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Elevated Granulocyte Colony-stimulating Factor Levels in Patients With Active Phase of Adult-onset Still Disease

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ABSTRACT. ***Objective.*** Neutrophilia is a hallmark of adult-onset Still disease (AOSD). We aimed to investigate the levels of granulocyte colony-stimulating factor (G-CSF), an essential regulator of neutrophil production and function, in the pathogenesis of AOSD.

Methods. Sera were collected from 70 patients with AOSD and 20 healthy controls (HCs). The levels of G-CSF were determined by ELISA. Low-density granulocytes (LDGs) were quantified by flow cytometry. Correlations between G-CSF levels and disease activity, laboratory variables, and LDG levels in patients with AOSD were analyzed by Spearman correlation test.

Results. Patients with active AOSD presented significantly higher levels of G-CSF compared to inactive AOSD patients ($P < 0.001$) and HCs ($P < 0.0001$). The G-CSF levels were significantly decreased after active AOSD patients achieved disease remission ($P = 0.0015$). The G-CSF levels were significantly correlated with C-reactive protein, erythrocyte sedimentation rate, ferritin, and systemic score in AOSD ($P < 0.0001$). Significant correlations between the levels of G-CSF and circulating neutrophils ($P < 0.0001$), neutrophil-to-lymphocyte ratio ($P < 0.0001$), percentages of LDGs in the peripheral blood mononuclear cells ($P = 0.004$), as well as absolute numbers of circulating LDGs ($P = 0.018$) were identified. Patients with fever, evanescent rash, sore throat, arthralgia, myalgia, lymphadenopathy, or hepatomegaly/elevated liver enzymes displayed significantly higher levels of G-CSF compared to patients without these manifestations ($P < 0.05$).

Conclusion. Our findings indicate that G-CSF is implicated in the pathogenesis of AOSD, and targeting G-CSF may have therapeutic potential for AOSD. In addition, introducing circulating G-CSF levels into the clinical assessment system may help to monitor disease activity.

Key Indexing Terms: adult-onset Still disease, disease activity, G-CSF, low-density granulocytes, neutrophils

Adult-onset Still disease (AOSD) is a rare systemic autoinflammatory disease, characterized by temporal fever, scattered rash, sore throat, and arthritis. Uncontrolled activation of innate immune system (monocytes/macrophages and neutrophils) and overproduction of several proinflammatory cytokines are central to the pathogenesis of AOSD[1]. Specifically, neutrophils with an enhanced proinflammatory state have been considered as the primary effector cells in the pathogenesis of AOSD.

The imbalance between neutrophil survival and clearance plays a critical role during the inflammatory process. A principal regulator of neutrophil production and survival is the granulocyte colony-stimulating factor (G-CSF). In homeostatic conditions, serum levels of G-CSF remain very low but rise dramatically under pathological conditions, such as rheumatoid arthritis[2], antineutrophil cytoplasmic antibody–associated vasculitis (AAV)[3], Sweet syndrome, and Behçet disease[4]. The proinflammatory role of G-CSF has been highlighted in several inflammatory diseases, such as AAV, wherein treatment with G-CSF can further exacerbate the underlying pathological conditions[3,5], whereas G-CSF deficiency protects mice from acute and chronic arthritis[5].

G-CSF has been shown to induce granulocytic hyperplasia[6]. A previous study examined bone marrow (BM) biopsies from patients with AOSD and found that all the BM biopsies exhibited features of granulocytic hyperplasia[7]. G-CSF is produced by different cell types, including monocytes, endothelial cells, and fibroblasts, upon stimulation with inflammatory mediators such as interleukin 6 and tumor necrosis factor-α, which are the cytokines critically implicated in AOSD. Given that neutrophilia is a hallmark of AOSD and given the critical role of G-CSF in neutrophil production, it is likely that G-CSF may be involved in the pathogenesis of AOSD.

Low-density granulocytes (LDGs), a subset of circulating proinflammatory neutrophils, were first described in systemic

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The authors declare no conflicts of interest.

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lupus erythematosus (SLE) as a population of granulocytes that sediment within the peripheral blood mononuclear cells (PBMCs) fraction upon gradient centrifugation of whole blood[8]. A recent study suggests that the levels of LDGs are elevated in patients with active AOSD[9]. Of note, increasing evidence suggests that G-CSF promotes expansion of LDGs[10], which may contribute to the elevated levels of LDGs in AOSD. Currently, the role of G-CSF in AOSD remains unclear. In view of the proinflammatory nature of G-CSF and the potential link between G-CSF and AOSD, we investigated the role of G-CSF in AOSD and presented a detailed analysis of the relationships between the levels of G-CSF and different clinical features or laboratory variables.

