# Cytokine signature profiles in acquired aplastic anemia and myelodysplastic syndromes

Xingmin Feng,[1] Phillip Scheinberg,[1] Colin O. Wu,[2] Leigh Samsel,[3] Olga Nunez,[1] Courtney Prince,[4] Rebecca D. Ganetzky,[5] J. Philip McCoy Jr,[3] Jaroslaw P. Maciejewski,[4] and Neal S. Young[1]

[1]Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; [2]Office of Biostatistics Research, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; [3]Flow Cytometry Core, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; [4]Department of Translational Hematology/Oncology Research, Taussig Cancer Center, Cleveland Clinic, Cleveland, OH; and [5]Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland Clinic, Cleveland, OH, USA

## ABSTRACT

Although aplastic anemia and myelodysplasia have been extensively investigated, little is known about their circulating cytokine patterns. We compared plasma soluble cytokines in 33 aplastic anemia, 57 myelodysplasia patients, and 48 healthy controls. High levels of thrombopoietin and granulocyte colony-stimulating factor, with low levels of CD40 ligand, chemokine (C-X-C motif) ligand 5, chemokine (C-C motif) ligand 5, chemokine (C-X-C motif) ligand 11, epidermal growth factor, vascular endothelial growth factor, and chemokine (C-C motif) ligand 11 were a signature profile for aplastic anemia. High levels of tumor necrosis factor-α, interleukin-6, chemokine (C-C motif) ligand 3, interleukin-1 receptor antagonist, and hepatocyte growth factor were a cytokine signature for myelodysplasia. Despite similar clinical presentations, distinct cytokine profiles were observed between aplastic anemia and hypocellular myelodysplasia. Future studies focusing on cytokines that better discriminate these two entities such as thrombopoietin and chemokine (C-C motif) ligand 3 may be useful tools in clinical practice.

Key words: cytokine, aplastic anemia, myelodysplastic syndromes.

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## Introduction

Aplastic anemia (AA) and myelodysplasia (MDS) are bone marrow (BM) failure syndromes in which impaired hematopoiesis results in single or multilineage cytopenias. AA usually affects younger patients. On presentation the marrow is hypocellular, the morphology of residual hematopoietic precursors is normal in AA, and cytogenetics shows normal chromosomes. Marrow failure in AA is usually secondary to an immunological mechanism, and most patients respond to immunosuppressive therapies (IST).[1] In contrast, MDS affects older patients. Marrow cellularity in MDS is often normal or increased, and dysmorphic changes are characteristic. Chromosomal defects are frequent in MDS. Despite these general differences, diagnostic confusion often arises. About 20% of MDS patients present with a hypocellular marrow, as in AA, and cytogenetics are normal in as many as 50% of MDS cases.[2] As the treatment toxicities and the long-term outcomes between AA and MDS differ substantially, a correct diagnosis at presentation is desirable for proper management.

Although bone marrow failure has been extensively investigated, little is known about the cytokines and chemokines in the blood. Cytokines and chemokines are soluble low-molecular-weight proteins secreted by immune cells that mediate inflammatory responses and regulate hematopoiesis by modulating bone marrow microenvironment. Some are essential for the viability, proliferation and differentiation of hematopoietic stem cells. *In vitro*, monolayers of marrow-adherent cells, including fibroblasts, endothelial cells, adipocytes, and macrophages, produce a variety of cytokines, which include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), and stem cell factor (SCF), either constitutively or after stimulation with IL-1 or tumor necrosis factor-α (TNF-α).[3,4]

To date, measurements of soluble circulating mediating factors in marrow failure have been limited largely to one or two cytokines in AA[5-9] and MDS.[10,11] Because of the complex array of proteins involved in an immune response, the measurement of a single cytokine is likely insufficient to assess alterations in disease. We conducted a comprehensive analysis of 31 cytokines, chemokines, and growth factors in the plasma of healthy volunteers, AA, and MDS patients. We describe differences in the cytokine profiles between those with marrow failure and healthy volunteers and propose a cytokine signature that might help to distinguish AA from hypocellular MDS.

