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

Miroslaw J. Szczepanski,[3] Marta Szajnik,[3] Malgorzata Czystowska,[3] Magis Mandapathil,[3] Laura Strauss,[3] Ann Welsh,[1,3] Kenneth A. Foon,[1,3] Theresa L. Whiteside,[2,3] and Michael Boyiadzis[1,3]

Abstract

Purpose: Regulatory $CD4^+CD25^{high}Foxp3^+$ 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.

Numerous studies show that tumors exert suppressive effects on the host immune system and that tumor progression is linked to functional impairments of immune cells (1, 2). Regulatory T cells (Treg) are a functionally heterogeneous subpopulation of T lymphocytes, which play a key role in maintaining tolerance (3). Treg are $CD4^+$ lymphocytes characterized by constitutive expression of high levels of the interleukin (IL)-2 receptor α chain (CD25). A majority of $CD4^+CD25^{high}$ Treg also express a forkhead family transcription factor (Foxp3) up to now considered to be essential for the development and function of Treg (4, 5). However, Foxp3 expression and high levels of Foxp3 mRNA expression have recently been shown in several cell lines derived from non-T-cell lineage cells, in tumor cells, and in activated effector T cells (6–8). Another transcription factor important for Treg function, the nuclear factor of activated T (NFAT) cells, forms a complex with Foxp3 and regulates the transcription of several genes including the IL-2 gene (9, 10). Treg also express CTL-associated antigen-4 (CTLA-4), CD45RO, CD39, CD73, and glucocorticoid-induced tumor necrosis factor (GITR), although the percentages of Treg expressing these markers are variable in patients with cancers (11, 12). Because most of these markers can be induced by activation of conventional $CD4^+$ T cells, they cannot discriminate between Treg and effector T cells (13). To date, no definite biomarker has been found for human Treg, and a "functional" definition remains their most reliable characteristic.

Authors' Affiliations: [1]Division of Hematology-Oncology, Department of Medicine, and [2]Department of Pathology, University of Pittsburgh School of Medicine, and [3]University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Received 11/17/08; revised 2/12/09; accepted 2/14/09; published OnlineFirst 5/5/09.

Grant support: Mario Lemieux Foundation (M. Boyiadzis) and National Cancer Institute grant PO1-CA109688 (T.L. Whiteside).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Current address for M.J. Szczepanski: Departments of Clinical Immunology and Otolaryngology, Poznan University of Medical Sciences, 61-701 Poznan, Poland.

Requests for reprints: Michael Boyiadzis, University of Pittsburgh Cancer Institute, 5150 Center Avenue, Suite 572, UPMC Cancer Pavilion, Pittsburgh, PA 15232. Phone: 412-648-6589; Fax: 412-648-6579; E-mail: boyiadzism@upmc.edu.

doi:10.1158/1078-0432.CCR-08-3010

Translational Relevance

This translational study reports on the phenotypic and functional properties of regulatory T cells (Treg) in patients with acute myelogenous leukemia (AML) at diagnosis and following induction chemotherapy. The data are likely to be important to the future therapy of cancer for two reasons: (*a*) the frequency and function of Treg at AML diagnosis correlate with response to chemotherapy and thus have prognostic significance and (*b*) the finding that AML patients who achieve complete remission following induction chemotherapy have an elevated frequency of Treg mediating high levels of suppressor activity suggests that Treg could contribute to leukemic relapse. Overall, the frequency and levels of Treg activity may be important biomarkers of AML response to therapy and disease progression. Future therapy aimed at Treg depletion could be beneficial for patients with AML.

A growing body of evidence suggests that Treg play an important role in human cancer progression. Treg-mediated immunosuppression has emerged as a crucial mechanism of tumor evasion, which may contribute to the observed lack of responses to immunotherapy in cancer patients (2, 14). Selective elimination of $CD4^+CD25^+$ T cells in mice results in tumor growth inhibition or, in some instances, tumor regression. In contrast, adoptive transfer of $CD4^+CD25^+$ T cells is associated with suppression of antitumor immunity and tumor progression (15–17).

