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Clinical significance of serum CXCL9 levels as a biomarker for systemic juvenile idiopathic arthritis associated macrophage activation syndrome

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ABSTRACT

To clarify cytokines involved in the development of systemic juvenile idiopathic arthritis (s-JIA) associated macrophage activation syndrome (MAS) and to identify the serum biomarkers for the diagnosis of s-JIA associated MAS, we employed an antibody array that simultaneously detects 174 cytokines. Fifteen s-JIA patients including 5 patients receiving tocilizumab (TCZ) were analyzed. The levels of five cytokines were significantly elevated in MAS phase compared to those in the active phase of s-JIA. CXCL9 showed the most significant increase following the development of s-JIA associated MAS. Next, to confirm clinical significance of serum CXCL9 levels as a biomarker for s-JIA associated MAS, serum CXCL9 levels in 56 patients with s-JIA including 20 with MAS were analyzed. Results were compared with the clinical features of s-JIA associated MAS. Serum CXCL9 levels correlated positively with disease activity. Monitoring of serum CXCL9 is useful for the evaluation of disease activity in s-JIA associated MAS.

1. Introduction

Systemic juvenile idiopathic arthritis (s-JIA) is characterized by chronic arthritis accompanied by high fever and other systemic symptoms, including salmon-pink evanescent rash, hepatosplenomegaly, lymphadenopathy, and serositis [1]. It has been suggested that s-JIA is an auto-inflammatory condition and aberrant induction of proinflammatory cytokines, such as interleukin (IL)-6, IL-1β, and IL-18, may be involved in the pathogenesis of s-JIA and correlate with disease activity [2].

Macrophage activation syndrome (MAS) is a severe complication of s-JIA, which is clinically characterized by fever, hepatosplenomegaly, lymphadenopathy, profound depression of all three blood cell lines, impaired liver function, intravascular coagulation, and central nervous system dysfunction [3]. Examination of bone marrow shows a feature of numerous macrophages exhibiting hemophagocytosis. MAS is considered as a secondary hemophagocytic lymphohistiocytosis (HLH) because of its close resemblance to a group of HLH syndromes [4,5].

The hallmark of MAS is an uncontrolled and dysfunctional immune response, which leads to marked hypercytokinemia [6]. There is accumulating evidence regarding the role of IL-18 as a key driver of both s-JIA and potentially its association with s-JIA associated MAS [7–11]. Although IL-1 and IL-6 are key cytokines in the pathogenesis of s-JIA, IL-18 may play a central role in the pathogenesis of s-JIA associated MAS. However, the cytokines involved in the pathogenesis of s-JIA associated remain to be determined.

Tocilizumab (TCZ) – a humanized anti-IL-6 receptor monoclonal antibody – is an effective cytokine inhibitor for the treatment of s-JIA, with demonstrated clinical efficacy [12,13]. Studies have reported that clinical symptoms and laboratory abnormalities were milder in patients with s-JIA receiving TCZ than in those not receiving TCZ [14,15]. In particular, the concentration of serum C-reactive protein (CRP) did not increase during TCZ therapy, even in patients with MAS. Inhibition of IL-6 by TCZ may induce suppression of the production of inflammatory cytokines [14]. However, the cytokine cascades affected by TCZ in s-JIA associated MAS remain to be determined.

MAS is a potentially life-threatening disease. Therefore, prompt diagnosis is essential to initiate life-saving treatment. However, distinguishing s-JIA associated MAS from s-JIA flares, sepsis, or other secondary HLH may be challenging. Differentiation of s-JIA associated MAS from these conditions is essential for the selection of an appropriate therapeutic intervention in a timely fashion. However, currently,

Abbreviations: s-JIA, systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome; IL, interleukin; HLH, hemophagocytic lymphohistiocytosis; TCZ, tocilizumab; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; HC, healthy children; sTNFR, soluble tumor necrosis factor receptor; IFN, interferon

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there are no definitive serum biomarkers establishing the diagnosis of s-JIA associated MAS.

Enzyme-linked immunosorbent assay (ELISA)-based methods are considered to be the most robust platforms for biomarker discovery and are characterized by a high degree of sensitivity [16]. A recent advancement in protein array technology has created a high-throughput platform for biomarker screening using ELISA.