*The online version of this article has a Supplementary Appendix.*

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*Correspondence: Xingmin Feng, Ph.D., Hematology Branch, NHLBI/NIH, Building 10-CRC, Rm 3E-5216, 9000 Rockville Pike, Bethesda, MD 20892 USA.*
*E-mail: fengx2@nhlbi.nih.gov*

## Design and Methods

Written informed consent from subjects was obtained in accordance with protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (Bethesda, MD, USA) and the Cleveland Clinic Foundation (Cleveland, OH, USA). Severe AA (SAA) was defined according to a previous report.[12] Fanconi anemia was excluded by chromosome breakage analysis. MDS was defined according to the World Health Organization (WHO) classification[13] and International Prognostic Scoring System (IPSS).[14] Healthy controls were recruited from the donors of the National Institutes of Health Blood Bank and the staff members of our laboratory. A total of 90 patient samples (33 SAA and 57 MDS) and 48 healthy control samples were tested in our study. Samples of all SAA patients were collected prior to IST; in 8 of them, serial measurements before and three and six months after horse antithymocyte globulin (ATG)/cyclosprine (CsA) treatment were also performed. MDS patients treated with IST or chemotherapy were excluded from this study. Patients did not have active infections as determined by clinical assessment at the time of sampling. Healthy controls and patients' characteristics are summarized in *Online Supplementary Table S1*.

Plasma was obtained by centrifugation of heparinized peripheral blood and stored in aliquots at -80°C until analysis. The following cytokines and growth factors were tested: CD40 ligand (CD40L), chemokine (C-X-C motif) ligand 5 (CXCL5), CXCL10, CXCL11, thrombopoietin (Tpo), TNF-α, G-CSF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL4, CCL5, CCL11, fibroblast growth factor basic (FGFb), GM-CSF, interferon-γ (INF-γ), IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, vascular endothelial growth factor (VEGF), and leptin. Measurement of all cytokines was performed by an immuno-bead-based multiplex assay (Luminex) according to the manufacturer's instructions. Panels of capture antibody-coated beads and labeled detection antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Assay sensitivity varied from 0.1 to 23.4 pg/mL, depending on the analyte.

## Results and Discussion

Due to the significant age difference between AA and MDS patients, we divided healthy controls into two groups: under 55 years of age (n=29) and 55 years and over (n=19), to better match the ages of AA and MDS patients, respec-