Mechanisms involved in Treg-mediated suppression of antitumor immunity are under investigation and recent studies have identified several distinct mechanisms Treg use for elimination of responder T cells, including activation of perforin-dependent or granzyme B-dependent pathways, production by Treg of IL-10 and transforming growth factor-β1 (TGF-β1), or up-regulation by Treg of indoleamine-2,3-dioxygenase expression in antigen-presenting cells (18, 19). ATP hydrolysis has recently emerged as another Treg-mediated mechanism of suppression. CD39 and CD73 expressed by Treg are enzymes belonging to the ectonucleoside triphosphate diphosphohydrolase family. Both enzymes work in sequence to generate the nucleoside, adenosine, with potent immunosuppressive effects on effector T cells (20, 21).

More recently, we and others have reported that the percentages of Treg are increased in the circulation of patients with solid tumors and hematologic malignancies relative to those in healthy controls (11, 12, 18, 22). Treg frequencies have been determined in these studies based on Foxp3 expression. More recently, the expression of $CD25^{high}$ has been used for definition of the human Treg subset-based clonal and functional analyses (11, 12). The accumulation of Treg and their increased suppressor function in cancer patients with advanced disease suggest that immune suppression is linked to tumor progression. Indeed, the increased proportion of Treg in the peripheral blood or at the tumor site has been reported to correlate with a poor disease outcome (23, 24).

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, CD45RA-FITC, anti-CD45RO-FITC, and 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-FITC, anti-CD39-FITC, and anti-IL-10-PE from eBioscience; anti-CD122-FITC (IL-2βR), anti-CCR7-FITC, anti-CD25-PE, and anti-CD132-PE (IL-2Rγ) from BD Bioscience; 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 nmol/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-FITC, 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 2×10^5 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 μmol/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 μg/mL; American Type Culture Collection) and soluble anti-CD28 mAb (1 μg/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.