In this study, we employed the RayBiotech C-Series 2000 antibody array system – an antibody array that simultaneously detects 174 cytokines – to identify the cytokines involved in the development of s-JIA associated MAS and those affected by TCZ, and also to identify potential predictive markers for s-JIA associated MAS. Furthermore, to confirm clinical significance of serum CXCL9 levels as a promising indicator of disease activity in s-JIA and MAS, these levels were measured, and determined their correlation with disease activity and severity.

2. Methods

2.1. Patients and samples

Fifteen patients with s-JIA associated MAS and 4 healthy controls (HCs) were enrolled in this study. Among these 15 patients, five patients developed s-JIA associated MAS during TCZ therapy. Serum samples were obtained in both active phase of s-JIA and MAS phase. The diagnosis of s-JIA was based on the criteria established by the International League of Associations for Rheumatology [1]. The diagnosis of MAS was based on the 2016 EULAR/ACR/PRINTO classification criteria [17]. The criteria for the acute phase of s-JIA were defined as follows: active arthritis, fever, rash, hepatosplenomegaly, generalized lymphadenopathy, and serositis, along with an increased erythrocyte sedimentation rate and CRP levels. At the onset of s-JIA, a proportion of patients had minimal joint disease and the presence of arthritis was confirmed at a later stage. The clinical characteristics of the patients during the active phase of s-JIA and MAS phase are shown in Table 1. Furthermore, we measured serum CXCL9 levels in 56 s-JIA patients (including 20 MAS patients) and 8 HCs, and compared them with clinical features (including serum IL-18 and ferritin levels) to confirm the clinical significance. The clinical characteristics of these patients are shown in Table 2.

The serum was separated from cells, divided into aliquots, frozen, and stored at −80 °C until analyzed. This study was approved by the Institutional Review Board of Kanazawa University, and all participants provided informed consent.

2.2. Quantification of cytokines in the serum

The RayBio human cytokine antibody array (C-Series 2000 antibody array, RayBiotech, Norcross, GA, USA) was used for the detection of 174 cytokines in frozen stock sera obtained from patients with s-JIA and HC, according to the manufacturer's protocol. Relative levels were determined by the ratio of the intensity of each sample over that of the internal positive control. The intensity of each sample and control was measured using the image processing software and analysis in Java ImageJ. The levels of CXCL9, IL-18, IL-6, soluble tumor necrosis factor receptor type I (sTNFR-I), and sTNFR type II (sTNFR-II) in the serum were evaluated using ELISA according to the manufacturer's instructions (CXCL9, IL-6, sTNFR-I, sTNFR-II: R&D Systems, Minneapolis, MN, USA; IL-18: MBL, Nagoya, Japan; neopterin: IBL, Hamburg, Germany).

2.3. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 7 software (GraphPad, San Diego, CA, USA). Cluster analysis was performed using the JMP 13 (SAS Institute Inc. Tokyo, Japan). Data were summarized as median and range. Comparisons between several groups were performed using one-way analysis of variance with Tukey's multiple comparisons test. Within-group comparisons were performed using the Mann–Whitney *U* test. The comparison of the levels of CXCL9 in the serum between the active and MAS phases was performed using a paired *t*-test. Correlations were expressed using the Spearman rank correlation coefficient. $P < 0.05$ denoted statistical significance.

3. Results

3.1. Cytokine expression in s-JIA patients not receiving TCZ

As shown in Fig. 1 and Supplement Tables 1 and 2, the levels of five cytokines (i.e., CXCL9, MIP-1δ, Axl, sTNF-RII, and sTNF-RI) were

Table 1
Clinical characteristics of the 15 patients with s-JIA included in this study.

