Increased Frequency and Suppression by Regulatory T Cells in Patients with Acute Myelogenous Leukemia

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Abstract Purpose: Regulatory CD4+CD25highFoxp3+ T cells (Treg) control peripheral immune tolerance. Patients with cancer, including those with hematologic malignancies, have elevated numbers of Treg in the peripheral circulation and in tumor tissues. However, mechanisms of suppression and clinical significance of Treg, especially in patients with acute myelogenous leukemia (AML), has not been well defined.

Experimental Design: We prospectively evaluated the phenotype, function, and mechanisms of suppression used by Treg in newly diagnosed untreated AML patients. The relationship between the frequency of circulating Treg and the disease status as well as treatment outcome was also evaluated.

Results: The percentage of circulating Treg was higher (P < 0.0001) and their phenotype was distinct in AML patients relative to normal controls. Suppression mediated by Treg coincubated with proliferating autologous responder cells was also higher (P < 0.001) in AML than that mediated by control Treg. Using Transwell inserts, we showed that interleukin-10 and transforming growth factor-β1 production as well as cell-to-cell contact were necessary for Treg-mediated suppression. Also, the pretreatment Treg frequency predicted response to chemotherapy. Unexpectedly, patients who achieved complete remission still had elevated frequency of Treg, which mediated high levels of suppressor activity.

Conclusions: Treg accumulating in the peripheral circulation of AML patients mediate vigorous suppression via contact-dependent and contact-independent mechanisms. Patients with lower Treg frequency at diagnosis have a better response to induction chemotherapy. During the post-induction period, the Treg frequency and suppressive activity remain elevated in complete remission, suggesting that Treg are resistant to conventional chemotherapy.
Acute myelogenous leukemia (AML) is a disease with considerable phenotypic and genotypic heterogeneity, characterized by acquisition of somatic mutations in hematopoietic progenitors, which acquire a proliferative and/or survival advantage, impair hematopoietic differentiation, and confer properties of limitless self-renewal. Several studies have described the presence of abnormalities in the immune system of patients with AML, including defective function of natural killer cells and dendritic cells (25, 26). In comparison with solid malignancies, relatively little information is available about functional characteristics of Treg or their clinical significance in patients with acute leukemia. In the present study, we investigate the phenotype, function, and mechanisms of suppression used by Treg in newly diagnosed untreated AML patients and evaluate the relationship between the frequency of circulating Treg and the disease status as well as treatment outcome.

Materials and Methods

AML patients and healthy volunteers. Samples of venous blood (20-50 mL) were obtained from 31 newly diagnosed AML patients (18 females and 13 males) before any treatment and 25 age-matched healthy volunteers. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. The blood was drawn into heparinized tubes, hand-carried to the laboratory, and immediately processed using Ficoll-Hypaque gradients. Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

Immunophenotypic analysis of cells and tissues. The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD3-ECD, anti-CD4-PC5, anti-HLA-DR-FITC, anti-CD62L-FITC, anti-CD122-FITC, anti-CD25-FITC all from Beckman Coulter; anti-GITR-FITC and anti-TGF-β1-PE from R&D Systems; anti-FoxP3-FITC, anti-CD152-PE (CTLA-4), anti-CD26-PE, anti-CD39-PE, and anti-IL-10-PE from eBioscience; anti-CD122-PEFITC (IL-2R), anti-CR7-FITC, anti-CD25-PE, and anti-CD132-PE (IL-2Ry) from BD Biosciences; anti-Fas-FITC and anti-Fasl-PE from BioLegend; and unconjugated anti-CD73 from Santa Cruz Biotechnology. The secondary antibody, goat anti-mouse FITC, was purchased from Jackson ImmunoResearch.

The following antibodies were used for immunostaining of cells for confocal microscopy: monoclonal mouse anti-human CD25 from R&D Systems; rat anti-human Foxp3 from eBioscience; polyclonal rabbit anti-human CD4 from Santa Cruz Biotechnology; and rabbit anti-NFAT1 from Upstate Biotechnology. Secondary antibodies were donkey anti-rabbit FITC-labeled from Santa Cruz Biotechnology and donkey anti-mouse Cy3-labeled, donkey anti-rat Cy5-labeled, and donkey anti-rabbit Cy5-labeled from Jackson ImmunoResearch. Before use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions. Appropriate isotype controls were included in all experiments.