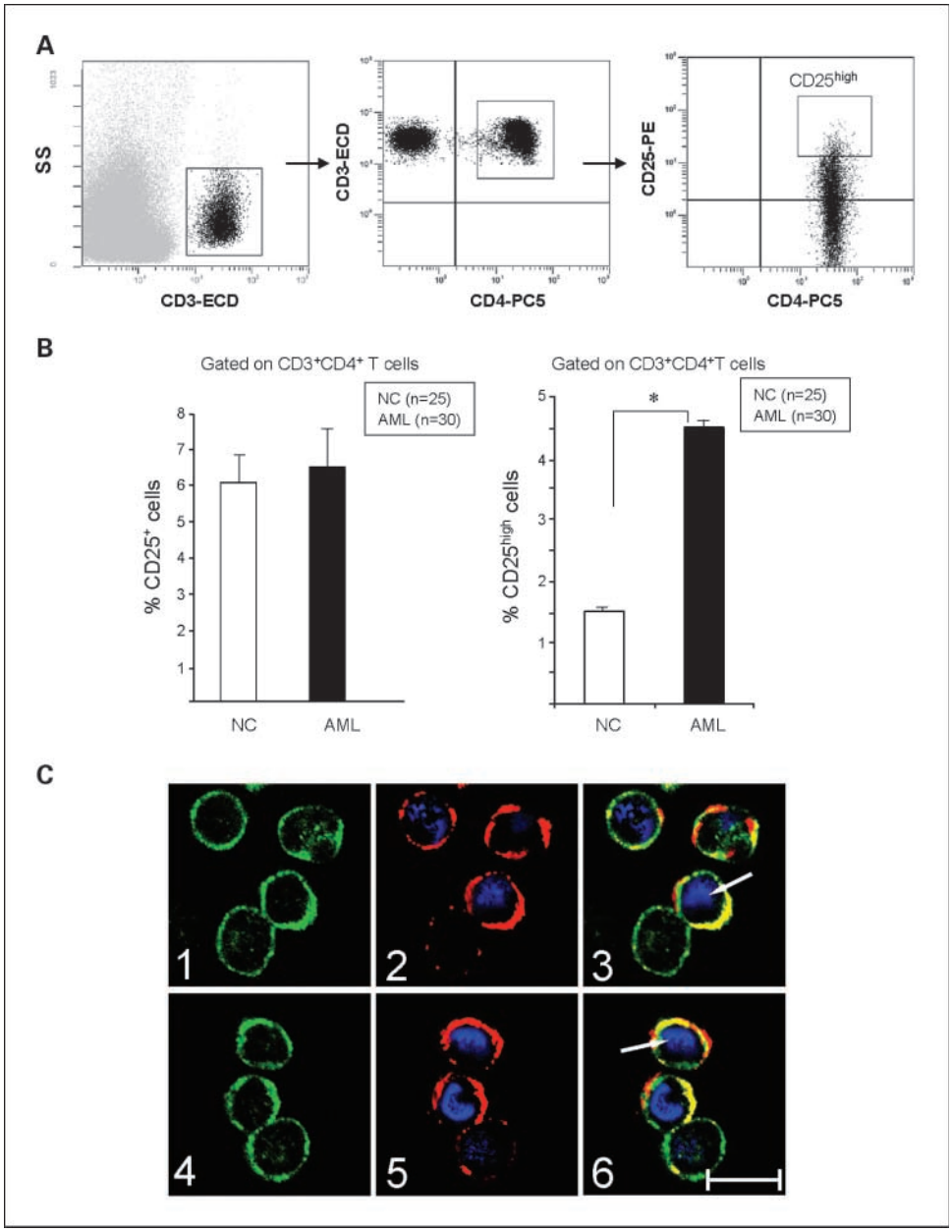

Fig. 1. Increased frequency of $CD4^+CD25^{high}$ T cells in the peripheral blood of patients with AML before treatment. *A,* gating strategy used to identify the $CD4^+CD25^{high}$ T reg. $CD4^+$ T cells with mean fluorescence intensity of CD25 expression ≥120 were classified as $CD25^{high}$ as reported 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 μm.

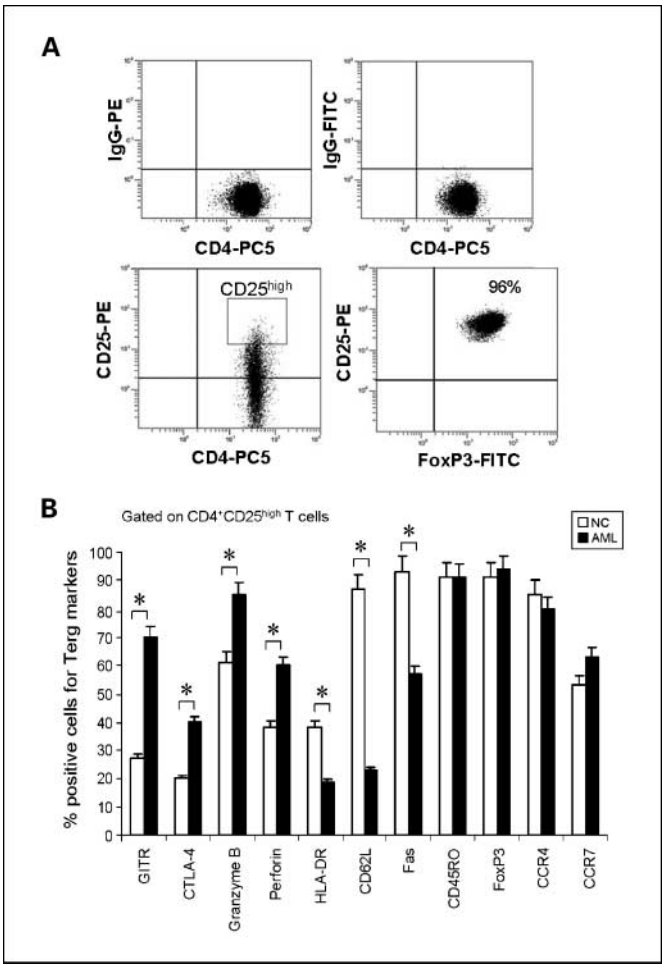

Fig. 2. Phenotypic characteristic of $CD4^+CD25^{high}$ Treg obtained from PBMC of AML patients and NC. *A,* gating strategy used to identify the $CD4^+CD25^{high}$ Treg and coexpression of Foxp3 in $CD4^+CD25^{high}$ cells. *B,* percentage of cells positive for various markers within the $CD4^+CD25^{high}$ T cell subsets in NC and AML patients. *Asterisks,* significant differences between NC and AML patients. *, $P < 0.05$.

