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ORIGINAL ARTICLE

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Intravenous gamma-globulin therapy improves hypercytokinemia in the acute phase of Kawasaki disease

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Abstract Proinflammatory cytokinemia and subsequent endothelial cell activation are the major pathological features of Kawasaki disease (KD), which progresses to systemic vasculitis and results in coronary artery lesions (CALs). We studied the serum levels of proinflammatory cytokines and of the soluble receptors before and after intravenous gamma-globulin (IVGG) treatment to investigate whether the anti-inflammatory effect of IVGG was due to the reduction of increased serum levels of their cytokines and soluble receptors. In the acute phase of KD, the serum levels of interleukin (IL)-2 and IL-5, as well as those of IL-6, IL-10, and interferon-γ, were markedly higher than those in controls. The level of tumor necrosis factor-α was higher than that in the control, but the difference was slight and not significant. The soluble IL-6 receptor levels were lower than those of the controls. After IVGG administration, the increased levels of these cytokines and soluble receptors were abruptly down-regulated to within their normal ranges. The patients enrolled in the present study were all effectively treated with IVGG, without a steroid, and improved without any residual CALs. Overall, IVGG administration to patients with KD was found to be effective in reducing inflammatory processes and in preventing CALs, and was followed by reduction of the serum levels of the proinflammatory cytokines and their receptors.

Key words Hypercytokinemia · Interleukin (IL)-6 · Intravenous gamma-globulin (IVGG) · Proinflammatory cytokine · Tumor necrosis factor (TNF)-alpha

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Introduction

Kawasaki disease (KD) is an acute illness of early childhood characterized by prolonged fever, diffuse mucosal inflammation, indurative edema of the hands and feet, polymorphous skin rash, and nonsuppurative lymphadenopathy. Immune activation and generalized vasculitis are two central features of KD, and coronary artery lesions (CALs) may develop in up to 25% of untreated patients.[1,2]

Recent observations have indicated that serum levels of cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β and IL-10, increased markedly during the acute phase of KD, a change which may induce endothelial cell activation and initial injury, thus triggering systemic vasculitis.[3–7] It is known that the administration of intravenous gamma-globulin (IVGG) improves the clinical status[8,9] and results in a decrease in the frequency of CALs from 25% to 5%.[10] Thus, we wondered whether the anti-inflammatory effect of IVGG was due to a reduction of serum cytokine levels.

Materials and methods

Subjects

All parents of patients enrolled in this study had given informed consent. The patients fulfilled the revised criteria for KD established in 1982.[11] Nine patients (6 boys and 3 girls) aged 21.6 ± 16.7 months (mean ± SD) were given 2 g/kg IVGG plus 30 mg/kg aspirin, but none received steroid therapy. The clinical characteristics, i.e., age, sex, total dose of IVGG, day of admission, initial date of IVGG treatment, laboratory findings (white blood cell count, percentage of neutrophils, and C-reactive protein (CRP)), and diameter of coronary arterial lumen estimated using echocardiography, are shown in Table 1. Eleven healthy age-matched children (5 boys and 6 girls) who had already recovered from previous inflammatory disease served as control subjects. The Institutional Review Board approved the study.