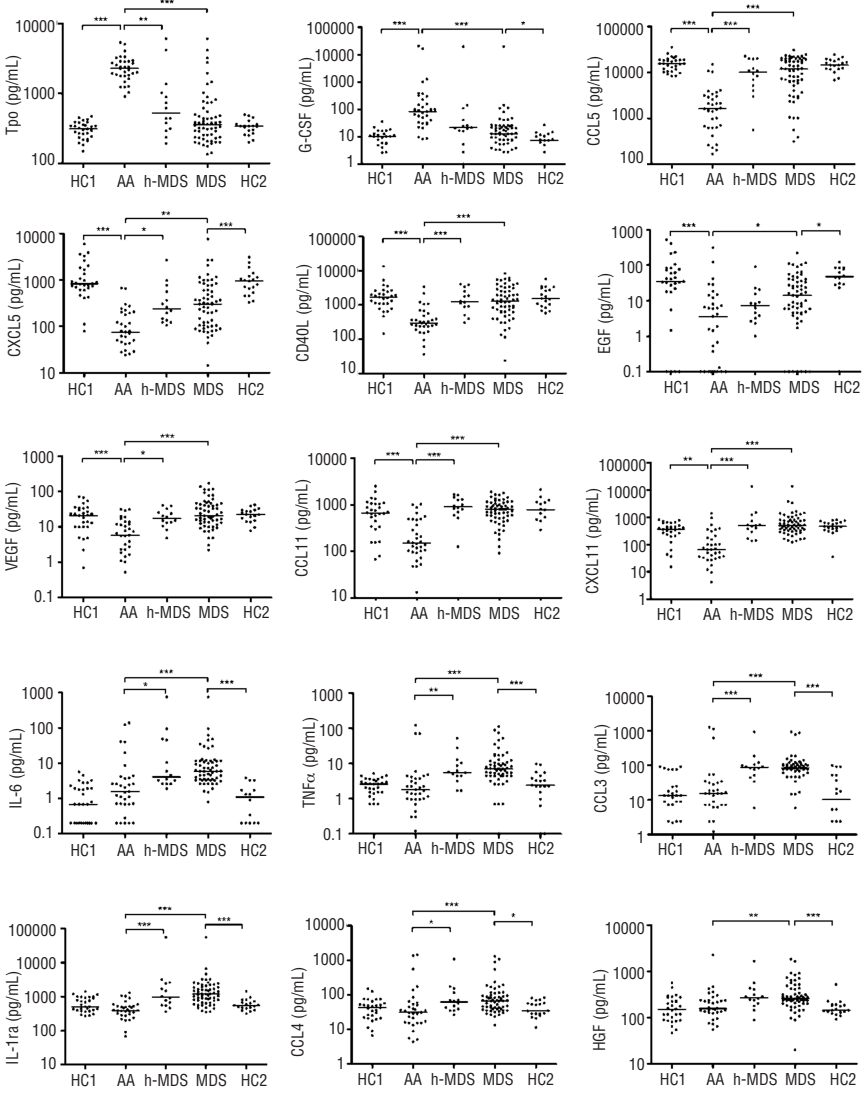


**Figure 1.** Comparison of cytokines and growth factors in the plasma of AA, MDS, and healthy controls. AA, aplastic anemia (n=33); MDS, myelodysplasia (n=57); HC-1, healthy control group-1 (< 55 yrs, n=29); HC-2, healthy control group-2 (≥55yrs, n =19); h-MDS, hypocellular MDS (n=14). The bars represent median values. The rank-based Kruskal-Wallis one-way analysis of variance (ANOVA) method was used to evaluate the differences in cytokine levels among AA, MDS patients, hypocellular MDS, and the healthy controls. ***$P$<0.001, **$P$< 0.01, *$P$<0.05.

tively. As shown in Figure 1, there was no difference in any cytokines measured between the younger and older healthy control groups ($P$>0.05). Correlations between the individual cytokines and age were further analyzed in the 48 healthy controls, 33 SAA patients, and 57 MDS patients. Among all cytokines, only a weak correlation with age was noted for TNF-α ($r^2$ = 0.1092, $P$=0.0266) in the healthy control group, and for CXCL5 ($r^2$ = 0.1856, $P$=0.0123) in SAA patients. Thus, plasma levels of the majority of cytokines tested did not vary with age. As shown in Figure 1, higher levels of Tpo and G-CSF and lower levels of CD40L, CCL5, CCL11, VEGF, CXCL5, EGF, and CXCL11 were present in AA, compared to healthy controls and to MDS patients. The higher levels of Tpo and G-CSF in AA are consistent with previous observations,[5-8] likely representing a compensatory physiological mechanism operating to counter impaired blood cell production. Plasma Tpo is strongly linked to the number of megakaryocytes in the bone marrow. In AA, a severe reduction in bone marrow megakaryocytes is present, while in MDS the number of megakaryocytes may be reduced, the platelet count is believed to be especially affected by the dysplasia. The difference in VEGF between AA and MDS might be linked to the increased microvessel density in the bone marrow of MDS patients. In both AA and MDS, lower

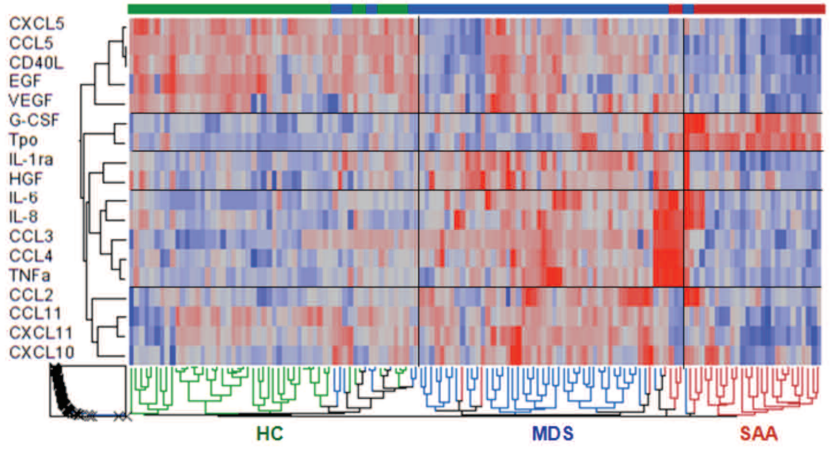


**Figure 2.** Different cytokine profiles of SAA, MDS and healthy controls (HC). Two-way hierarchical cluster analysis was performed on log-transformed cytokine levels of SAA, MDS and HC samples using Ward's method. Color scales represent cytokine levels (red indicates high levels; blue, low levels). The dendrogram under the figure classifies 3 clusters according to the cytokine profiles. Red lines and marks represent SAA, blue lines and marks represent MDS, and green lines and marks represent HC. The dendrogram on the left side of the figure reflects the proximities of cytokines.

