# Aberrant expression of Treg-associated cytokine IL-35 along with IL-10 and TGF-β in acute myeloid leukemia

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**Abstract.** Acute myeloid leukemia (AML) is the most common hematological malignancy in adults, characterized by distorted proliferation and the development of myeloid cells and their precursors in the blood and bone marrow. Interleukin 35 (IL-35), a novel inhibitory cytokine secreted by regulatory T (Treg) cells is a novel potential target used for the therapeutic manipulation of Treg activity in order to treat cancer and autoimmune diseases. To investigate the role and imbalance of Treg-related cytokines in the pathogenesis of AML, we measured the plasma concentration of three Treg-associated cytokines [IL-35, IL-10 and transforming growth factor-β (TGF-β)] and evaluated their clinical relevance. The concentration of IL-35, IL-10 and TGF-β in plasma specimens from 55 patients with AML [27 newly diagnosed (ND) patients and 28 in complete remission (CR)] and 24 controls was analyzed using the enzyme-linked immunosorbent assay method. Significantly higher levels of plasma IL-35 and IL-10 were observed in AML ND patients compared with healthy controls or AML CR patients. IL-10 concentrations were positively correlated with TGF-β, whereas no correlations were found between the other cytokines. IL-10 levels were positively correlated with white blood cell (WBC) and neutrophil (NEU) count but there were no correlations between IL-35 and TGF-β with WBC and NEU count. In conclusion, we demonstrated for the first time that AML ND patients have increased plasma concentrations of IL-35, suggesting that this cytokine is involved in the pathophysiological process of the disease, and that further research is required to address this issue.

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## Introduction

Acute myeloid leukemia (AML) is a life-threatening hematopoietic stem cell neoplasm characterized by an increase in the number of myeloid cells in the bone marrow and an arrest in their maturation, frequently resulting in fatal infection, bleeding or organ infiltration, with or without leukocytosis (1-3). The etiology of AML is heterogeneous and complex, but it is widely accepted that both environmental and genetic factors play significant roles in the development of the disease. Immune system disorders have increased our understanding of leukemogenesis (4). However, little is known about the pathogenic events leading to the initiation and progression of this disease. Previously, elevated levels of regulatory T (Treg) cells in a variety of hematological malignancies including AML have been reported (5-8). Patients with a lower Treg cell frequency at diagnosis have a better response to induction chemotherapy and a favorable prognosis (6,8).

Treg cells, a subpopulation of $CD4^+$ T cells, inhibit the immune response by influencing the activity of other cell types. Typically, Treg cells are classified into naturally occurring $CD4^+CD25^+$ Treg cells, interleukin 10 (IL-10)-secreting Treg cells, and transforming growth factor-β (TGF-β)-secreting Treg cells based on the types of cytokines they produce. IL-10-secreting Treg cells, known as type 1 T regulatory cells (Tr1), are produced *in vitro* by the antigenic stimulation of naive cells in the presence of IL-10 (9). TGF-β-secreting Treg cells, also known as T helper 3 cells (Th3), are propagated from animals via oral tolerance and are readily accepted (9). Naturally occurring $CD4^+CD25^+$ Treg cells, which are present in the normal immune system, engage in the maintenance of natural self-tolerance and also the control of immune responses to foreign antigens (9).

IL-35, a member of the IL-12 family, is a recently identified heterodimeric cytokine consisting of Epstein-Barr virus-induced gene protein 3 (EBI3) and the p35 subunit of IL-12 (10). In contrast to all other known IL-12 family members, which are not expressed by T cells, IL-35 is secreted by Treg cells and contributes to their suppressive activity, rather than acting in an immunostimulatory or proinflammatory manner (11). Secreted exclusively by Treg cells and other cell populations with regulatory potential, IL-35 is a novel potential target

Table I. Clinical and laboratory parameters of AML ND patients.