Table 1. Profile of patients

Pt. No.	Age (months)	Sex	Day of admission	Criteria score	WBCs on admission (/μl)	Neutrophils on admission (%)	CRP on admission (mg/dl)	Initial day of IVGG	WBC in convalescent phase (/μl)	Neutrophils in convalescent phase (%)	CRP in convalescent phase (mg/dl)	Int. diam. of cor. art. at discharge (mm)
1	6	Male	3	5/6	18 400	51.1	7.11	5	8 700	43.3	0.33	2.4
2	8	Male	4	5/6	18 500	61.0	10.95	5	7 400	47.4	0.13	2.1
3	12	Female	6	5/6	9 800	44.7	11.19	6	4 800	42.0	0.23	1.9
4	27	Male	4	6/6	11 100	81.0	6.19	5	5 500	44.9	0.14	2.4
5	43	Male	4	5/6	15 000	86.0	6.70	5	9 600	57.0	0.80	2.7
6	49	Male	4	5/6	14 100	86.0	6.32	6	4 100	27.0	0.00	2.2
7	32	Male	4	6/6	6 400	84.5	1.60	5	10 400	45.8	0.20	3.0
8	5	Female	5	6/6	17 400	65.5	6.19	5	9 500	25.1	0.20	1.6
9	12	Female	4	5/6	15 800	55.0	8.29	4	8 800	37.8	0.11	2.3
Mean ± SD	21.6 ± 16.7		4.2 ± 0.8		14 056 ± 4 171	68.3 ± 16.4	7.2 ± 2.9	5.1 ± 0.6	7 644 ± 2 801	41.4 ± 10.0	0.2 ± 0.2	2.3 ± 0.4

Pt., patient; int. diam., internal diameter; cor. art., coronary artery

Detection of serum cytokines

Serum samples were stored at −20°C until use. The concentrations of cytokines in the sera before and 3 weeks after the start of IVGG administration were determined using a cytometric bead array (CBA) system (BD Biosciences, San Jose, CA, USA) and an enzyme-linked immunosorbent assay (ELISA).

The serum levels of IL-2, IL-4, IL-5, IL-10, and TNF-α were measured with the CBA system (Th1/Th2 kit) according to the manufacturer's instructions. Briefly, five bead populations with distinct fluorescence intensities and coated with antibodies specific for each cytokine were mixed with PE-conjugated detection antibodies and then incubated with recombinant standards or 50 μl of serum for 3 h at room temperature while protecting them from direct exposure to light. After incubation, both standards and samples were washed with CBA wash buffer and then centrifuged. The supernatants were discarded from each assay tube, and the bead pellets were resuspended with wash buffer. Then acquisition of the samples was performed using CellQuest software with a FACSCalibur flow cytometer, and the sample data were analyzed using CBA Software (BD Biosciences).

The concentrations of IL-6, IFN-γ, soluble IL-6 receptor, and soluble tumor necrosis factor receptor-I (sTNFR-I) were determined by ELISA using an OptEIA kit (PharMingen, San Jose, CA, USA), a human IL-6 ELISA kit (PharMingen), a human IFN-γ ELISA kit (Endogen, Woburn, MA, USA), a Quantikine human IL-6 sR kit (R&D systems, Minneapolis, MN, USA), and a Quantikine human sTNF RI kit (R&D systems), according to the manufacturer's instructions. Briefly, these assays employ the quantitative sandwich enzyme-linked immunosorbent assay technique. A monoclonal antibody for a specific cytokine or cytokine receptor had been precoated onto a 96-well plate. Standards (recombinant cytokines or cytokine receptors) and samples are pipetted into the wells, and any cytokine or cytokine receptor present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokine or cytokine receptor is added to the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of cytokine or cytokine receptor bound in the initial step. The color development is stopped and the intensity of the color is measured at 450 nm within 30 min.

Statistical analysis

The quantitative variables were tested for statistical significance by the Mann–Whitney *U*-test. Values of $P < 0.05$ were considered to be significant.