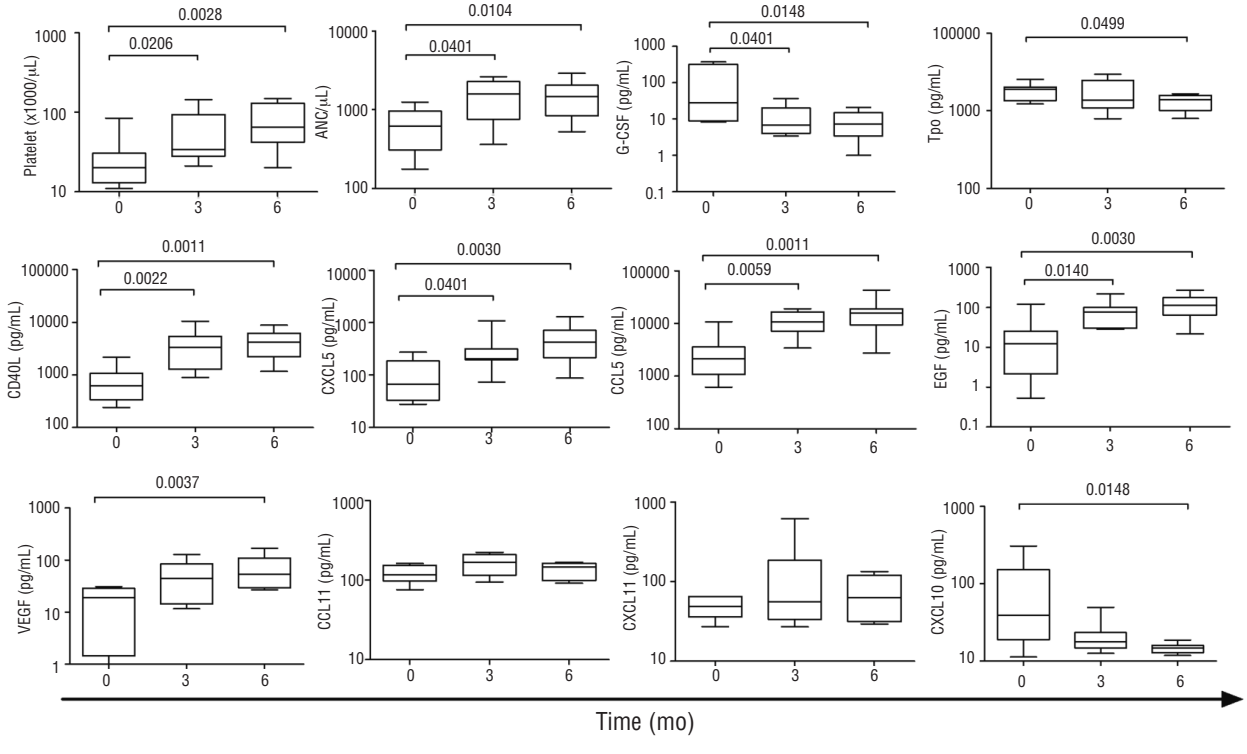


**Figure 3.** Variations of blood cell counts and cytokine levels in SAA patients after immunosuppressive therapy (IST). In 8 SAA patients who responded to horse ATG + cyclosporine, plasma cytokines were measured before, and at three and six months after IST. Box-and-whiskers plot with the whiskers showing the range of the data. Differences in cytokines and cell counts before IST *vs*. three months, and six months post IST were evaluated by Mann-Whitney U test, respectively.

levels of EGF and CXCL5 compared to healthy controls were observed. TNF-α, IL-6, CCL3, IL-1ra, HGF, and CCL4 were higher in MDS patients only, compared to AA patients and healthy controls (Figure 1). MDS patients also showed slightly higher levels of CCL2 than AA ($P<0.05$, *data not shown*). These pro-inflammatory cytokines may reflect an inflammatory microenvironment in MDS. Levels of INF-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17, FGFb, and GM-CSF were undetectable, while there was no significant difference in CXCL10, IL-8, and leptin between patients and healthy controls. Clinically, hypocellular MDS and AA may be difficult to distinguish, due to shared clinical, laboratory, and bone marrow findings. When analyzed separately, hypocellular MDS patients showed a much lower level of Tpo and higher levels of CCL3, CXCL11, CCL5, CD40L, CCL11, TNFα, IL-1ra, IL-6, CXCL5, VEGF, and CCL4 than did AA patients (similar to that observed in all MDS patients, Figure 1). Among these cytokines, Tpo and CCL3 appeared more discriminatory between hypocellular MDS and AA due to the larger difference in levels and minimal overlap between them. Further testing of Tpo and CCL3 by enzyme-linked immunosorbent assay (ELISA) confirmed the large difference in plasma levels between hypocellular MDS and AA. Of note, the majority of hypocellular MDS patients (71%, 10 of 14) had low levels of Tpo and high levels of CCL3, and conversely, the majority of AA patients (91%, 30 of 33) had high levels of Tpo and low levels of CCL3. Therefore, measurement of Tpo and CCL3 cytokines might help distinguish between these two entities. CCL3 is an inhibitor of hematopoietic stem cell proliferation and is potentially important in leukemias associated with RUNX1 or MOZ chromosomal rearrangements,[15] which suggests a role for this cytokine in MDS pathogenesis.