Characteristics	s-JIA not receiving TCZ (n = 10)		s-JIA receiving TCZ (n = 5)	
	Active	MAS	Active	MAS
Age at disease onset (median/range, years)	4 (0.67–15)		2 (0.67–12)	
Disease duration (median/range, month)	0 (0–1)		20 (1–102)	
Sex (male/female, n)	4/6		2/3	
Laboratory findings (median/range)				
Ferritin (ng/ml)	2273.5 (405–17,484)	9667 (729.6–46,256)	89 (88.4–292)	1100 (63.3–5498)
Plt ($\times 10^4$/μl)	35.5 (21.9–45.5)	12.3 (5.8–22.1)	22 (16.7–30.5)	9.8 (5.8–14)
AST (IU/l)	38 (25–431)	302 (39–1382)	29 (18–43)	73 (45–385)
Fibrinogen (mg/dl)	435 (279–664)	273 (77–512)	204 (197–498)	141 (125–172)
TG (mg/dl)	123.5 (69–136)	191 (143–287)	298.5 (128–469)	606 (112–1100)
IL-18 (pg/ml)	91,250 (25,200–377,000)	259,000 (74,000–830,000)	14,200 (6350–31,500)	87,000 (41,800–183,000)
IL-6 (pg/ml)	10 (2–180)	8.9 (3–152)	22 (3–34)	132 (3–704)
Clinical symptoms (n, %)				
Fever	10 (100)	7 (100)	3 (60)	3 (75)
Rash	7 (70)	3 (43)	1 (20)	1 (25)
Hepatomegaly	0 (0)	0 (0)	0 (0)	0 (0)
Splenomegaly	0 (0)	0 (0)	0 (0)	0 (0)
Arthritis	2 (20)	0 (0)	0 (0)	0 (0)
Serositis	0 (0)	0 (0)	0 (0)	0 (0)
Treatments (n, %)				
PSL	2 (20)	8 (80)	5 (100)	2 (40)
CsA	1 (10)	2 (20)	2 (40)	2 (40)
Others	1 (10)	0 (0)	5 (100)	2 (40)

Table 2
Clinical characteristics of the 56 patients with s-JIA whose CXCL9 levels in sera were determined.

Characteristics	s-JIA not receiving TCZ		s-JIA receiving TCZ	
	Active (n = 46)	MAS (n = 15)	Active (n = 5)	MAS (n = 5)
Age at disease onset (median/range, years)	5 (0.67–15)	4 (0.67–15)	2 (0.67–12)	
Duration from disease onset (median/range, month)	0 (0–82)	0 (0–60)	20 (1–102)	
Sex (male/female, n)	24/22	5/10	2/3	
Laboratory findings (median/range)				
Ferritin (ng/ml)	754.3 (113–17,484)	10,351 (729.6–66,030)	89 (88.4–292)	1100 (63.3–5498)
Plt ($\times 10^4$/μl)	36.2 (16.3–60.8)	11.7 (2.9–22.1)	22 (16.7–30.5)	9.8 (5.8–14)
AST (IU/l)	36 (15–431)	302 (39–1382)	29 (18–43)	73 (45–385)
Fibrinogen (mg/dl)	605 (279–1019)	298.5 (77–512)	204 (197–498)	141 (125–172)
TG (mg/dl)	101 (56–136)	191 (81–563)	298.5 (128–469)	606 (112–1100)
IL-18 (pg/ml)	46,650 (3050–377,000)	255,000 (53,000–830,000)	14,200 (6350–31,500)	87,000 (41,800–183,000)
IL-6 (pg/ml)	45 (2–770)	16 (3–352)	22 (3–34)	132 (3–704)
Clinical symptoms (n, %)				
Fever	45 (98)	12 (100)	3 (60)	3 (75)
Rash	30 (65)	3 (25)	1 (20)	1 (25)
Hepatomegaly	4 (9)	0 (0)	0 (0)	0 (0)
Splenomegaly	2 (4)	0 (0)	0 (0)	0 (0)
Arthritis	20 (43)	0 (0)	0 (0)	0 (0)
Serositis	4 (9)	0 (0)	0 (0)	0 (0)
Treatments (n, %)				
PSL	9 (20)	10 (66)	5 (100)	2 (40)
CsA	1 (2)	2 (13)	2 (40)	2 (40)
Others	1 (2)	0 (0)	5 (100)	2 (40)

significantly increased during the MAS phase compared with those observed during the active phase. Of note, in these patients, the levels of two cytokines (i.e., MMP-9 and PDGF-BB) were significantly increased during the active phase compared with those reported during the MAS phase.

3.2. Cytokine expressions in s-JIA patients receiving TCZ

As shown in Fig. 1 and Supplement Tables 1 and 3, the levels of 10 cytokines (i.e., CXCL9, GROα, ICAM-1, IL-8, MSPα, sTNFR-II, sTNFR-I, Endoglin, ICAM-2, and IL-18BPα) were significantly decreased during the MAS phase in patients receiving TCZ compared with those in patients not receiving TCZ. On the other hand, the levels of five cytokines (i.e., BTC, IGFBP3, IL-6R, MIF, and MIP-3β) were significantly increased during the MAS phase in patients receiving TCZ compared with those in patients not receiving TCZ.