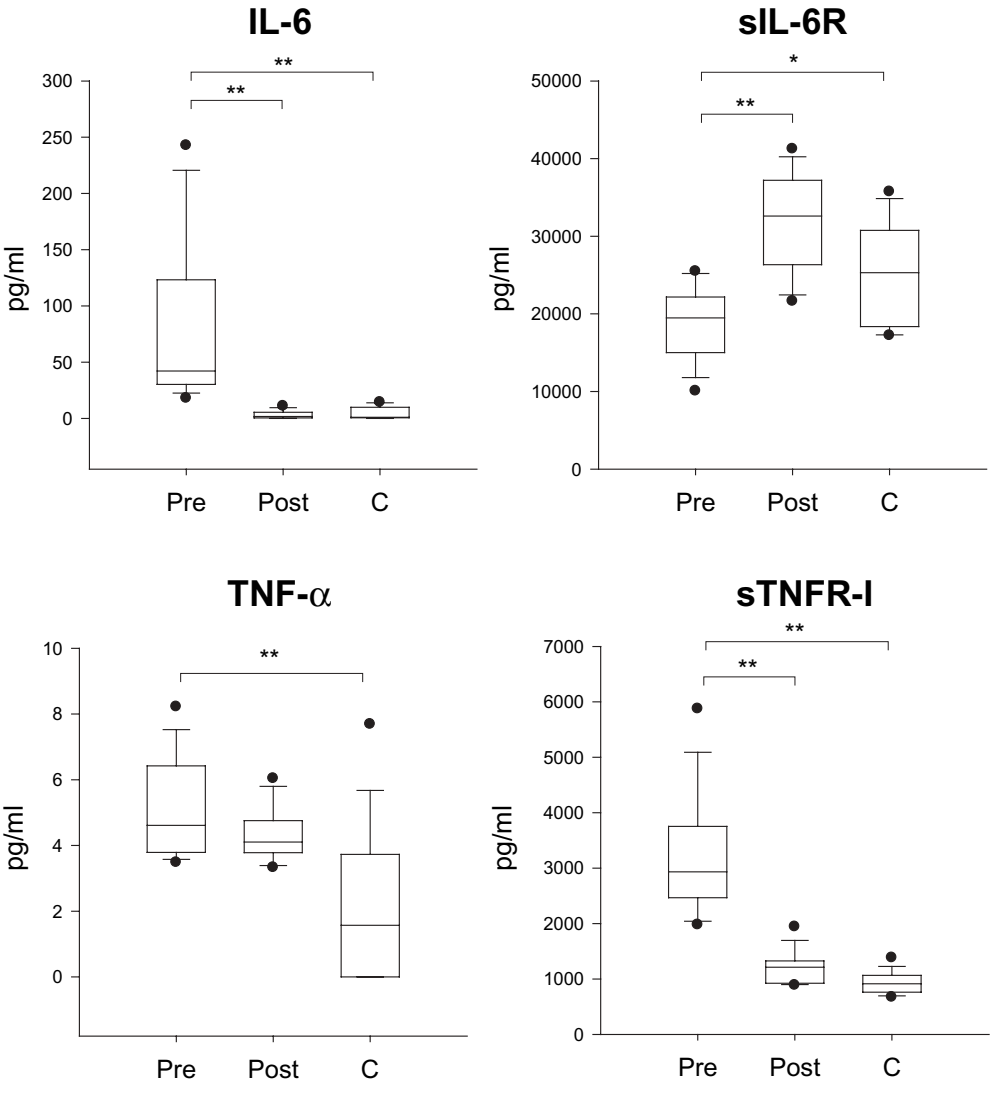

Fig. 1. Proinflammatory cytokines and their soluble receptor levels in sera of Kawasaki disease (KD) patients before and after intravenous gamma-globulin (IVGG) therapy. Serum cytokine levels in KD patients and in healthy children used as controls were measured with the cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA) systems. Each box represents statistical values. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. The whiskers above and below the box indicate the 90th and 10th percentiles. The significance is expressed by *P* values (**P* < 0.05 and ***P* < 0.01 by the *U*-test). *Pre*, KD in the pre-IVGG treatment; *Post*, KD in the post-IVGG treatment; *C*, healthy children as controls

