Elevated Levels of G-CSF in Patients with Active Phase of Adult-onset Still's Disease

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Running Title: Elevated levels of G-CSF in active AoSD

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ABSTRACT.

Objective. Neutrophilia is a hallmark of adult-onset Still's 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 parameters, or LDGs levels in patients with AoSD were analyzed by Spearman's correlation test.

Results. Active AoSD patients presented significantly higher levels of G-CSF compared to inactive AoSD patients (p<0.001) and HCs (p<0.0001). The levels of G-CSF were significantly decreased after active AoSD patients achieved disease remission (p=0.0015). The levels of G-CSF were significantly correlated with CRP, ESR, 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 PBMCs (p=0.0042) as well as absolute numbers of circulating LDGs (p=0.0180) 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, G-CSF, DISEASE ACTIVITY, NEUTROPHILS, LOW-DENSITY GRANULOCYTES

Adult-onset Still's 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 pro-inflammatory cytokines are central to the pathogenesis of AoSD (1). Specifically, neutrophils with an enhanced pro-inflammatory 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 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 (ANCA) vasculitis (3), Sweet syndrome and Behçet disease (4). The proinflammatory role of G-CSF has been highlighted in several inflammatory diseases, such as ANCA vasculitis, as treatment with G-CSF can further exacerbate the underlying pathological conditions (3, 5), while of 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 (IL-6) and tumor necrosis factor alpha (TNF-α), the cytokines critically implicated in AoSD. Given neutrophilia is a hallmark of AoSD and 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 proinflammatory neutrophils in the

circulation, were first described in systemic 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 suggest that G-CSF promotes expansion of the 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 different laboratory parameters.

MATERIALS AND METHODS

Materials and methods are included in the online supplementary material.

Subjects. We included 70 patients with AoSD (44 patients with active AoSD and 26 patients with inactive AoSD), who fulfilled the Yamaguchi's diagnostic criteria (11). The disease activity of AoSD 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).

RESULTS

The 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. 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 levels of G-CSF were elevated in active AOSD patients. Overall, patients with AoSD displayed significantly higher levels of G-CSF compared to HCs (p<0.0001) (Supplemental Figure 1A). Patients with AoSD were further divided into active AoSD group and inactive AoSD group 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*) showed a strong prognostic impact in identifying patients at risk of AOSD-related death (14). As such, we compared the levels of G-CSF between patients with systemic score of ≥ 7.0 and patients with systemic score of < 7.0. Significantly higher levels of G-CSF were identified in active patients with systemic score of ≥ 7.0 compared to active patients with systemic score of < 7.0 (p=0.317) (Supplemental Figures 1B and C). A total of 12 patients with active AoSD patients were followed-up until they achieved disease remission. A 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 levels of G-CSF were correlated with disease activity and circulating neutrophils and LDGs. As shown in Figure 1, the levels of G-CSF were significantly correlated with the acute phase reactants, including C-reactive protein (CRP) (r=0.6829, p<0.0001) and erythrocyte sedimentation rate (ESR) (r=0.6000, p<0.0001) (Figures 1C and 1D). Serum ferritin have been shown as a marker of disease activity in AoSD (15). The levels of G-CSF were significantly associated with the levels of ferritin as well as the disease activity

score, as determined by a modified Pouchot score (r=0.5490, p<0.0001; r=0.6388, p<0.0001, respectively) (Figures 1E and 1F). 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 levels of G-CSF were significantly associated with neutrophil-to-lymphocyte ratio, a parameter 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=0.0042) as well as between G-CSF levels and the absolute numbers of circulating LDGs (r=0.3208, p=0.0180) were identified (Figure 2C and 2D).

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, which was consistent with findings

from a very recent study (16). We further show that the levels of G-CSF were significantly correlated with systemic score and laboratory parameters reflecting disease activity. Importantly, we found that patients with systemic score of ≥ 7.0 displayed significantly higher levels of G-CSF compared to patients with 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 a 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 reduced when the patient reached clinical remission in a follow-up study, suggesting that dynamic monitoring changes of G-CSF levels may help to predict disease relapse and remission.