| Patient no. | Gender/age (years) | WBC ($10^9$/l) | NEU ($10^9$/l) | Marrow blast (%) | Blood blast (%) |
|---|---|---|---|---|---|
| 1 | F/25 | 70.55 | | 96 | 28 |
| 2 | F/56 | 52.25 | 35.62 | 80 | 66 |
| 3 | F/46 | 0.85 | 0.16 | 95 | 11 |
| 4 | F/56 | 1.81 | 0.05 | 78 | 68 |
| 5 | M/45 | 2.89 | 0.17 | 70 | 30 |
| 6 | F/56 | | | | |
| 7 | F/80 | 1.97 | 0.56 | 28 | |
| 8 | F/74 | 2.16 | 1.29 | 54 | 3 |
| 9 | F/40 | 25.3 | 11.3 | 68 | 32 |
| 10 | F/32 | 1.28 | 0.27 | 37.50 | 6 |
| 11 | F/15 | 4.58 | 0.03 | 53.50 | 51 |
| 12 | M/61 | 4.58 | 2.95 | 94 | 64 |
| 13 | M/76 | 196.57 | | 98 | 92 |
| 14 | M/69 | 16.16 | 3.49 | 93 | 82 |
| 15 | M/40 | 76.67 | 20.72 | 85 | 75 |
| 16 | M/21 | 45.06 | 18.74 | 44 | 40 |
| 17 | F/59 | 4.14 | 0.3 | 58 | 36 |
| 18 | F/53 | 123.3 | 28.83 | 71 | 64 |
| 19 | M/44 | 11.7 | 45.2 | 13 | 70 |
| 20 | M/21 | 24.77 | 75.8 | 79 | 68 |
| 21 | M/29 | 105.4 | 50.5 | 86 | 95 |
| 22 | F/45 | 97.81 | 5.3 | 96 | 95 |
| 23 | M/42 | 5.03 | 10.2 | 70 | 34 |
| 24 | M/55 | 66.25 | 15.54 | 80 | 80 |
| 25 | M/20 | 39.32 | 1.25 | 95 | 93 |
| 26 | F/49 | 5.1 | 3.58 | 86 | 65 |
| 27 | F/47 | 6.91 | 5.42 | 95 | 65 |

AML, acute myeloid leukemia; ND, newly diagnosed; WBC, white blood cell; NEU, neutrophil.

used for the therapeutic manipulation of Treg activity in order to treat cancer and autoimmune diseases (11).

However, the exact roles of Treg cells in AML remain unknown. To investigate the role and imbalance of Treg-related cytokines in the pathogenesis of AML, we measured the plasma concentration of the three Treg-associated cytokines (IL-35, IL-10 and TGF-β) and evaluated their clinical relevance.

## Materials and methods

*Study population*. Following approval by the institutional review board, 55 adult AML patients visiting the Qilu Hospital of Shandong University between September 2009 and June 2010 were studied. Twenty-seven of these patients were newly diagnosed (ND) (12 males and 15 females; median age, 46; range, 15-80 years) and 28 were in complete remission (CR) (15 males and 13 females; median age, 40; range, 20-63 years). The AML patients were diagnosed according to the French-American-British (FAB) classification system (12,13). CR was defined based on International Working Group criteria (14). Clinical and laboratory observations regarding the patients are summarized in Table I. Twenty-four healthy adults (13 males and 11 females; median age, 56; range, 18-76 years) without any evidence of hematological disease served as the control group. Informed consent was obtained from all participants.

*Plasma samples*. Heparinized venous peripheral blood (20 ml) was collected from controls and patients prior to induction chemotherapy. Plasma samples were obtained following centrifugation and preserved at -80˚C in aliquots for cytokine assays, and thawed only once before use to avoid degradation.