Results

The serum levels of proinflammatory cytokines (IL-6, INF-γ, IL-10, and TNF-α), Th1/Th2 cytokines (IL-2, IL-4, and IL-5), and cytokine receptors (sIL-6R and sTNFR-I) in KD were determined by CBA and ELISA. These data are shown as the vertical boxes with the error bars in Figs. 1 and 2. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. The whiskers above and below the box indicate the 90th and 10th percentiles. The significance is expressed by *P* values (**P* < 0.05 and ***P* < 0.01 by the *U*-test). Before IVGG treatment, all the measured cytokine levels except TNF-α in the acute phase of the disease were markedly increased compared with those in normal subjects (IL-6, 87.5 ± 79.1 pg/ml; IFN-γ, 17.0 ± 14.8 pg/ml; IL-10, 45.9 ± 47.9 pg/ml; IL-2, 18.6 ± 10.9 pg/ml; IL-4, 10.7 ± 5.4 pg/ml; IL-5, 14.2 ± 9.8 pg/ml) (Figs. 1 and 2). These findings confirmed the previous reports that hypercytokinemia of IL-6, INF-γ, IL-10, IL-2, and IL-4 might play a pathological role in the acute phase of KD, and increased levels of IL-5 in this study will add new findings to hypercytokinemia in KD.

The profile of TNF-α concentration was unique in that serum levels in the acute phase were slightly increased, but the increase was not significant (KD, 5.1 ± 1.6 pg/ml; control, 2.1 ± 0.9 pg/ml), and that it was coupled with remarkable increases in sTNFR-I concentration (KD, 3271 ± 1183 pg/ml; control, 938 ± 334 pg/ml) (Fig. 1).

Surprisingly, sIL-6R levels in the acute phase of the disease were lower than those in the controls (KD, 18 682 ± 4970 pg/ml; control, 25 625 ± 6788 pg/ml) (Fig. 1), which was in contrast with the marked increases of sTNFR, indicating that a different system of ligand–receptor regulation may exist, operating in IL-6/sIL-6R and in TNF-α/sTNFR regulation.

After IVGG administration, the high fever in all patients subsided, and inflammatory markers (CRP, ESR, white blood cell count) and serum albumin were normalized within 2–4 days (data not shown). Sera obtained from