MATERIALS AND METHODS

Subjects. We included 70 patients with AOSD (44 patients with active AOSD and 26 patients with inactive AOSD) who fulfilled the Yamaguchi diagnostic criteria[11]. Patients with infectious, neoplastic disease, pregnancy or puerperium, and autoimmune disorders at the time of evaluation were excluded. Among 44 patients with active AOSD, 12 patients were followed up for 3–6 months when the patients were in stable remission status. All of those 12 patients were treated with steroid and conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), except 1 patient who was also treated with biologic DMARDs. The AOSD disease activity of each patient was evaluated by a modified Pouchot score[12]. Active AOSD was defined as systemic score ≥ 4 and inactive AOSD was defined as systemic score ≤ 2, as previously described[13].

Macrophage activation syndrome (MAS) was diagnosed and confirmed by the findings of hemophagocytosis in the BM aspiration. We also included 20 age- and sex-matched healthy controls (HCs). HCs were individuals who went to Peking University People's Hospital for annual physical examination. HCs who displayed abnormality in blood differential tests, biochemistry profile tests, or autoantibody profile tests, or who had a history of systemic, neoplastic, and/or autoimmune/autoinflammatory diseases, were excluded. Demographic and clinical characteristics were recorded. The study protocol was reviewed and approved by the Ethical Committee of Peking University People's Hospital (PKUPH protocol number: 2019PHB244) and informed consent was obtained from all participants.

Determination of serum levels of G-CSF. Serum levels of G-CSF were determined by ELISA (R&D Systems Inc.) according to the manufacturer's instructions.

Flow cytometry analysis of LDGs. Peripheral blood from patients and control subjects was collected into EDTA tubes, and PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare). LDGs were determined by multiparametric flow cytometry as CD10−/+/CD14−/lo/CD15+ cells in the PBMC fraction. All the antibodies used in flow cytometry were from Biolegend. Data were collected with BD Canto (BD Biosciences) and analyzed with FlowJo (FlowJo, LLC).

Statistical analysis. Continuous variables were compared using the Mann-Whitney *U* test or Kruskal-Wallis test. The correlations were evaluated with Spearman nonparametric test. The Wilcoxon matched-pairs signed-rank test was utilized to compare the changes of G-CSF levels in patients who underwent follow-up study. All statistical analyses were performed by GraphPad Prism 6 (GraphPad Software Inc.). *P* values < 0.05 were considered statistically significant.

RESULTS

Demographic and clinical characteristics of patients with AOSD. The detailed demographic and clinical characteristics of patients with active AOSD, patients with inactive AOSD, and HCs are depicted in Supplementary Table 1 (available from the authors on request). All active AOSD patients had high-spiking fevers (> 39°C); evanescent rash, arthralgia, and lymphadenopathy were present in 93.2%, 75.0% and 68.2% of active AOSD patients, respectively. The systemic score, as determined by a modified Pouchot score[12], was 6.57 ± 1.18 in active AOSD patients.

The G-CSF levels were elevated in active AOSD patients. Overall, patients with AOSD displayed significantly higher levels of G-CSF compared to HCs ($P < 0.0001$; Supplementary Figure 1A, available from the authors on request). Patients with AOSD were further divided into active and inactive AOSD groups based on systemic scores. Patients with active AOSD exhibited significantly enhanced levels of G-CSF compared to patients with inactive AOSD ($P < 0.001$; Figure 1A). Interestingly, inactive AOSD patients also showed significantly elevated levels of G-CSF compared to HCs ($P < 0.01$; Figure 1A). Ruscitti, *et al* reported that a systemic score of ≥ 7.0 (based on score system proposed by Pouchot, *et al*[13]) showed a strong prognostic effect in identifying patients at risk of AOSD-related death[14]. As such, we compared the levels of G-CSF between patients with a systemic score of ≥ 7.0 vs < 7.0. Significantly higher levels of G-CSF were identified in active patients with a systemic score of ≥ 7.0 compared to active patients with a systemic score of < 7.0 ($P = 0.317$; Supplementary Figures 1B,C, available from the authors on request). A total of 12 patients with active AOSD were followed until they achieved disease remission. Significantly decreased levels of G-CSF were noted during the follow-up ($P = 0.0015$; Figure 1B). These findings suggest that G-CSF is implicated in active AOSD.