In this study, we found that AoSD patients with fever displayed significantly enhanced levels of G-CSF. Interestingly, clinical evidence show that healthy subjects treated with G-CSF develop fever and bone pain, which can be ameliorated by the usual dose of nonsteroidal anti-inflammatory drugs (NSAIDs) (17), suggesting a direct link between G-CSF and fever. Indeed, a recent study confirms that G-CSF can provoke fever by promoting catecholamine production, which further activates neutrophils to release prostaglandin E2 (PGE2), a critical mediator of fever (18). In addition, the levels of G-CSF were significantly elevated in patients with arthralgia and myalgia in comparison to patients without these manifestations. Our findings are consistent with previous observation that patient developed polyarthralgia and myalgia with rising fever after 5 consecutive days subcutaneous administration of G-CSF (19). Taken together, these results suggest that most of characteristics in AOSD, including evanescent rash, liver dysfunction, and inflammatory arthritis, may result from the aberrant production of G-CSF.

Macrophage activation syndrome (MAS) has been considered as the most severe complication of AoSD (20). In this study, 7 active AoSD patients simultaneously presenting

with MAS. Interestingly, no significant differences in the levels of G-CSF were identified between active AoSD patients with or without MAS, which may be due to the small sample size. Further studies with large sample size, 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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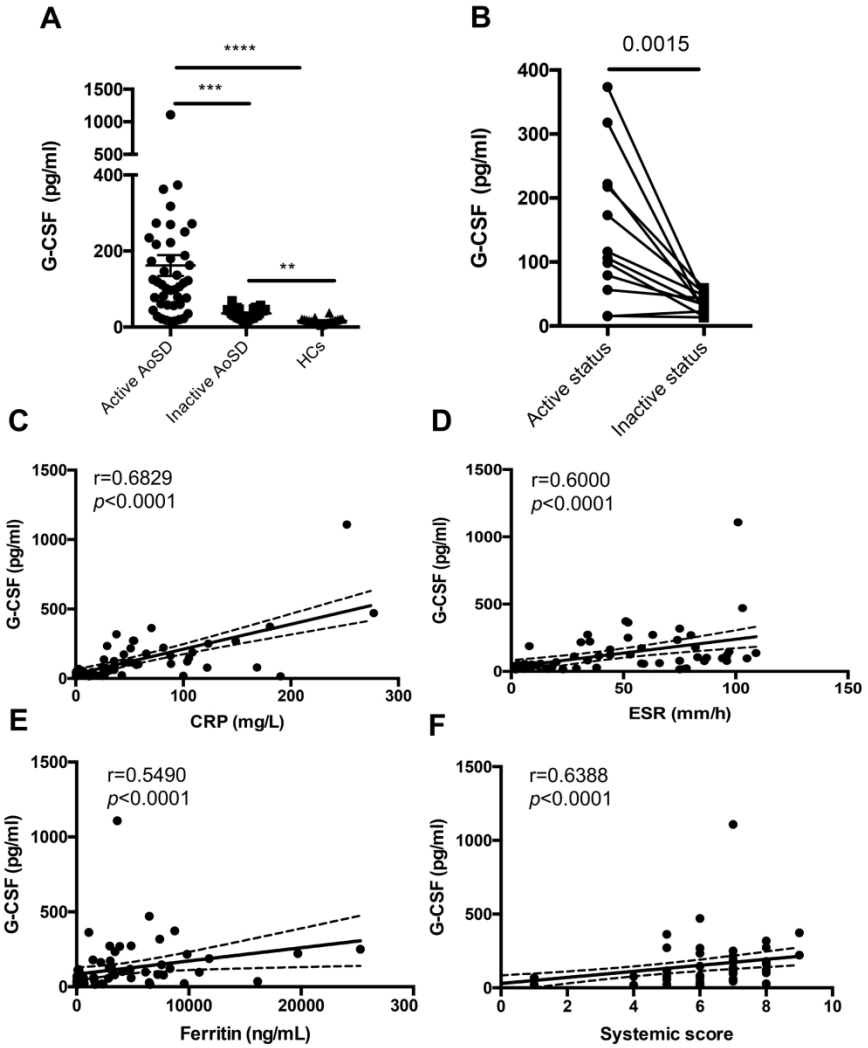
Figure 1. The levels of G-CSF were elevated in active adult-onset Still's disease (AoSD) patients. A. The levels of G-CSF in patients with active AoSD (n=44), inactive AoSD (n=26) and healthy controls (HCs) (n=20). B. Changes in the levels of G-CSF in patients with AoSD at active phase and at inactive phase (n=12). C. Associations between the levels of G-CSF with C-reactive protein (CRP). (D). Associations between the levels of G-CSF with erythrocyte sedimentation rate (ESR). (E). Associations between the levels of G-CSF with ferritin. (F) Associations between the levels of G-CSF with systemic score. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$