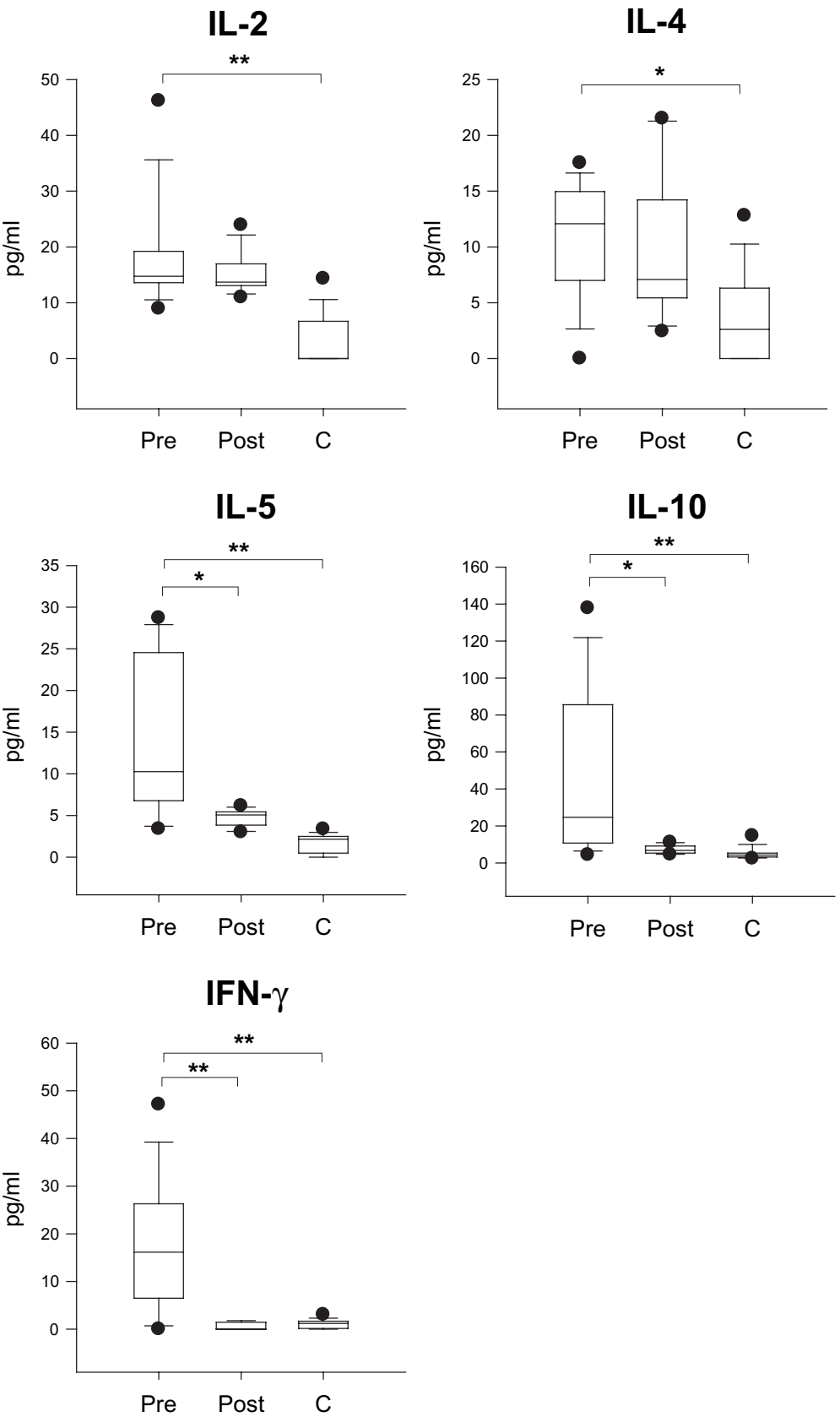

Fig. 2. Th1/Th2 cytokine levels in sera of KD patients before and after IVGG therapy. Serum cytokine levels in KD patients and in healthy children used as controls were measured with the CBA and ELISA systems. Each box represents statistical values. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. The whiskers above and below the box indicate the 90th and 10th percentiles. The significance is expressed by P values ($^{*}P < 0.05$ and $^{**}P < 0.01$ by the U-test). *Pre*, KD in the pre-IVGG treatment; *Post*, KD in the post-IVGG treatment; *C*, healthy children as controls

patients at this stage were used to determine their contents of cytokines and their receptors. Serum levels of IL-6, IFN-γ, IL-10, and IL-5 decreased, and returned to the normal range. However, serum levels of IL-2 and IL-4 were persistently high. TNF-α levels were again unremarkable. The down-regulated sIL-6R recovered to levels equivalent to those of the control subjects, accompanied by decreases in IL-6 levels. The concentration of sTNFR had also decreased to the normal range.

Discussion

In inflammatory diseases, including KD, the mode of action of IVGG seems to be complex, involving modulation of the expression and function of Fc receptors, interference with the activation of complement and the cytokine network, the provision of anti-idiotypic antibodies, regulation of cell growth, and effects on the activation differentiation and effector functions of T and B cells.[12,13] In sera of the acute phase of KD in particular, increased levels of proinflammatory cytokines were detected. Since a clinical improvement and a marked reduction in proinflammatory cytokine levels were achieved simultaneously after IVGG administration, hypercytokinemia will probably prove to be the major pathological sign in KD.[14,15] Sakata et al.[15] demonstrated that cytokines and antiendothelial cell antibodies are important in restructuring and destroying vessel walls in KD by enhancing the migration of endothelial cells, and IVGG may be therapeutically effective for this disease by suppressing this endothelial cell migration. The precise mechanism of the anti-inflammatory effect of IVGG has still not been elucidated, but clinically, IVGG was associated with reduced cytokine levels in the sera,[16,17] which presumably stabilizes the cytokine-producing cells in vasculitis lesions.