Transwell assays. To assess whether cell-to-cell contact was necessary for Treg to mediate suppression, polycarbonate 24-well Transwell inserts (0.4 μmol/L; Corning Costar) were used to separate responder cells from suppressor cells. At least 5×10^4 carboxyfluorescein diacetate succinimidylester-labeled $CD4^+CD25^-$ T cells were stimulated with OKT3 and anti-CD28 mAb (each at 1 μg/mL) in the presence of 150 IU IL-2/mL in lower chambers. The same number of autologous Treg or control cells ($CD4^+CD25^-$) was added to upper chambers at the suppressor to responder cell ratio of 1:1.

ATP hydrolysis. $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T cells were incubated with 10 μmol/L exogenous ATP (Sigma-Aldrich) for 1 and 2 h in wells of flat-bottomed 96-well plates. An ectonucleotidase inhibitor, ARL67156 (Sigma-Aldrich), was added to some cells at the final concentration of 250 μmol/L for 30 min before the addition of exogenous ATP. The concentration of "unhydrolyzed" ATP was determined by measuring the frequency of luminescent events (counts/min) in a luciferase-based detection system (ATP Lite Luminescence ATP Detection Assay System from Perkin-Elmer). The plates were examined in a Microplate Scintillation and Luminescence Counter (Packard). The average counts/min were determined from triplicate wells.

Confocal microscopy. To detect $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+NFAT1^+$ Treg in the peripheral blood of AML patients, PBMC (1×10^4) 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.

Statistical analysis. Data were summarized by descriptive statistics (mean ± SE for continued variables and frequency and percentage for categorical variables). Statistical analyses were done using the paired and unpaired two-tailed Student's *t* tests. $P < 0.05$ was considered to be significant.

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 $CD4^+CD25^{high}$ Treg was higher ($P < 0.0001$) in the AML patients (4.5 ± 0.2%; range, 1.7-8.2%) compared with NC (1.5 ± 0.08%; 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 is NC (1.4 ± 0.1%). The majority of $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ Treg subset. On staining for intracellular and cell surface markers in $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ T cells in AML patients. To evaluate the suppression mediated by $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ T cells isolated from AML patients were cocultured with autologous $CD4^+CD25^-$ responder cells, the mean suppression was significantly higher at 82 ± 8% ($P \leq 0.001$; 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).

$CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ 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.

$CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ cells based on the emerging evidence that Foxp3 is not a selective marker for human Treg (8) and strong suppressive activity of $CD4^+CD25^{high}$ clones (12).

In comparison with NC, the newly diagnosed AML patients had an increased frequency of Treg in the peripheral blood.