The G-CSF levels were correlated with disease activity, and circulating neutrophils and LDGs. As shown in Figure 1, the G-CSF levels were significantly correlated with the acute-phase reactants, including C-reactive protein ($r = 0.6829$, $P < 0.0001$; Figure 1C) and erythrocyte sedimentation rate ($r = 0.6000$, $P < 0.0001$; Figure 1D). Serum ferritin have been shown as a marker of disease activity in AOSD[15]. The G-CSF levels were significantly associated with the levels of ferritin and the disease activity score, as determined by a modified Pouchot score[12] ($r = 0.5490$, $P < 0.0001$ and $r = 0.6388$, $P < 0.0001$, respectively; Figures 1E,F). Consistent with a critical role of G-CSF in controlling neutrophil numbers, significant correlations between the levels of G-CSF and circulating neutrophils were identified ($r = 0.6400$, $P < 0.0001$; Figure 2A). In addition, the G-CSF levels were significantly associated with neutrophil-to-lymphocyte ratio, a variable indicative of systemic inflammatory response ($r = 0.6351$, $P < 0.0001$; Figure 2B).

A recent study highlights the involvement of LDGs in active AOSD[9]. As previous studies have suggested that G-CSF is implicated in expanding LDGs[10], the associations between levels of G-CSF and the levels of LDGs were assessed. Significant correlations between G-CSF levels and percentages of LDGs in the PBMCs ($r = 0.3839$, $P = 0.004$), as well as between G-CSF levels and the absolute numbers of circulating LDGs ($r = 0.3208$, $P = 0.018$), were identified (Figure 2C,D).