The initial vascular lesions in KD are associated with endothelial cell activation and the up-regulation of cytokine-induced leukocyte adhesion molecules, accompanied by the infiltration of neutrophils and mononuclear cells. The mononuclear cell infiltrate consists of both CD4+ and CD8+ T cells, as well as macrophages.[18] This state of immune activation is accompanied by elevated levels of proinflammatory cytokines such as IFN-γ, TNF-α, IL-1β, and IL-6.

Several studies of cytokines in KD have been reported. Leung[19] reported that KD patients in the acute phase had circulating antibodies lytic for vascular endothelial cells stimulated with IL-1, TNF, or IFN-γ, and Leung et al.[7] reported that skin biopsies from KD patients have shown endothelial activation by the elevated expression of new surface antigens induced by IL-1β or TNF. In addition, it has been reported that IL-1β and TNF produced by mononuclear cells increased during the acute phase of KD, although these findings were based on a phenomenon observed in vitro.[20] Furukawa and co-workers[3] suggested that the serum TNF, IL-2R, IL-6, and IFN-γ levels were increased during the acute phase, and that these cytokine levels in patients with CALs were significantly higher than those found in patients without CALs.

In this study, the serum levels of IL-2 and IL-4 did not decrease, or decreased temporarily and then returned to elevated levels, between IVGG administrations. The reason for this may have been that IL-2 mRNAs had been stable in activated T cells.[21,22] Some cytokine genes, including IL-2, have base sequences that contribute to the instability of mRNA in their untranslated regions. These sequences prevent the continuous secretion of cytokines, and hence serve to regulate their activity. However, it has been reported that IL-2 mRNAs are stabilized by signaling mediated by CD28, which is a receptor for B7 known as a co-stimulating molecule on the surface of the T cell.[21,22]

In the acute phase of KD, the serum IL-6 levels were increased, but the sIL-6R levels were paradoxically decreased, and sank to lower levels than those of the controls. After IVGG treatment, sIL-6R returned to the level of the controls. IL-6 binds both the membrane-bound and the soluble-form receptors, and the IL-6–sIL-6R complex is capable of activating cells via interaction with membrane-bound gp130.[23] Thus, the decrease of sIL-6R in the acute phase may have been the negative feedback pathway for down-regulating the IL-6/sIL-6R complexes. Bauer et al.[24] reported that a decrease in IL-6R mRNA levels is accompanied by IL-6 secretion by monocytes under inflammatory conditions.[24] Thus, our findings may have been telling the same story as Bauer's study.

Serum sTNFR-I levels were significantly increased in the acute phase of the disease. In contrast to sIL-6R, sTNFR is capable of neutralizing the cytotoxicity of TNF-α by forming a TNF-α/sTNFR complex in peripheral blood. In KD, therefore, elevated levels of serum sTNFR may represent the severe inflammatory responses at the acute phase.[25]

We have suggested that IVGG treatment results in the reduction of high proinflammatory cytokine levels in the serum in the acute phase of KD. However, this leads to the question, where do the increased cytokine levels originate? Although investigators have speculated that hypercytokinemia originated from peripheral circulating mononuclear cells, the de novo expression of several cytokine mRNAs was traced in the peripheral blood mononuclear cells (PBMC) of KD by RPA analysis (data not shown). Therefore, the additional cytokines might, more probably, have originated not from the circulating mononuclear cells, but from lymphocytes and macrophages located in inflammatory lesions such as those of vasculitis.

In conclusion, we have shown that the main role of IVGG in the treatment of KD may be to reduce the elevated levels of the proinflammatory cytokines. More observations of more patients will be needed in order to confirm these findings.

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