As shown in Fig. 1 and Supplement Tables 1 and 4, the levels of seven cytokines (i.e., Axl, ICAM-1, MIP-3β, OPG, MCSF-R, MMP-1, and Siglec5) increased in patients receiving TCZ during the MAS phase compared with those in the active phase. On the other hand, the levels of 26 cytokines (i.e., Fraltalkine, GCP2, GM-CSF, I-309, IFN-γ, IGFBP1, IGFBP4, IGF-1, IL-13, IL-1α, IL-1β, IL-2, Light, MIP-α, SDF-1α, b-NGF, HGF, IL-8, THPO, TRAIL3, DR6, IL-2Rγ, MRIF-1, NGF-R, SCF-R, and TIMP4) were significantly decreased in patients receiving TCZ during the MAS phase compared with those during the active phase.

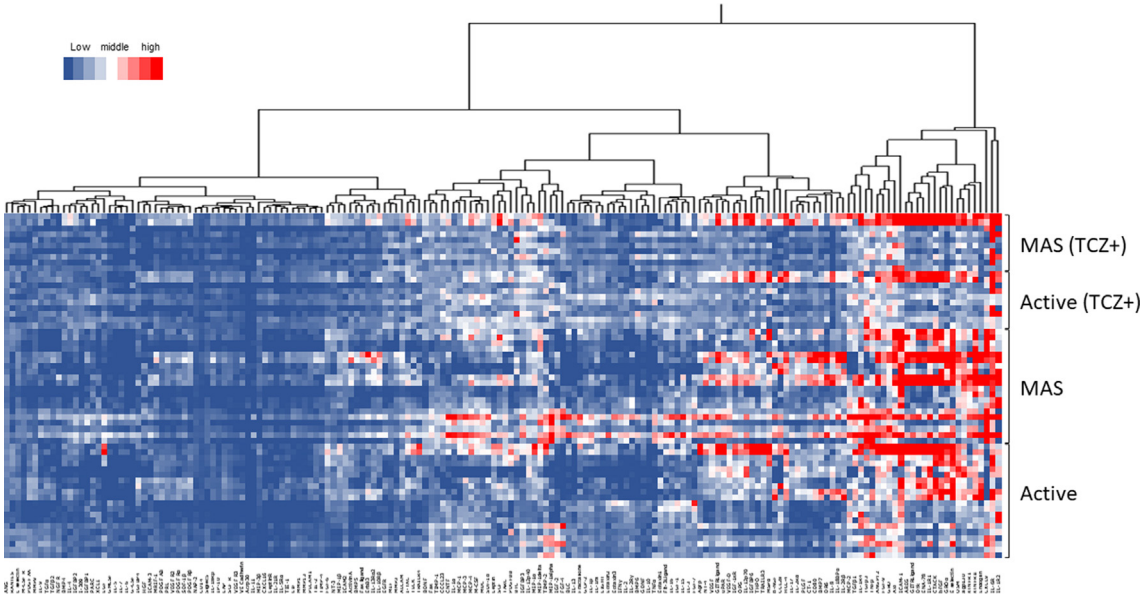

Fig. 1. The expressions of cytokines in patients with systemic juvenile idiopathic arthritis and macrophage activation syndrome. Heat map of the expressions of 174 cytokines in patients with systemic juvenile idiopathic arthritis and macrophage activation syndrome. Data are shown as relative expression to healthy controls. MAS, macrophage activation syndrome; s-JIA, systemic juvenile idiopathic arthritis; TCZ, tocilizumab.