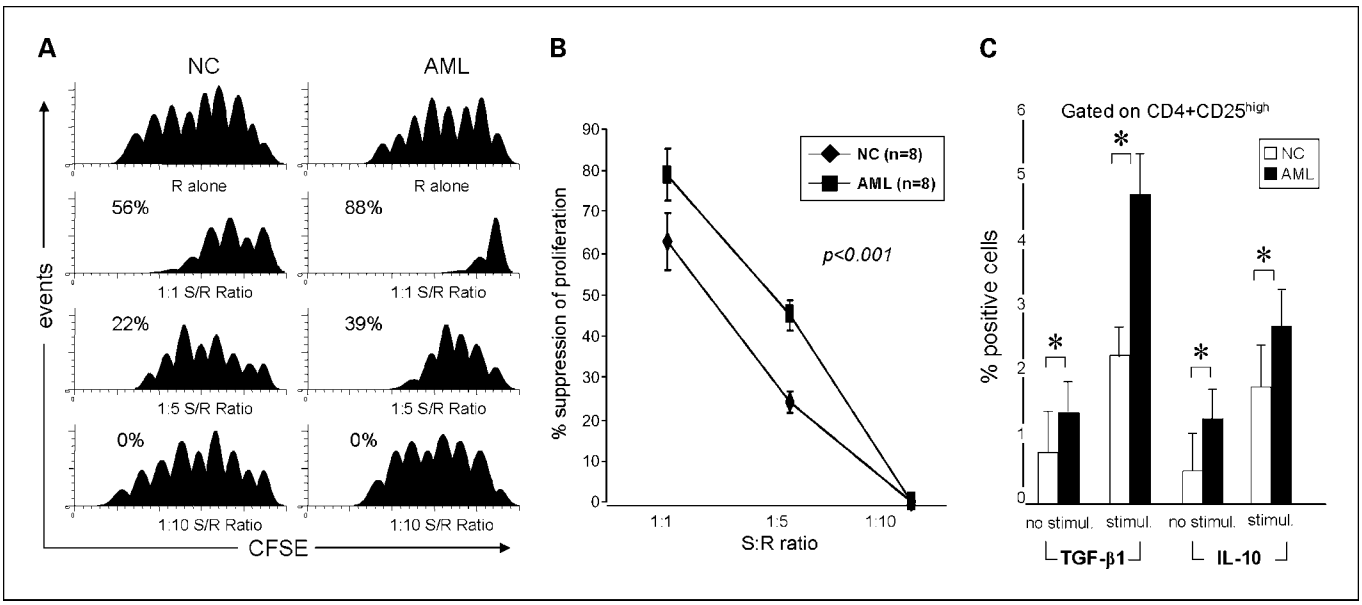

Fig. 3. Suppression of proliferation by $CD4^+CD25^{high}$ Treg. *A,* $CD4^+CD25^-$ responder T cells (R) isolated from PBMC of a representative NC and an AML patient before any treatment. R cells were labeled with carboxyfluorescein diacetate succinimidylester and stimulated with OKT3, anti-CD28 antibody, and IL-2 in the absence of Treg (R alone). The addition of $CD4^+CD25^{high}$ Treg (S) from PBMC of NC or AML patient at different suppressor to responder cell ratios inhibited R cell proliferation, with the highest inhibition observed at the 1:1 suppressor to responder cell ratio. *B,* suppression of proliferation by $CD4^+CD25^{high}$ Treg in the newly diagnosed AML patients was significantly higher compared with NC. *C,* intracellular expression of TGF-β1 and IL-10 in NC and AML patients. Although the levels of expression were low, the expression was significantly different. The intracellular expression of TGF-β1 and IL-10 after stimulation with phorbol 12-myristate 13-acetate and ionomycin was significantly higher in the AML patients compared with NC. *Asterisks,* significant differences between NC and AML patients at $P < 0.001$.

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 $CD4^+$ $CD25^{high}$ 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.