Intracellular staining for Foxp3, CD152 (CTLA-4), and GITR was done as described previously (27). Intracytoplasmic expression of TGF-β1 and IL-10 was assessed before and after stimulation of PBMC for 4 h with phorbol 12-myristate 13-acetate (20 μg/mL) and ionomycin (1 μmol/L/mL). Briefly, samples were first incubated with mAbs against surface markers CD4, CD3, and CD25. After washing, cells were fixed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, washed once with PBS containing 0.5% (v/v) bovine serum albumin and 2 mmol/L EDTA, permeabilized with PBS containing 0.5% bovine serum albumin and 0.1% (v/v) saponin, and stained with anti-CTLA-4-PE, anti-Foxp3-PE, anti-TGF-β1-PE, or anti-IL-10-PE mAb for 30 min at room temperature. Cells were further washed twice with PBS containing 0.5% bovine serum albumin and 0.2% (v/v)
saponin, resuspended in fluorescence-activated cell sorting flow solution, and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

Flow cytometry. A Beckman Coulter flow cytometer equipped with Expo32 software was used to acquire at least $2 \times 10^5$ lymphocytes in each sample. The acquisition and analysis gates were set on lymphocytes based on forward scatter and side scatter properties of the cells. Forward and side scatter were set in a linear scale. For more extensive analysis, gates were set on CD3$^+$CD4$^-$ and CD4$^+$CD25$^{high}$ T-cell subsets as appropriate. CD4$^+$ T cells with mean fluorescence intensity of CD25 expression $>120$ were classified as CD25$^{high}$ based on the data previously reported by us (27).

Suppressor activity. For use in suppression assays, CD4$^+$CD25$^{high}$ T cells were single-cell sorted from the populations of CD4$^+$CD25$^+$ T cells obtained by positive selection of PBMC using the Regulatory T Cell Separation Kit and AutoMACS (Miltenyi Biotech). CD4$^+$CD25$^{high}$ T cells were tested for regulatory function in coculture assays as described previously (11). Briefly, CD4$^+$CD25$^+$ responder cells were labeled with 1.5 $\mu$mol/L carboxyfluorescein diacetate succinimidylester (Molecular Probes/Invitrogen) and cocultured with autologous Treg at the suppressor to responder cell ratios of 1:1, 1:5, and 1:10 for 5 days. Bead-bound OKT3 (1 $\mu$g/mL; American Type Culture Collection) and soluble anti-CD28 mAb (1 $\mu$g/mL) (Miltenyi Biotec) were used for T-cell stimulation in the presence of 150 IU IL-2/mL. T-cell populations were classified as suppressive if they inhibited proliferation of the CD4$^+$CD25$^+$ responder cells in the coculture assay and if decreasing the number of CD4$^+$CD25$^{high}$ cells relative to the number of CD4$^+$CD25$^+$ responder cells in coculture restored proliferation. All carboxyfluorescein diacetate succinimidylester data were analyzed using the ModFit software provided by Verity Software House. The percentage of suppression was calculated based on the proliferation index of responder cells alone compared with the proliferation index of cultures containing responder cells and Treg. The program determines the percent of cells within each peak, and the sum of all peaks in the control culture is taken as 100% of proliferation or 0% of suppression.

![Fig. 1.](image-url) Increased frequency of CD4$^+$CD25$^{high}$ T cells in the peripheral blood of patients with AML before treatment. A, gating strategy used to identify the CD4$^+$CD25$^{high}$ T reg. CD4$^+$ T cells with mean fluorescence intensity of CD25 expression $>120$ were classified as CD25$^{high}$ as reported previously (26). Only CD4$^+$CD25$^{high}$ T cells with this or higher mean fluorescence intensity in PBMC have suppressor activity. B, percentages of CD4$^+$CD25$^+$ (left) and CD4$^+$CD25$^{high}$ (right) T cells in the peripheral blood of NC and AML patients. The percentage of Treg was increased in AML patients relative to that of NC $P < 0.001$. C, multicolor confocal microscopy shows the presence of CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$CD25$^+$NFAT1$^+$ in the peripheral blood of a representative AML patient. Green, CD4$^+$ cells (1 and 4); red, CD25$^+$ cells (2 and 5); blue, Foxp3$^+$ cells (2); blue, NFAT1$^+$ cells (5). Merged pictures show CD4$^+$CD25$^+$Foxp3$^+$ (3) and CD4$^+$CD25$^+$NFAT1$^+$ (6). Bar, 10 $\mu$m.
The concentration of 250 μmol/L for 30 min before the addition of exogenous ATP. 