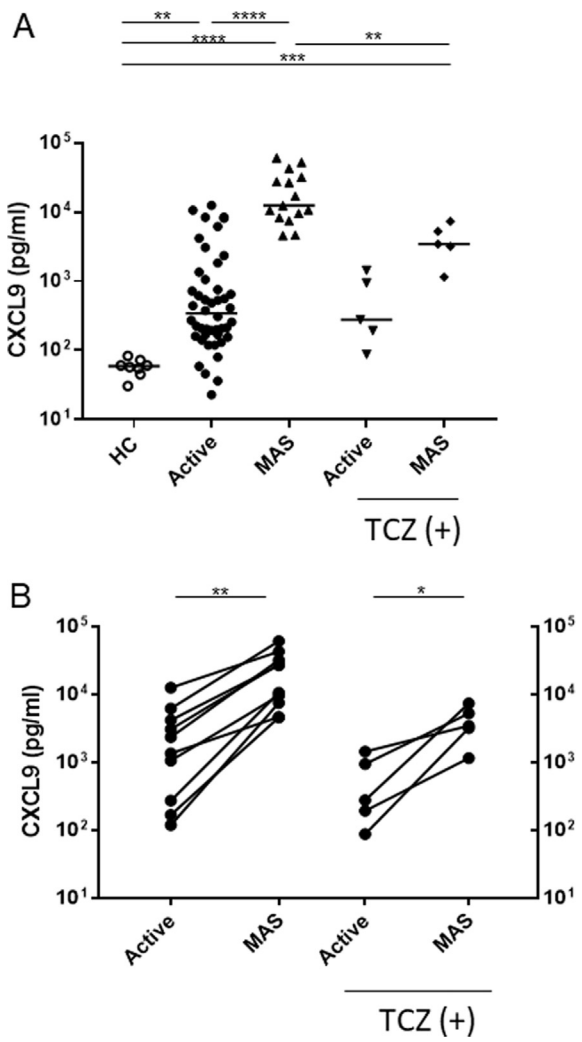

Fig. 2. The levels of CXCL9 in the sera of patients with systemic juvenile idiopathic arthritis and macrophage activation syndrome. A. The levels of CXCL9 in patients with systemic juvenile idiopathic arthritis and macrophage activation syndrome. Bars represent median values. Statistically significant differences between each patient group are shown as $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. B. Changes in the levels of CXCL9 in the serum between the active phase and the MAS phase. Statistically significant differences between each patient group are shown as $^{**}P < .01$. Active, the active phase of systemic juvenile idiopathic arthritis; MAS, the phase of macrophage activation syndrome.

3.3. Clinical significance of serum CXCL9 levels in s-JIA and MAS

The levels of CXCL9 exhibited the most statistically significant increase during the MAS phase compared with the active phase of s-JIA. We measured the levels of CXCL9 in the sera of 56 s-JIA patients (including 20 MAS patients) using ELISA and compared them with clinical features of MAS/s-JIA (including serum IL-18 and ferritin levels) to confirm the clinical significance.

As shown in Fig. 2A, serum CXC9 levels in s-JIA patients not receiving TCZ were significantly elevated during the MAS phase (median: 12,461 pg/mL; range: 4576–60,961 pg/mL) compared with those during the active phase (median: 342 pg/mL, range: 23–12,539 pg/mL) ($P < .0001$) and HCs (median: 59 pg/mL; range: 31–83 pg/mL) ($P < .0001$). Receiver operating characteristic curve analysis revealed the cut off value and the area under the ROC curve value of CXCL9 was 4379 pg/ml and 0.9609, respectively.

Serum CXCL9 levels in s-JIA patients receiving TCZ (median: 3448 pg/mL, range: 1155–7375 pg/mL) were significantly elevated during the MAS phase compared with those reported in HCs ($P < .001$). Serum CXCL9 levels in s-JIA patients receiving TCZ were higher during the MAS phase than during the active phase (median: 276 pg/mL; range: 88–1438 pg/mL). However, the difference was not statistically significant. During the MAS phase, serum CXCL9 levels were significantly decreased in patients receiving TCZ compared with those in patients not receiving TCZ ($P < .01$).

Subsequently, we evaluated changes in serum CXCL9 levels from the active phase to the MAS phase in 10 s-JIA patients not receiving TCZ and 5 s-JIA patients receiving TCZ. The levels of CXCL9 were significantly increased from the active phase to the MAS phase in s-JIA patients not receiving TCZ ($P < .01$) (Fig. 2B). Similarly, the levels of CXCL9 were significantly increased from the active phase to the MAS