Figure 2. Associations between the levels of G-CSF with circulating levels of neutrophils (A) (n=70), neutrophil-to-lymphocyte ratio (B) (n=70), percentages of low-density granulocytes (LDGs) in the PBMC fraction (C) (n=54), and absolute numbers of circulating LDGs (D) in patients with AoSD (n=54).

Figure 1



The levels of G-CSF were elevated in active adult-onset Still's disease (AoSD) patients.

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Figure 2

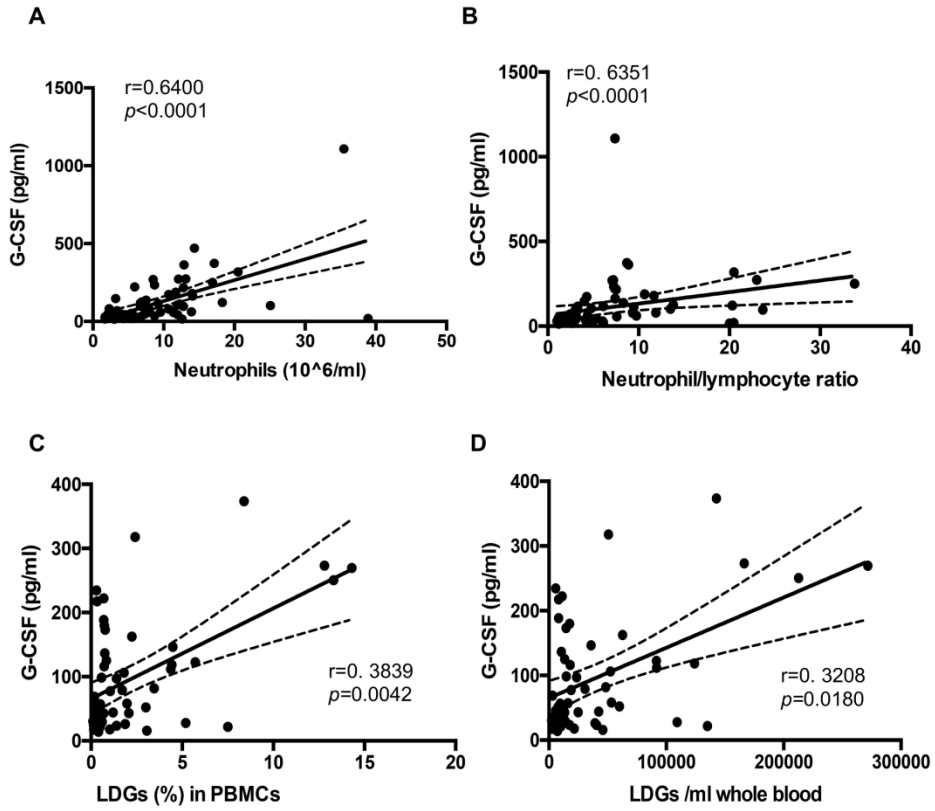

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Table 1. Comparison of serum levels of granulocyte colony-stimulating factor (G-CSF) based on disease manifestations in patients with adult-onset Still's disease (AoSD).

Manifestations	Status (n)	G-CSF (pg/ml)	*p*-value
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	0.0179
	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	0.1694
	Absence (61)	105.4±156.4	
Lymphadenopathy	Presence (30)	155.9±198.3	0.0079
	Absence (40)	84.5±102.7	
Hepatomegaly/elevated liver enzymes	Presence (28)	161.4±205.5	0.0157
	Absence (42)	84.2±98.4	

All values are presented as number (percent) or mean ± SD. Differences between two groups were performed with Mann-Whitney U test.