*Determination of cytokines in plasma*. Plasma IL-35 concentration in AML patients and control subjects was measured by enzyme-linked immunosorbent assay (ELISA) using a kit from Uscn Life Science Inc. (Wuhan, China). Plasma IL-10 and TGF-β were measured using ELISA kits from Bender MedSystems (Vienna, Austria). The assay was performed in triplicate and the concentrations were calculated from a standard curve according to the manufacturer's instructions. The minimum detectable doses (MDD) of the assays were as follows: IL-35, 7.1 pg/ml; IL-10, 1.0 pg/ml; TGF-β, 9 pg/ml.

Table II. Cytokine concentrations in AML patients and controls.

| Cytokine | Median (range) (pg/ml) | | | P-value | | |
|---|---|---|---|---|---|---|
| | AML ND | AML CR | Controls | ND vs. CR | ND vs. controls | CR vs. controls |
| IL-35 | 107.40 (74.95-468.22) | 68.80 (48.07-252.47) | 63.23 (24.46-489.45) | 0.001[a] | 0.000[a] | 0.114 |
| IL-10 | 14.41 (6.39-59.20) | 8.47 (5.78-17.82) | 5.87 (4.29-7.78) | 0.000[a] | 0.000[a] | 0.000[a] |
| TGF-β | 377.31 (83.68-7220.11) | 285.39 (42.54-2172.26) | 1008.86 (250.28-33793.68) | 0.216 | 0.001[a] | 0.000[a] |

The Mann-Whitney test was used to compare the difference of cytokine concentrations between the two groups. [a]P<0.05 was considered statistically significant. AML, acute myeloid leukemia; ND, newly diagnosed; CR, complete remission.

*Statistical analysis*. Statistical analyses were performed using SPSS version 17.0 software (SPSS, Chicago, IL, USA). Due to the abnormal distribution and heterogeneity of variance, the data are presented as medians (range). Statistical significance among cases with AML ND, AML CR and normal controls was determined using the Kruskal-Wallis test and the difference between the two groups was determined using the Mann-Whitney test. Spearman's test was used for correlation analysis. P<0.05 was considered to indicate a statistically significant result.

## Results

*Plasma concentration of Treg cytokines in AML and controls*. Plasma concentrations of IL-35 were found to be significantly higher in AML ND patients (median, 107.40 pg/ml; range, 74.95-468.22) compared to AML CR patients (median, 68.80 pg/ml; range, 48.07-252.47; P=0.001) and the control group (median, 63.23 pg/ml; range, 24.46-489.45; P<0.001) (Table II). Plasma IL-10 levels were also significantly higher in AML ND patients (median, 14.41 pg/ml; range, 6.39-59.20) compared to AML CR patients (median, 8.47 pg/ml; range, 5.78-17.82; P<0.001) and the control group (median, 5.87 pg/ml; range, 4.29-7.78; P<0.001).

However, the plasma concentrations of TGF-β were significantly higher in the control group (median, 1008.86 pg/ml; range, 250.28-33793.68) compared to the AML ND patients (median, 377.31 pg/ml; range, 83.68-7220.11; P=0.001) or AML CR patients (median, 285.39 pg/ml; range, 42.54-2172.26; P<0.001). No significant difference was found in the plasma levels of TGF-β between AML ND and AML CR patients (P=0.216). Additionally, no significant difference was found in the plasma levels of IL-35 between AML CR patients and the control group (P=0.114).

*Correlations between plasma cytokine levels in AML ND patients*. Correlations between the plasma concentrations were analyzed in AML ND patients. The data demonstrated that the plasma IL-10 concentration level positively correlated with the plasma TGF-β concentration level (r=0.435, P=0.023), whereas no correlations were found between the other cytokines.

*Correlations of cytokine levels with clinical and laboratory parameters in AML ND patients*. Among the AML ND patients, there were no significant differences in Treg cytokine concentrations between males and females (Table III). Correlations between age and cytokine concentrations were analyzed in AML ND patients, and no significant correlations were found (r=-0.032, P=0.875 for IL-35; r=-0.092, P=0.647 for IL-10; r=-0.017, P=0.935 for TGF-β).