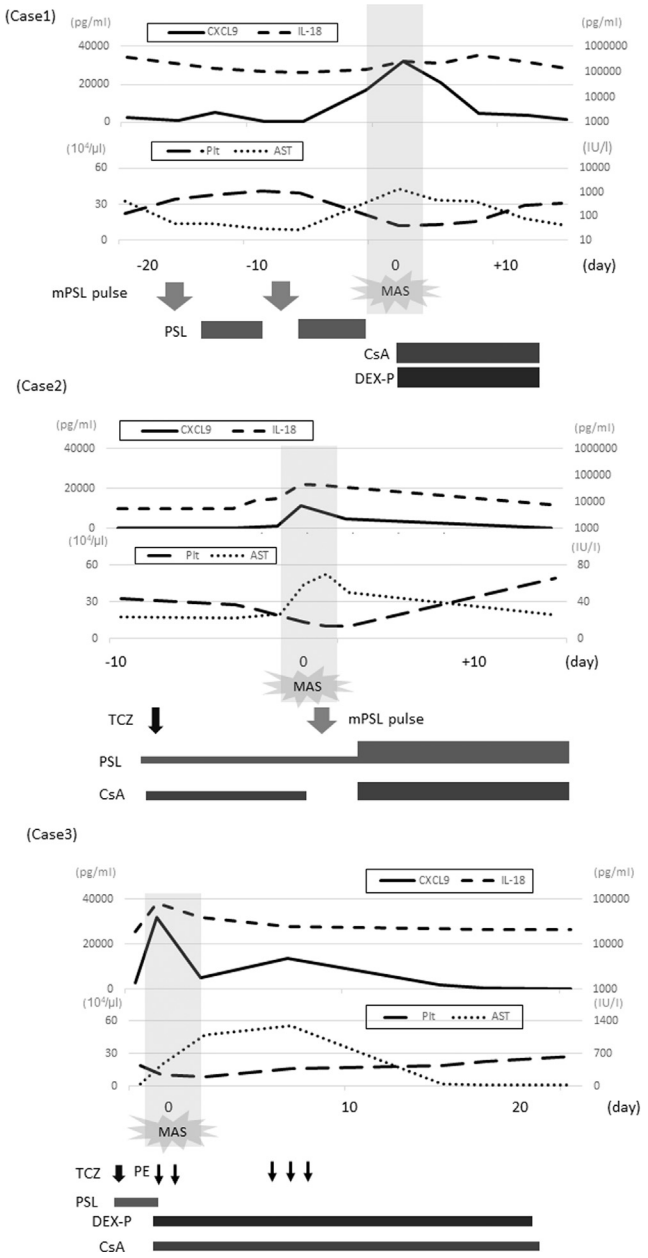

Fig. 3. Longitudinal follow-up of serum CXCL9 levels in three patients with MAS. Changes in serum CXCL9 levels (solid lines) and serum IL-18 levels (dot lines) are shown in upper panels and platelets counts (solid lines) and AST levels (dot lines) are shown in lower panels. MAS, macrophage activation syndrome; AST, aspartate aminotransferase; mPSL, methylprednisolone; CsA, cyclosporineA; DEX-P, dexamethasone palmitate.