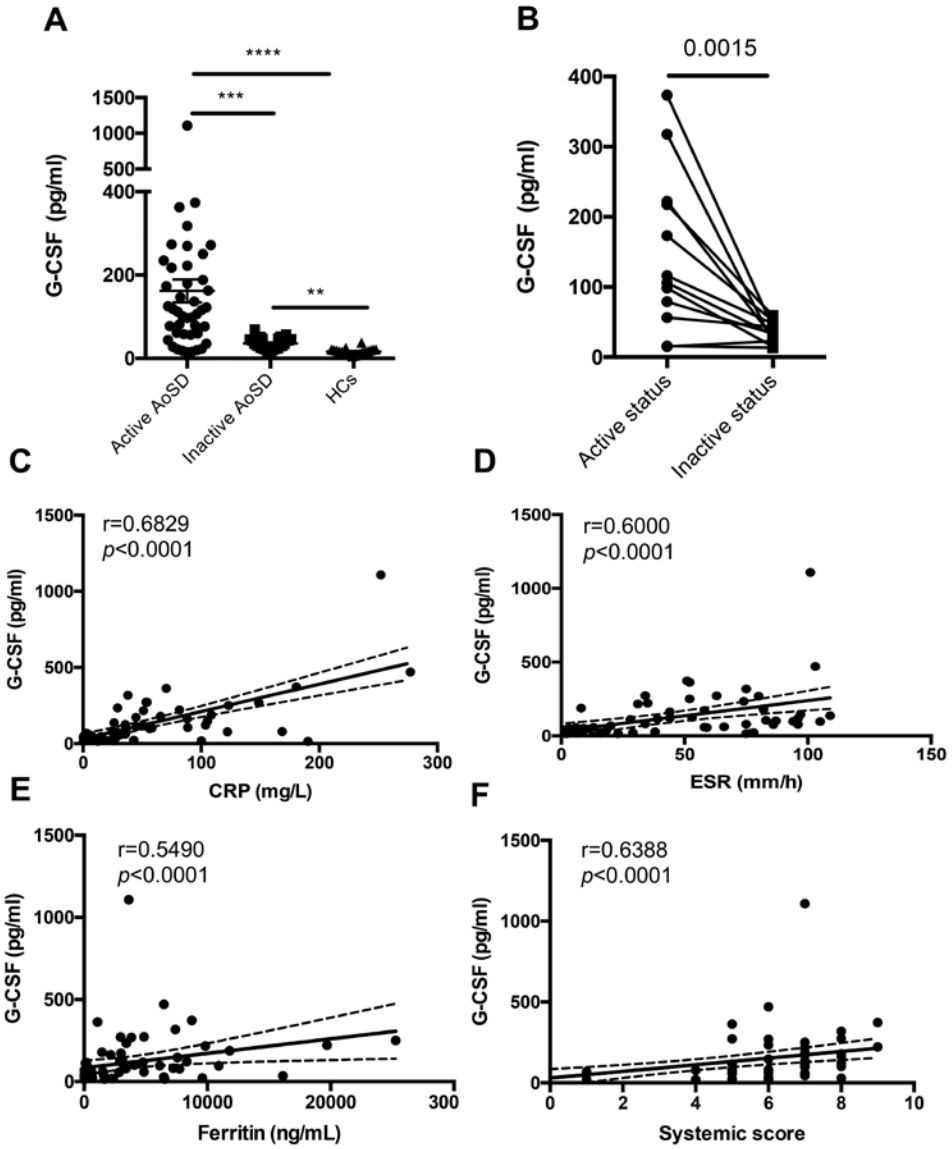

Figure 1. The levels of G-CSF were elevated in active AOSD patients. A. The levels of G-CSF in patients with active AOSD (n = 44), inactive AOSD (n = 26) and HC (n = 20). B. Changes in the levels of G-CSF in patients with AOSD at active phase and at inactive phase (n = 12). C. Associations between G-CSF levels with (C) CRP, (D) ESR, (E) ferritin, and (F) systemic score. **** $P < 0.0001$. *** $P < 0.001$. ** $P < 0.01$. AOSD: adult-onset Still disease; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; G-CSF: granulocyte colony-stimulating factor; HC: healthy controls.

Serum G-CSF levels in AOSD patients with different clinical features. Circulating G-CSF levels were compared between AOSD patients in the presence and absence of certain clinical features. Patients with fever displayed significantly higher levels of G-CSF than patients without fever (162.0 ± 179.9 pg/mL vs 35.7 ± 13.7 pg/mL, $P < 0.0001$). Similarly, patients with evanescent rash, sore throat, arthralgia, myalgia, lymphadenopathy, or hepatomegaly/elevated liver enzymes had significantly higher levels of G-CSF compared to patients without these symptoms ($P < 0.05$; Table 1).

DISCUSSION

Although neutrophilia is one of the major features of AOSD, little is known regarding the role of G-CSF in the pathogenesis of AOSD. Here, we report that the serum levels of G-CSF were significantly elevated in patients with AOSD; this was consistent with findings from a recent study[16]. We further show that G-CSF levels were significantly correlated with systemic scores and laboratory variables reflecting disease activity. Importantly, we found that patients with a systemic score of ≥ 7.0 displayed significantly higher levels of G-CSF compared to those with a systemic score of < 7.0. In addition, we found that serum levels of G-CSF were significantly correlated with blood neutrophil counts as well as circulating LDGs, supporting the role of G-CSF in controlling neutrophil release from BM and expansion of LDGs[10]. Further, we showed that serum levels of G-CSF were significantly

