive signal. Six KD patients and 2 control (Ctr) subjects were screened using protein arrays. Ctr-1 was a pediatric subject with a non-KD fever. Ctr-2 was a normal healthy subject. Only the KD/Ctr ratios (the average of KD patients/average of ctr cases) of chemokines and CAMs exceeding 1.3 are shown.