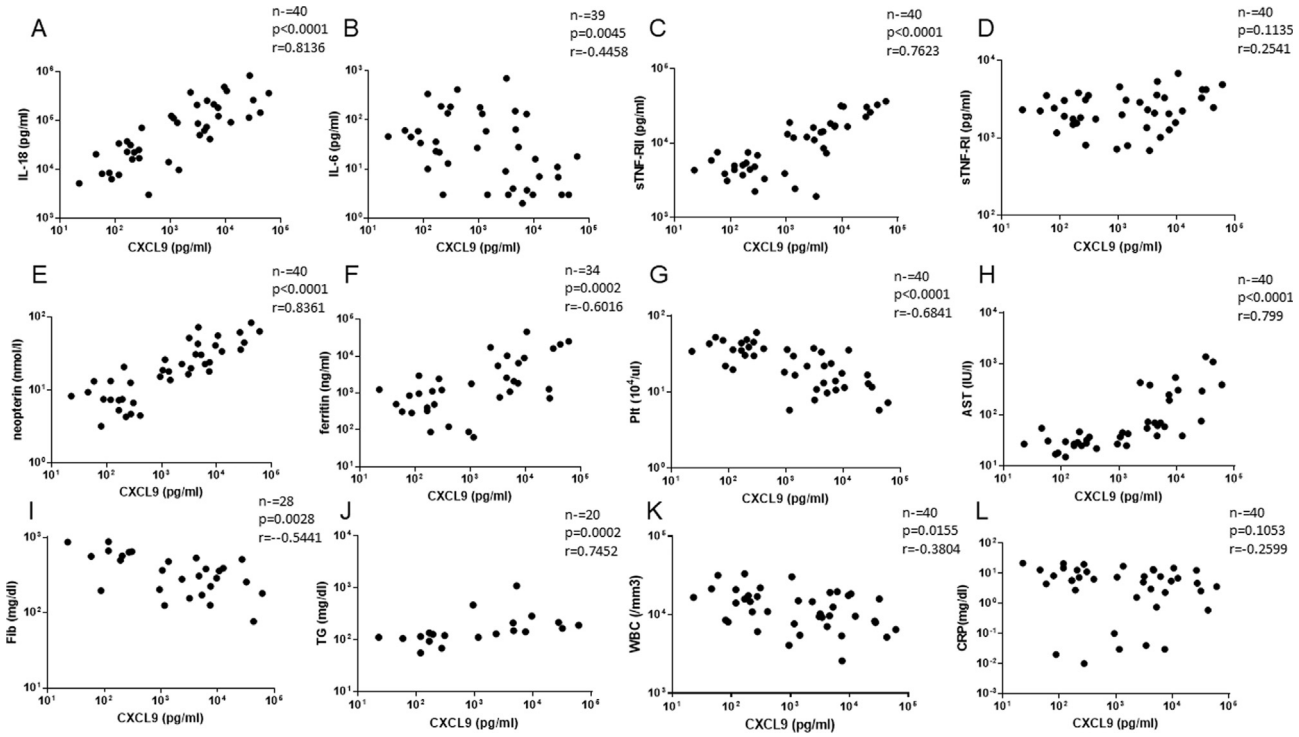

Fig. 4. Correlations between the levels of CXCL9 in the serum and measures of disease activity of macrophage activation syndrome. (A) IL-18, (B) IL-6, (C) sTNFR-I, (D) sTNFR-II, (E) neopterin, (F) ferritin, (G) platelets, (H) AST, (I) fibrinogen, (J) triglyceride, (K) white blood cell counts, and (L) CRP.

phase in s-JIA patients receiving TCZ ($P < .05$).

Furthermore, serum CXCL9 levels were serially monitored in three cases with MAS complicating s-JIA, to investigate the relevance of these levels in the pathogenesis of MAS (Fig. 3) serum CXCL9 levels markedly and rapidly increased as MAS developed, whereas the levels of IL-18 remained elevated from the active phase to the MAS phase.

3.4. Correlation between serum CXCL9 levels and measures of disease activity in patients with s-JIA

The levels of IL-18, IL-6, sTNFR-II, sTNFR-I, neopterin, ferritin, platelets, aspartate aminotransferase (AST), fibrinogen, triglyceride, white blood cell (WBC) counts, and CRP in the serum are used as clinical indicators of disease activity in s-JIA and MAS. An assessment of the correlation between the serum CXCL9 levels and these indicators in the active and MAS phases revealed that the CXCL9 levels positively correlated with IL-18, IL-6, sTNFR-II, neopterin, ferritin, platelets, AST, fibrinogen, triglyceride, and WBC (Fig. 4).

4. Discussion

In this study, we compared the profile of cytokines in the serum between the active phase of s-JIA and the MAS phase in MAS + s-JIA patients. CXCL9, sTNF-RI, sTNF-RII, Axl, and MIP-1δ were significantly increased from the active phase to the MAS phase. In MAS patients, CXCL9 was the most sensitive and specific of these cytokines. Furthermore, we confirmed these findings using ELISA. The production of CXCL9 is induced specifically and exclusively by IFN-γ [18]. IFN-γ plays a central role in the pathogenesis of MAS and pHLH [19,20]. We previously reported that the levels of neopterin – a catabolite of guanosine triphosphate synthesized by macrophages upon IFN-γ stimulation – were higher in patients with MAS compared with those observed in patients with active s-JIA without MAS [7]. Recently, Bracaglia et al. showed that high levels of IFN-γ and of IFN-γ-induced chemokines were present in patients with MAS complicating s-JIA [20]. Additionally, the levels of CXCL9 in the serum strongly correlated with laboratory parameters related to the severity of MAS [20]. Consistent with the findings of that study, in the present study, the levels of CXCL9 in the serum were significantly elevated during the MAS phase, reflecting the disease activity of MAS. Furthermore, these levels strongly correlated with other inflammatory markers. These results indicate that serum CXCL9 levels may be a useful biomarker for the diagnosis of MAS.

In our study, the levels of sTNFR-I and sTNFR-II in the serum were also significantly elevated in the MAS phase compared with those observed in the active phase of s-JIA. Apart from IFN-γ, TNF-α also plays an important role in the pathogenesis of MAS [21]. The sTNFR-I and sTNFR-II are important modulators of the biological function of TNF-α. In various pathologic conditions, sTNFR-I and sTNFR-II mediate the host response and determine the course and outcome of the disease by interacting with TNF-α. We previously reported that the levels of TNFR-I and sTNFR-II in the serum were significantly increased in the MAS phase compared with those reported in the active phase of s-JIA [22]. Furthermore, the sTNFR-II/I ratio profoundly increased as MAS developed and correlated positively with disease activity. These results indicate that the serum sTNFR-II/I ratio may also be a useful biomarker for the diagnosis of MAS. Further larger studies comparing the sensitivity and specificity of potential biomarkers (e.g., CXCL9, sTNFR-II/I ratio, and neopterin) are warranted to confirm their clinical significance in the diagnosis of MAS.