Table III. Cytokine concentrations of male and female AML ND patients.

| Cytokine | Median (range) (pg/ml) | | P-value |
|---|---|---|---|
| | Male | Female | |
| IL-35 | 133.40 (74.95-468.22) | 95.13 (81.33-462.10) | 0.373 |
| IL-10 | 16.57 (7.00-36.08) | 12.65 (6.39-59.20 ) | 0.399 |
| TGF-β | 356.04 (152.71-1891.20) | 491.74 (83.68-7220.11) | 0.755 |

The Mann-Whitney test was used to compare the difference of cytokine concentrations between the genders of AML ND patients. AML, acute myeloid leukemia; ND, newly diagnosed.

To evaluate whether the Treg cytokine levels correlated with the FAB subtype, the Kruskal-Wallis test was used. Results showed that there were no significant differences in Treg cytokine concentrations among the different FAB subtypes (data not shown). Correlations between cytokine concentrations and white blood cell (WBC) and neutrophil (NEU) count were analyzed in AML ND patients; the data demonstrated that IL-10 levels were positively correlated with WBC or NEU count (r=-0.438, P=0.025 for WBC; r=-0.581, P=0.003 for NEU). However, there were no correlations between IL-35 and TGF-β with WBC and NEU count (Table IV).

When evaluating correlations between cytokine concentrations and the marrow/blood blast percentage, the correlation between IL-10 and the marrow blast percentage almost reached statistical significance (r=0.351, P=0.079), whereas no other correlations were observed for the other cytokines (Table IV).

Table IV. Correlations of the cytokine concentrations with laboratory parameters in AML ND patients.

| Cytokine | | WBC | NEU | Marrow blast (%) | Blood blast (%) |
|---|---|---|---|---|---|
| IL-35 | r | 0.083 | 0.029 | 0.038 | -0.015 |
| | P | 0.685 | 0.894 | 0.845 | 0.945 |
| IL-10 | r | 0.438 | 0.581 | 0.351 | 0.326 |
| | P | 0.025[a] | 0.003[a] | 0.079 | 0.111 |
| TGF-β | r | 0.169 | 0.090 | 0.179 | 0.173 |
| | P | 0.410 | 0.677 | 0.382 | 0.408 |

[a]P<0.05 was considered statistically significant. AML, acute myeloid leukemia; ND, newly diagnosed; WBC, white blood cell; NEU, neutrophil.

## Discussion

Treg cells prevent autoimmune diseases by suppressing host immune responses. Previous studies have demonstrated that the prevalence of Treg cells is increased in cancer patients, and that tumor cells recruit these Treg cells to inhibit antitumor immunity in the tumor microenvironment, thus limiting the efficiency of cancer immunotherapy (15,16). In previous studies, elevated percentages or levels of Treg cells were reported in the total T-cell population isolated from tumor tissues or peripheral blood in a variety of hematological malignancies, including B-cell non-Hodgkin lymphoma (17), Hodgkin lymphoma (18), chronic lymphocytic leukemia (19-21), multiple myeloma (22) and AML (5-8). Treg cells accumulating in the peripheral circulation of AML patients mediate vigorous suppression via IL-10 and TGF-β as well as contact-dependent mechanisms (6). Patients with a lower Treg cell frequency at diagnosis have a better response to induction chemotherapy and a good prognosis (6,8). We investigated the role and imbalance of Treg-related cytokines IL-35, IL-10 and TGF-β in the pathogenesis of AML.