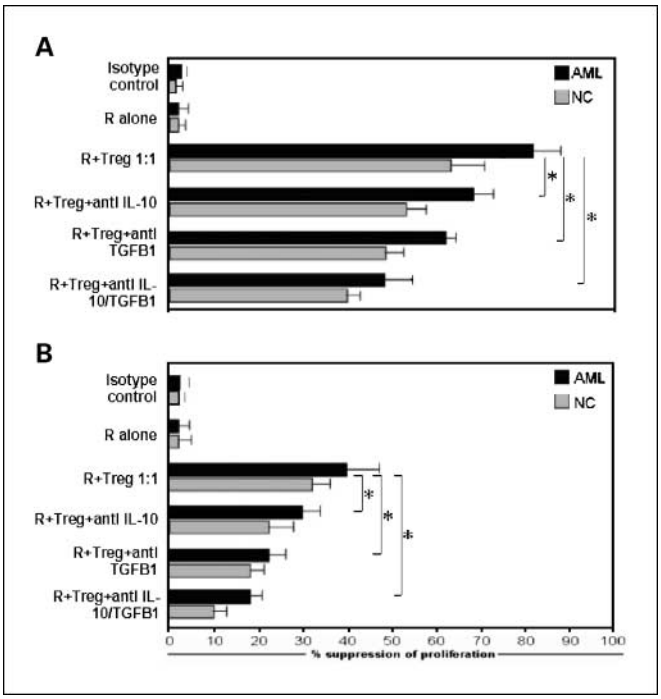

Fig. 4. Suppression mediated by $CD4^+CD25^{high}$ T cells is cell contact and cytokine dependent. $CD4^+CD25^{high}$ T cells (S) from AML patients were coincubated with carboxyfluorescein diacetate succinimidylester-labeled autologous $CD4^+CD25^-$ T cells (R) responding to OKT3, anti-CD28 antibodies, and IL-2. Neutralizing anti-IL-10 and/or anti-TGF-β1 antibodies were added to the assays. *A,* in the absence of Transwell inserts, the addition of anti-IL-10 or anti-TGF-β1 antibody to cocultures resulted in a significant reduction of suppression; the addition of both neutralizing antibodies resulted in an even greater reduction of Treg-mediated suppression ($P < 0.001$). *B,* in the presence of Transwell inserts, suppression was reduced relative to cultures without inserts (as in *A*). Nevertheless, suppression levels remained at 40%, indicating that the cell-to-cell contact was in part necessary. When neutralizing antibodies to IL-10 or TGF-β1 were added to these cocultures, suppression was further reduced with the greatest inhibition of Treg suppression observed when Transwell inserts were combined with neutralizing anti-IL-10 and anti-TGF-β1 antibodies ($P < 0.00001$). *Asterisks,* significant differences between R + Treg and R + Treg + anti-IL-10, R + Treg + anti-TGF-β1, and R + Treg + anti-IL-10 + anti-TGF-β1 ± Transwell inserts.

Not only was the frequency of circulating $CD4^+CD25^{high}$ 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.