We previously reported that treatment with TCZ masks the clinical symptoms of s-JIA and MAS in patients with s-JIA [14]. Furthermore, Schulert GS et al. revealed that patients who developed MAS while treated with canakinumab – a humanized anti-IL-1β monoclonal antibody – showed a trend towards lower levels of ferritin at the onset of MAS, but did not demonstrate differences in other cardinal clinical or laboratory features [15]. In comparison, patients who developed MAS while treated with TCZ were less likely to be febrile and had notably lower levels of ferritin [15]. Other features of MAS, including lower platelet counts, lower levels of fibrinogen, and higher levels of AST, were also more pronounced in patients treated with TCZ [15]. In this study, the expressions of 15 cytokines were significantly affected by TCZ during the MAS phase. The changes in the expressions of these

cytokine induced by TCZ may be associated with characteristic clinical features of MAS in s-JIA patients receiving TCZ.

Serum CXCL9 levels were significantly lower in patients receiving TCZ. These findings indicate that the expression of CXCL9 may also be suppressed by inhibition of IL-6. However, these levels reflected the disease activity of MAS in both patients receiving TCZ and those not receiving TCZ. Thus, it is necessary to perform larger-scale studies to confirm the significance of CXCL9 serum levels even in patients receiving TCZ.

Recent investigations into the pathophysiology of s-JIA have focused on mediators of the innate immune system. In particular, the levels of IL-1, IL-6, and IL-18 in the serum are correlated with disease activity and secondary complications [2]. Biological agents inhibiting IL-1 and IL-6 have already changed the approach for the treatment of s-JIA [12,13,23]. There is accumulating evidence suggesting that inhibition of IL-1 or IL-6 is highly efficacious in a considerable number of patients with s-JIA, with improvements observed in both systemic symptoms and arthritis [12,13,23]. We previously reported that the levels of IL-18 in the serum are increased in active s-JIA [7]. Thus, IL-18 is increasingly used as a biomarker for the diagnosis of s-JIA and therapeutic response in s-JIA [7]. The levels of IL-18 are increased further in patients with s-JIA-related MAS [7,11]. Furthermore, we reported two subsets of patients with s-JIA comprising certain distinct clinical features based on their IL-6 and IL-18 levels [10,11]. Patients in the IL-18-dominant subset (IL-18/IL-6 > 1000) were more likely to develop MAS. These findings indicate that IL-18 is causatively involved in the development of MAS. IL-18 is a well-known IFN-γ-inducing cytokine. Recent studies revealed high levels of free IL-18 (that is, IL-18 not bound to IL-18 binding protein) increases the risk of developing MAS [24,25]. Increase of free IL-18 and induction of IFN-γ might be closely associated with the development of MAS in the pathogenesis of s-JIA.

Bracaglia et al. showed serum CXCL9 levels were also significantly elevated in patients with secondary HLH other than s-JIA associated MAS [20]. On the other hand, serum IL-18 levels were significantly elevated in patients with s-JIA associated MAS compared to those in patients with Epstein-Barr virus associated HLH, the most common cause of secondary HLH in children [22]. Furthermore, serum IL-18 levels during active phase in patients with MAS were significantly higher than those without MAS [11]. Serum IL-18 levels > 47,750 pg/ml might be useful to predict MAS development [11]. From these findings, monitoring of serum IL-18 levels in conjunction with serum CXCL9 levels might be useful for the prediction and the diagnosis of MAS.

The limitation of the present study was the small number of patients with s-JIA, in particular, those receiving TCZ. However, despite this limitation, the elevated levels of CXCL9 and their correlation with disease activity of MAS indicate a pivotal role of IFN-γ in MAS. Monitoring of serum CXCL9 levels may be useful for the evaluation of disease activity in patients with s-JIA and MAS. The expression of numerous inflammatory cytokines (including CXCL-9) was altered in patients receiving TCZ. These changes may be related to the modification of clinical symptoms of MAS during therapy with TCZ.

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Declaration of interest

The authors received no financial support or other benefits from commercial sources for the work described in the manuscript and declare no financial interests that could create a potential conflict of interest or the appearance of a conflict of interest with regard to this study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.03.018.

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