To detect CD4+CD25*Foxp3+ and CD4+CD25+NEAT1+ Treg in the peripheral blood of AML patients, PBMC (1 × 10⁸) were cytocentrifuged onto glass slides, air dried, and fixed in acetone and ethanol 1:1 for 8 min. Smears were blocked with 10% donkey serum in PBS for 45 min. Cells were incubated with primary antibodies for 1 h at room temperature in a moist chamber, washed in PBS, and then incubated with the secondary antibodies under the same conditions and in the dark. Controls were treated with isotype control IgG in place of primary antibodies. Cells were examined using an inverted Olympus Fluoview 1000 laser scanning confocal microscope under an oil immersion objective. For digital image analysis, the software Adobe Photoshop 7.0 version was used.

### Results

**Treg frequency in the peripheral blood of AML patients.** The percentages of circulating CD4+CD25*T cells were similar in the AML patients and NC (mean ± SD, 12.8 ± 1.8% versus 12.1 ± 0.7%). However, the percentage of circulating CD4+CD25* Treg was higher (P < 0.0001) in the AML patients (4.5 ± 0.2%; range, 1.7-8.2%) compared with NC (1.5 ± 0.8%; range, 0.9-3.1%; Fig. 1A and B). Similarly, the frequency of CD4+Foxp3*Treg was higher in the AML patients (4.2 ± 0.3%) than in NC (1.4 ± 0.1%). The majority of CD4+CD25* Treg (>95%) in both AML and NC expressed intracellular Foxp3 and were negative for CD127. CD4+CD25*Foxp3* cells were CD4+CD25*NFAT1+ in the peripheral blood of AML patients (Fig. 1C).

**Phenotypic analysis of the CD4+CD25* Treg subset.** On staining for intracellular and cell surface markers in CD4+CD25* T cells, considerable phenotypic differences were observed between AML patients and NC (Fig. 2A and B). Surface expression of GITR and CTLA-4 was increased in Treg of AML patients (P < 0.001 for both) relative to NC. This was also noted for GITR and CTLA-4 expression within the CD4+Foxp3* subset (data not shown) and the intracellular expression of perforin and granzyme B (P < 0.01 for both). In contrast, HLA-DR, CD62L, and CD95 expression was reduced in Treg of the AML patients compared with NC (P < 0.001 for all). Cell surface expression of CD45RO and chemokine receptors CCR7 and CCR4 or intracellular expression of Foxp3 was not significantly different.

**Suppressor function of circulating CD4+CD25* T cells in AML patients.** To evaluate the suppression mediated by CD4+CD25* T cells (S), suppression assays were done. At the suppressor to responder cell ratio of 1:1, the mean ± SD suppression of responder cell proliferation was 63 ± 7% in NC. However, when CD4+CD25* T cells isolated from AML patients were cocultured with autologous CD4+CD25* responder cells, the mean suppression was significantly higher at 82 ± 8% (P < 0.001; Fig. 3A and B).

Having established that Treg from AML patients have higher suppressive activity, we evaluated the intracellular expression of TGF-β1 and IL-10, cytokines that have been shown to play a role in Treg-mediated suppression (11). Compared with NC, the levels of expression of TGF-β1 and IL-10 were higher in Treg obtained from AML before and after stimulation with phorbol 12-myristate 13-acetate and ionomycin (Fig. 3C).