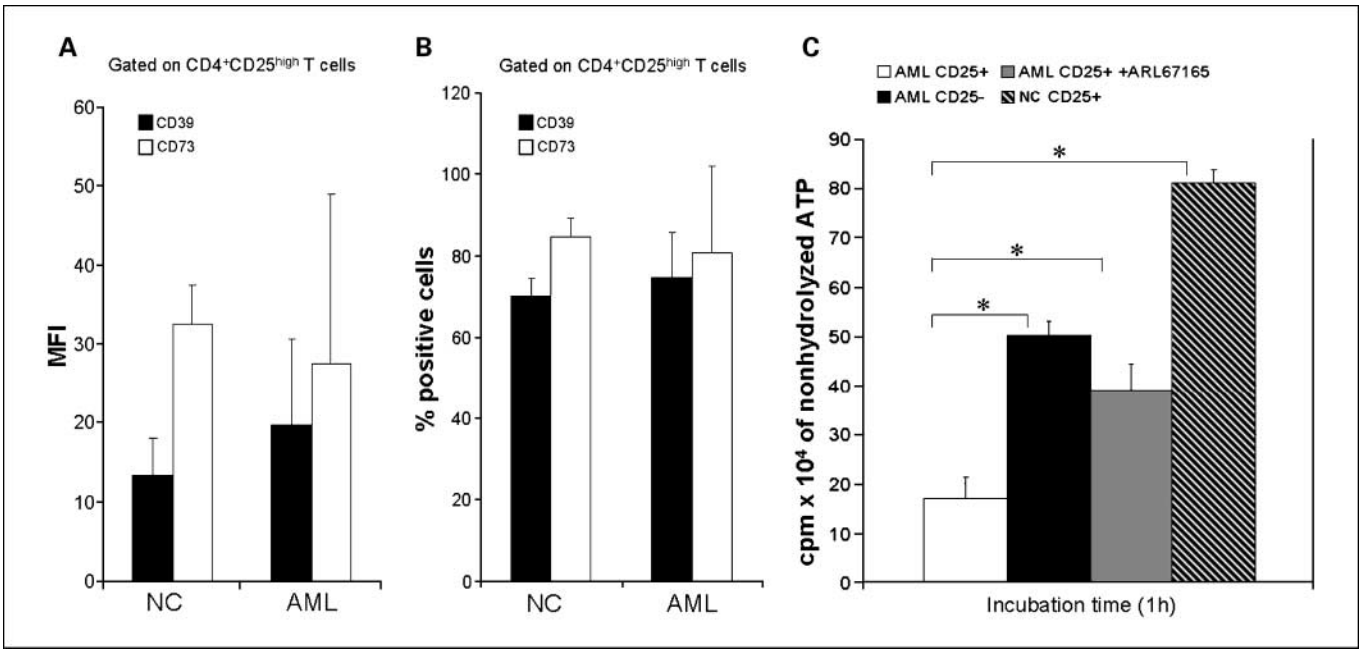

Fig. 5. Constitutive expression of CD39 and CD73 by Treg and adenosine generation. *A,* expression levels of CD39 and CD73 proteins on $CD4^+CD25^{high}$ T cells in AML patients and NC. *B,* percentages of $CD4^+CD25^{high}$ T cells coexpressing CD39 and CD73 in the circulation of AML patients and NC. 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 $P < 0.001$.

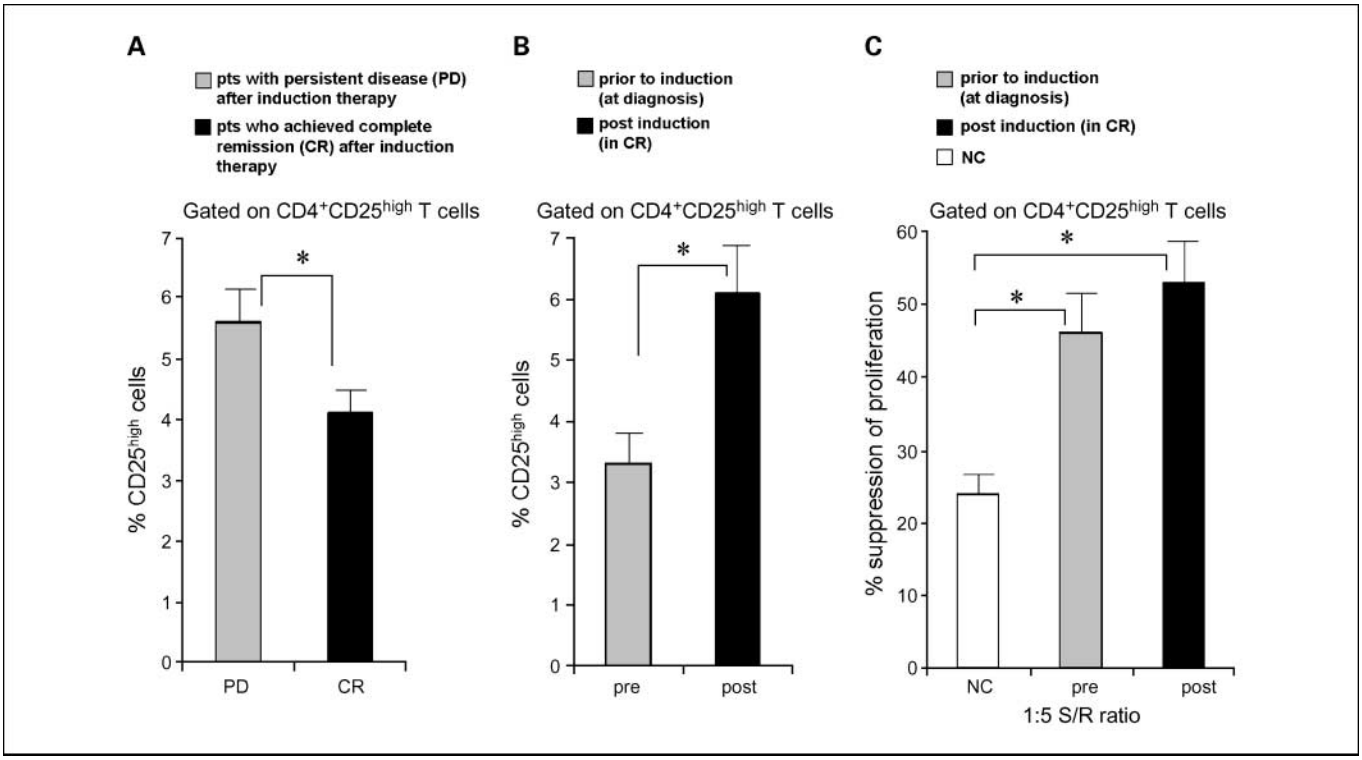

Fig. 6. Frequency and suppressor function of Treg in AML patients at diagnosis and after achieving CR. *A,* frequency of Treg was evaluated in all patients at diagnosis. Patients who achieved CR after induction chemotherapy had lower Treg levels compared with patients that had persistent leukemia. *B,* 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