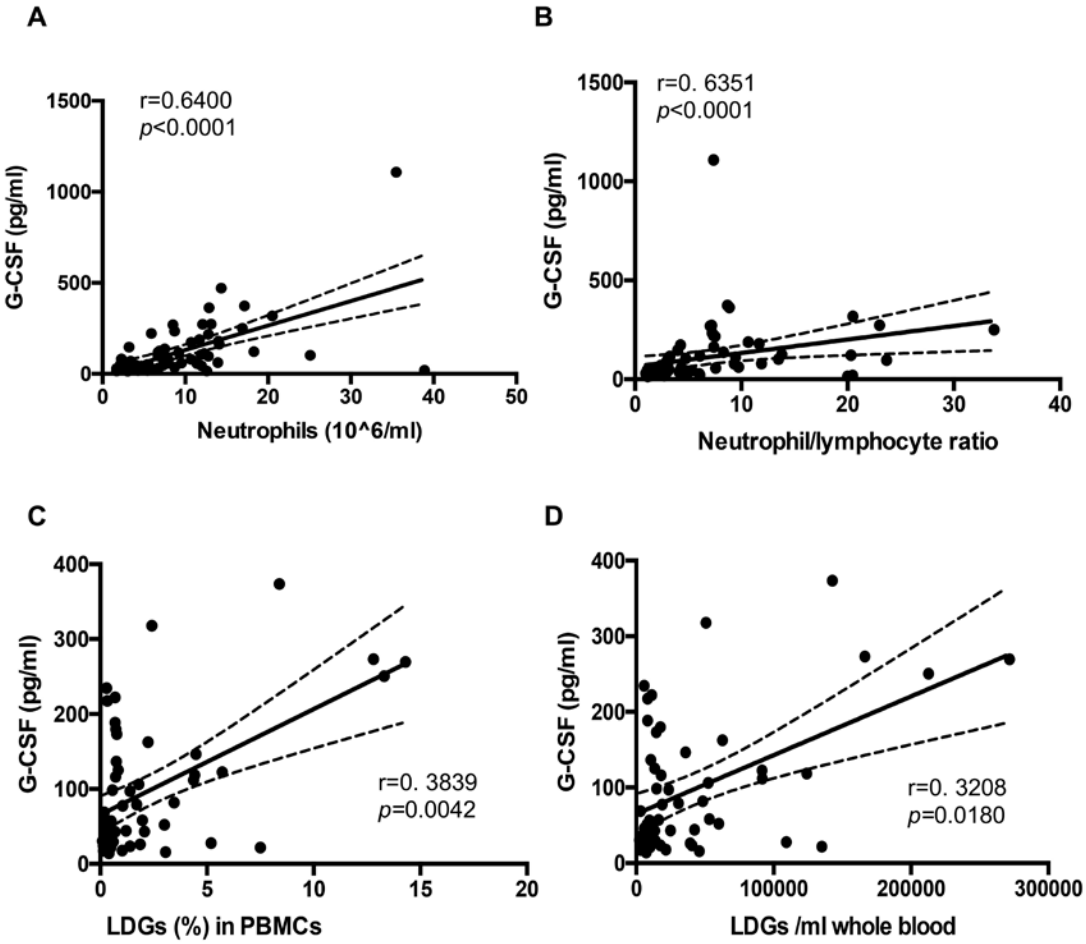

Figure 2. Associations between the levels of G-CSF with (A) circulating levels of neutrophils (n = 70), (B) neutrophil-to-lymphocyte ratio (n = 70), (C) percentages of LDGs in the PBMC fraction (n = 54), and (D) absolute numbers of circulating LDGs in patients with AOSD (n = 54). G-CSF: granulocyte colony-stimulating factor; LDG: low-density granulocytes; PBMC: peripheral blood mononuclear cells.

Table 1. Comparison of serum G-CSF levels based on disease manifestations in patients with AOSD.

Manifestations	Status (n)	G-CSF, pg/mL	*P*
Fever	Presence (44)	162.0 ± 179.9	< 0.0001
	Absence (26)	35.7 ± 13.7	
Evanescent rash	Presence (43)	153.0 ± 176.1	< 0.0001
	Absence (27)	54.8 ± 85.1	
Sore throat	Presence (28)	131.5 ± 108.4	0.018
	Absence (42)	104.2 ± 179.2	
Arthralgia	Presence (34)	171.2 ± 192.2	< 0.0001
	Absence (36)	62.1 ± 79.1	
Myalgia	Presence (27)	158.4 ± 110.9	< 0.0001
	Absence (43)	88.0 ± 172.2	
Pneumonitis	Presence (9)	180.8 ± 130.2	0.169
	Absence (61)	105.4 ± 156.4	
Lymphadenopathy	Presence (30)	155.9 ± 198.3	0.008
	Absence (40)	84.5 ± 102.7	
Hepatomegaly/elevated liver enzymes	Presence (28)	161.4 ± 205.5	0.016
	Absence (42)	84.2 ± 98.4	

All values are presented as n or mean ± SD. Differences between 2 groups were performed with Mann-Whitney *U* test. G-CSF: granulocyte colony-stimulating factor; AOSD: adult-onset Still disease.

reduced when the patient reached clinical remission, suggesting that dynamic monitoring changes of G-CSF levels may help to predict disease relapse and remission.

In the present study, we found that AOSD patients with fever displayed significantly enhanced levels of G-CSF. Interestingly, clinical evidence shows that healthy subjects treated with G-CSF develop fever and bone pain, which can be ameliorated by the usual dose of nonsteroidal antiinflammatory drugs[17], suggesting a direct link between G-CSF and fever. Indeed, a previous study confirms that G-CSF can provoke fever by promoting catecholamine production, which further activates neutrophils to release prostaglandin E2, a critical mediator of fever[18]. In addition, G-CSF levels were significantly elevated in patients with arthralgia and myalgia in comparison to patients without these manifestations. Our findings are consistent with previous observations that patients developed polyarthralgia and myalgia with rising fever after 5 consecutive days of subcutaneous administration of G-CSF[19]. Taken together, these results suggest that most of the characteristics in AOSD, including evanescent rash, liver dysfunction, and inflammatory arthritis, may result from the aberrant production of G-CSF.

MAS has been considered to be the most severe complication of AOSD[20]. In the present study, 7 active AOSD patients simultaneously presented with MAS. Interestingly, no significant differences in the levels of G-CSF were identified between active AOSD patients with or without MAS; this may be due to the small sample size. Further studies with large sample sizes, especially with more MAS patients, will be of great importance to investigate the role of G-CSF in AOSD patients with MAS.

Our findings indicate that G-CSF is implicated in the disease pathogenesis, and targeting G-CSF may have therapeutic potential for AOSD. In addition, adding G-CSF into the assessment system may help monitor disease activity in clinical practice.

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