CD4+CD25* T cells mediate suppression through cytokine production and cell-to-cell contact. To further evaluate the role
of cytokines produced by Treg in suppression of responder cell proliferation, the cocultured cells were separated by a Transwell membrane. Suppression mediated by CD4+CD25high T cells (S) isolated from AML patients in the presence of Transwell membranes was significantly inhibited (Fig. 4). The addition of neutralizing antibodies to IL-10 and/or TGF-β1 in the absence of the Transwell inserts resulted in reduction of the Treg-mediated suppression, with the greatest inhibition observed when the combination of these antibodies was used (P < 0.001). The addition to cocultures of neutralizing antibodies to IL-10 and TGF-β1 in the presence of Transwell inserts resulted in the greatest reduction of the Treg-mediated suppression (P < 0.00001; Fig. 4B). In aggregate, these data suggest that both cell-to-cell contact and cytokine production contribute to suppression mediated by Treg obtained from AML patients.

CD4+CD25high Treg isolated from AML patients hydrolyzed ATP. Adenosine generation catalyzed by ectoenzymes CD39 and CD73 has been shown to contribute to Treg-mediated suppression (20, 21). Both CD39 and CD73 were expressed in CD4+CD25high Treg from AML patients (Fig. 5A and B). Compared with Treg of NC, those obtained from AML patients more efficiently hydrolyzed ATP to adenosine (Fig. 5C).

Frequency and suppressor function of circulating CD4+CD25high T cells and clinical outcome in AML patients. Cytogenetic analysis was done in all newly diagnosed AML patients, and cytogenetic abnormalities were grouped according to the published criteria (28). We have not detected any significant difference in the percentage of circulating Treg between the patients who have an unfavorable karyotype and those with favorable and intermediate karyotypes at presentation. The mean percentage of circulating CD4+CD25high Treg at diagnosis in patients who had achieved complete remission (CR) was lower than that in the peripheral circulation of patients who had persistent leukemia after induction chemotherapy (4.1 ± 0.3% versus 5.6 ± 0.6%; P < 0.04; Fig. 6A). Similarly, the CD4+Foxp3+ population was lower (3.8 ± 0.4% versus 5.2 ± 0.6%) in these AML patients.

We also evaluated the frequency, phenotype, and suppressor activity of Treg in 7 AML patients who achieved CR. The percentages of their CD4+CD25high T cells as well as CD4+Foxp3+ T cells increased (P < 0.001) relative to the pretreatment values (6.1 ± 0.7% versus 3.3 ± 0.4%). Expression of various surface and intracellular Treg markers that were evaluated at diagnosis did not significantly change once patients achieved CR. Compared with NC, the mean percent suppression was significantly higher in Treg of CR patients, and these post-treatment suppression levels were similar to those determined before chemotherapy (Fig. 6B and C). The data suggest that, in AML patients who achieve CR after induction therapy, the frequency of Treg remains elevated, as does the suppressor activity these cells mediate.

Discussion

In this study, we evaluated the frequency, phenotype, and functional characteristics of Treg obtained from the peripheral circulation of patients with AML at diagnosis and in a subset of these patients who achieved CR after induction chemotherapy. The subset of Treg was defined as CD4+CD25high Treg based on the emerging evidence that Foxp3 is not a selective marker for human Treg (8) and strong suppressive activity of CD4+CD25high clones (12).

In comparison with NC, the newly diagnosed AML patients had an increased frequency of Treg in the peripheral blood.
Further, these cells had a phenotypic profile that was distinct from that of Treg in the blood of NC and mediated significantly higher suppression. An elevated frequency of Treg in the peripheral blood has been previously reported in various solid cancers and hematologic malignancies (18). Wang et al. have shown an increased frequency of Treg in the peripheral blood of AML patients and a concomitant increase in their frequency in the bone marrow (29). Surprisingly, AML patients had a higher percentage of apoptotic cells in the CD4+CD25<sup>high</sup> T-cell subset relative to NC and a higher proliferative rate, suggesting a rapid turnover of Treg, which compensated for the higher apoptotic loss (29).

Increases of Treg at the tumor site or in the peripheral circulation of cancer patients have been previously associated with poor treatment outcomes in some cancer patients and with more advanced disease stages (23, 24). To gain further insights into the role Treg play in acute leukemia, we also evaluated the frequency of Treg after induction chemotherapy. We found that patients who achieved CR after induction chemotherapy had a significantly lower Treg frequency at diagnosis compared with patients who did not respond and had persistent leukemia. Counterintuitively, the Treg frequency remained elevated in patients who achieved CR after induction therapy. Furthermore, Treg suppressor activity remained persistently high in these CR patients. These data suggest that Treg are resistant to induction chemotherapy and that this therapy may have enhancing effects on the frequency and/or function of Treg. The high frequency of Treg post-therapy could represent a secondary response to inflammation caused by induction chemotherapy and to cytokine secretion promoting the expansion and proliferation of peripheral Treg. Whether the observed high suppressor activity levels of Treg in patients with CR are related to higher relapse rates will need to be further evaluated in a larger cohort of AML patients.