To classify the cytokine patterns and the relationships among the individual cytokines, two-way hierarchical cluster analysis was performed on log-transformed cytokine levels of AA, MDS, and healthy samples using Ward's method (Figure 2). The dendrogram under the figure distinguished 3 clusters according to the cytokine profiles, with the great majority of samples matching their corresponding groups, indicating AA, MDS, and healthy controls possessed different cytokine patterns; the dendrogram on the left of the hierarchical cluster shows the proximity between the different cytokines (Figure 2). Among the individual cytokines, five distinct groups were observed: 1) CXCL5, CCL5, CD40L, EGF, and VEGF; 2) G-CSF and Tpo; 3) IL-1ra and HGF; 4) IL-6, IL-8, CCL3, CCL4, and TNFα; 5) CCL2, CCL11, CXCL11, and CXCL10. Cytokines in each of these groups may share the same origin and/or exert similar functions. The chemokine clustering may also share common transcriptional regulation (as through the NF- kappaB pathway).

Due to the heterogeneity of MDS, we investigated variations of cytokine levels in different subgroups (*Online Supplementary Figure S1*). Among the MDS subtypes (*Online Supplementary Figure S1A*), CCL5 and EGF were lower in RCMD and RAEB patients compared to those with RA-RS. CXCL5 was lower in RCMD and RAEB than in 5q-, it appeared that the levels of CCL5, EGF, and CXCL5 in different subtypes were proportional to the platelet counts they had. In high-risk MDS, decreased levels of CXCL5 ($P<0.01$), CCL5 ($P<0.001$), CD40L ($P<0.01$), EGF ($P<0.01$), and VEGF ($P<0.05$) and an elevated level of CCL4 ($P<0.05$) were observed compared to low-risk MDS (*Online Supplementary Figure S1B*). We then studied variations of cytokine levels in MDS patients with different marrow cellularity (*Online Supplementary Figure S1C*), most cytokine levels were again similar, with the exception of Tpo, which was higher in hypocellular compared to hypercellular marrow ($P<0.05$).

To determine if the cytokines that were decreased or increased in AA (compared to healthy controls) changed after IST, levels were measured before and at three and six months after ATG and CsA in 8 patients who were hematologic responders (Figure 3). Response was defined as no longer meeting criteria for SAA and was determined at three and six months following ATG.[16] Statistically significant increases of CD40L, CXCL5, CCL5, EGF, and VEGF were present at six months after IST compared to pre-treatment levels. An increase was not seen for CCL11 and CXCL11 after IST. Tpo and G-CSF levels that were elevated in AA decreased after IST. Of the remaining cytokines that showed no difference in untreated AA compared to healthy controls, no difference was noted after IST compared to pre-treatment levels, with the exception of CXCL10. In an additional 3 AA patients who were non-responders to IST, no changes of cytokines were observed at three and six months post treatment (*data not shown*).

To determine if higher Tpo levels observed in AA patients were a consequence of a lower platelet count, Tpo levels between AA and MDS patients with comparable low platelet counts were evaluated (platelet count on the day of the plasma collection in all patients). In patients with a platelet count less than $40\times10^9$ /L, Tpo levels were higher in AA with a median 2263 (interquartile range 1760-2865) pg/ml compared to MDS where the median was 409 (interquartile range 216-2991) pg/mL ($P<0.05$).

To analyze correlations of cytokines with different cell types, multivariate analysis was performed, CXCL5 (r=0.7895), CCL5 (r=0.7895), CD40L (r=0.7757), EGF (r=0.5722), VEGF (r=0.6626), and Tpo levels (r=-0.4187) showed a stronger correlation with the platelet count compared to ANC, absolute lymphocyte count (ALC), absolute monocyte count (AMC), or ARC. AMC had a higher correlation with IL-1ra (r=0.6006) and HGF (r=0.5127) than observed for other cell types. The high correlation of cytokine levels with different blood cell types may indicate the source of the cytokines.

In summary, we show that differences in cytokine profiles exist between AA and MDS, suggesting differences in pathophysiology. More importantly, despite similar clinical presentations, differences in cytokine profiles were also present between AA and hypocellular MDS. Future studies focusing on those cytokines that differed significantly between these two entities (for example Tpo and CCL3) should define those relevant for laboratory discrimination and potentially provide an important tool in clinical practice. A more targeted evaluation of these cytokines using standard assays such as an ELISA could be more widely adopted when evaluating these cases and help in correct diagnosis and management.

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