In the present study, plasma concentrations of three Treg-associated cytokines IL-35, IL-10 and TGF-β were determined. IL-35 is secreted by Treg cells and contributes to their suppressive activity (11). In turn, treatment of naive human or mouse T cells with IL-35 induces a regulatory population, known as iT(R)35 cells. iT(R)35 cells constitute a key mediator of infectious tolerance and contribute to Treg cell-mediated tumor progression (23,24). By expanding regulatory T cells and inhibiting the differentiation of Th17 cells, IL-35 may have therapeutic effects against collagen-induced arthritis (25,26). In this study, we have demonstrated that the plasma concentrations of IL-35 in AML ND patients were significantly higher than those in AML CR patients and a control group. Increased IL-35 levels decreased when patients achieved CR following chemotherapy, suggesting that the measurement of IL-35 concentrations may be valuable in the evaluation of therapeutic effect. However, further investigations are required to determine whether regulating IL-35 or specific combinations of IL-35 and other Treg cytokines has additional value in animals and patients.

Consistent with higher plasma IL-35 levels, our data have demonstrated that AML ND patients also had increased plasma IL-10 levels compared with AML CR patients and the control group, although these cytokines were secreted by different subtypes of Treg cells. IL-10 is known to inhibit cytokine production by T cells, and exerts anti-inflammatory and immunosuppressive activities. It inhibits the production of IL-2, IFNγ and granulocyte macrophage colony-stimulating factors (GM-CSF) as well as the proliferative response of T helper (Th) 1 cells (27). The serum levels of IL-10 in patients with adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) infection were elevated and IL-10 protein was detected in the culture medium of leukemic cells from ATL patients (28). Our findings showed that the concentration of IL-35 was also increased in AML patients. IL-10 has been shown to inhibit the proliferation of AML cells *in vitro* by suppressing the production of IL-1α, IL-1β, granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-6, and tumor necrosis factor α (TNFα), and promoting the production of IL-1ra (29-31). In accordance with the *in vitro* studies, IL-10 was found to increase serum IL-1ra *in vivo* (32). However, it increased serum IL-1β and TNFα levels and had no effect on GM-CSF levels (32). The *in vitro* effects of IL-10 do not necessarily reflect its *in vivo* effects, and the complex effects of IL-10 on serum cytokine levels render it necessary to conduct more research to address this issue.

TGF-β signaling controls a diverse set of cellular functions, including cell proliferation, recognition, apoptosis, tumorigenesis and cell differentiation, during embryogenesis as well as in mature tissues (33). A growing body of evidence supports deregulated TGF-β signaling in leukemogenesis. In the erythroleukemia TF1 cell line, the aberrant expression of Smad5β is likely to alter the erythroid differentiation response to TGF-β/BMP ligands (34). A missense mutation in the MH1 domain (P102L) and a frameshift mutation resulting in termination in the MH2 domain [Δ(483-552)] in Smad4 results in a disruption of TGF-β signaling, and thus leads to AML (35). AML1-ETO, an AML-associated fusion protein, cooperates with Smads, blocking the response to TGF-β1 (36) and inducing the expression of C-KIT gene mutation (37). The production and secretion of an active form of TGF-β and stimulation of collagen synthesis in a paracrine manner results in bone marrow fibroblasts, which are often associated with acute megakaryoblastic leukemia (AMKBL) (38). Combined treatment with TGF-β and 1,25-dihydroxyvitamin D3 (D3) may cause terminal monocytic maturation in human monocytic (U-937) and promyelocytic (HL-60 and AML-193) leukemic cell lines (39). In the present study, the plasma concentrations of TGF-β in the control group were found to be significantly higher than in the AML ND and AML CR patients. No significant difference was found in the plasma levels of TGF-β between AML ND and AML CR patients. However, Wu *et al* indicated that the concentration of plasma TGF-β was increased significantly in peripheral blood samples with AML (51.37±11.30 versus 14.35±4.00 ng/ml, P<0.01) (40). These different findings may result from the investigation of relatively low sample numbers and thus, larger scale case-control studies should be implemented.

In conclusion, we have demonstrated for the first time that AML ND patients had increased plasma concentrations of IL-35 and IL-10, suggesting that they are involved in the pathophysiological process of the disease, and that their modulation may provide a new immunotherapy for AML. However, the precise

involvement of IL-35 and IL-10 in leukemogenesis should be clarified and further research is required to address this issue.

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