Not only was the frequency of circulating CD4<sup>+</sup>CD25<sup>high</sup> T cells increased in AML patients in our study, but these Treg mediated high suppression levels, inhibiting proliferation of autologous effector cells. As Treg can use different mechanisms of suppression, initially we used the Transwell system to show that Treg of AML patients mediated suppression via cell-to-cell contact. Indeed, in the presence of a Transwell membrane, suppression was reduced by ∼50%, confirming that the cell-to-cell contact is important. In addition, neutralizing antibodies to IL-10 and TGF-β1 further inhibited Treg-mediated suppression in the Transwell experiments, indicating that these cytokines also contribute to Treg-mediated suppression. Another mechanism of Treg-mediated suppression that we investigated was enzymatic hydrolysis of ATP to adenosine (20, 21). Our data showed for the first time that Treg in AML patients express endonucleotidases, CD39 and CD73, and consequently can hydrolyze ATP more efficiently than their counterparts in NC.

Treg suppressor function is controlled by the constitutive expression of several receptors. Compared with NC, expression levels of these biomarkers on Treg, including CTLA-4 and GITR, were elevated in AML patients. Studies in mouse models and in cancer patients have shown that blocking of Treg receptors results in a decrease of Treg suppressor function, improves antitumor immunity, and induces tumor regression (30–33). These data suggest that the distinct receptor repertoire of Treg in AML patients could be related to suppressor activity mediated by these T cells and that blocking of these receptors on Treg could restore antitumor immunity.

In summary, our data provide insights into the regulatory mechanisms used by Treg in patients with AML. The increased frequency of Treg with high suppressor activity in AML patients at diagnosis indicates that these cells likely play a role in host antitumor immune responses. However, following induction chemotherapy, the frequency and suppression mediated by Treg remain elevated even in AML patients who achieve a CR. These data suggest that chemotherapy does not reduce the frequency or function of Treg and that their persistence could influence leukemia recurrence. Although further studies are required to confirm this hypothesis, it might be possible to suggest that immunotherapies that down-regulate functional activity of Treg should be considered for patients with AML.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Fig. 5. Constitutive expression of CD39 and CD73 by Treg and adenosine generation. A, expression levels of CD39 and CD73 proteins on CD4+CD25^high T cells in AML patients and NC. B, percentages of CD4+CD25^high T cells coexpressing CD39 and CD73 in the circulation of AML patients and NC. Mean ± SD (A and B). C, ATP hydrolysis by CD4+CD25^+ or CD4+CD25^- cells isolated from a representative AML patient or a NC. Note that CD4+CD25^- T cells from AML hydrolyzed more exogenous ATP than CD4+CD25^- from NC or CD4+CD25^+ T cells. In the presence of an inhibitor of ectonucleotidases, ARL67165, the ability of CD4+CD25^- cells to hydrolyze ATP was reduced. Representative of five experiments done. Asterisks, significant differences between triplicate samples tested at P < 0.001.

Fig. 6. Frequency and suppressor function of Treg in AML patients at diagnosis and after achieving CR. A, frequency of Treg was evaluated in all patients at diagnosis. Patients who achieved CR after induction chemotherapy had lower Treg levels compared with patients that had persistent leukemia. B, frequency of Treg after induction chemotherapy was evaluated in 7 patients who had achieved CR and was found to be significantly higher at the time of CR relative to that determined before treatment. C, compared with NC, the suppressor activity of CD4+CD25^high Treg remained elevated in AML patients who achieved CR. Elevated suppression levels were similar to those determined before treatment levels but were significantly higher relative to suppression mediated by Treg from NC. Mean ± SD. Asterisks, significant differences in suppression mediated by Treg of AML patients or NC at P < 0.001.
References


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