1. Ferrone S, Whiteside TL. Tumor microenvironment and immune escape. Surg Oncol Clin N Am 2007;16:755–74, viii.
2. Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. Semin Cancer Biol 2006;16:3–15.
3. Sakaguchi S. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531–62.
4. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299: 1057–61.
5. Sakaguchi S. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005;6:345–52.
6. Hinz S, Pagerols-Raluy L, Oberg HH, et al. Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. Cancer Res 2007;67:8344–50.
7. Yamamoto M, Tsuji-Takayama K, Suzuki M, et al. Comprehensive analysis of FOXP3 mRNA expression in leukemia and transformed cell lines. Leuk Res 2008;32:651–8.
8. Ziegler SF. Foxp3: not just for regulatory T cells anymore. Eur J Immunol 2007;37:21–3.
9. Rudensky AY, Gavin M, Zheng Y. FOXP3 and NFAT: partners in tolerance. Cell 2006;126:253–6.
10. Wu Y, Borde M, Heissmeyer V, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell 2006;126:375–87.
11. Strauss L, Bergmann C, Gooding W, Johnson JT, Whiteside TL. The frequency and suppressor function of $CD4^+CD25^{high}Foxp3^+$ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. Clin Cancer Res 2007;13:6301–11.
12. Strauss L, Bergmann C, Whiteside TL. Functional and phenotypic characteristics of $CD4^+CD25^{high}Foxp3^+$ Treg clones obtained from peripheral blood of patients with cancer. Int J Cancer 2007;121:2473–83.
13. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood 2008;112:1557–69.
14. Gattinoni L, Powell DJ, Jr., Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 2006;6:383–93.
15. Antony PA, Piccirillo CA, Akpinarli A, et al. $CD8^+$ T cell immunity against a tumor/self-antigen is augmented by $CD4^+$ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 2005;174:2591–601.
16. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. Cancer Res 1999;59: 3128–33.
17. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 1999;163:5211–8.
18. Beyer M, Schultze JL. Regulatory T cells in cancer. Blood 2006;108:804–11.
19. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006;6: 295–307.
20. Borsellino G, Kleinewietfeld M, Di Mitri D, et al. Expression of ectonucleotidase CD39 by $Foxp3^+$ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110:1225–32.
21. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 2007;204:1257–65.
22. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 2001;61: 4766–72.
23. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942–9.
24. Griffiths RW, Elkord E, Gilham DE, et al. Frequency of regulatory T cells in renal cell carcinoma patients and investigation of correlation with survival. Cancer Immunol Immunother 2007;56: 1743–53.
25. Fauriat C, Just-Landi S, Mallet F, et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCR dull phenotype induction. Blood 2007;109: 323–30.
26. Fauriat C, Moretta A, Olive D, Costello RT. Defective killing of dendritic cells by autologous natural killer cells from acute myeloid leukemia patients. Blood 2005;106:2186–8.
27. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human $CD4^+CD25^+Foxp3^+$ regulatory T cells cultured with rapamycin. J Immunol 2007;178:320–9.
28. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood 2000;96:4075–83.
29. Wang X, Zheng J, Liu J, et al. Increased population of CD4(+)CD25(high), regulatory T cells with their higher apoptotic and proliferating status in peripheral blood of acute myeloid leukemia patients. Eur J Haematol 2005;75:468–76.
30. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 2002;3:135–42.
31. Levings MK, Sangregorio R, Sartirana C, et al. Human $CD25^+CD4^+$ T suppressor cell clones produce transforming growth factor β, but not interleukin 10, and are distinct from type 1 T regulatory cells. J Exp Med 2002;196:1335–46.
32. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996;271:1734–6.
33. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003;100:8372–7.