Reducing TNF Receptor 2\(^+\) Regulatory T Cells via the Combined Action of Azacitidine and the HDAC Inhibitor, Panobinostat for Clinical Benefit in Acute Myeloid Leukemia Patients

Chindu Govindaraj\(^1\), Peter Tan\(^2\), Patricia Walker\(^2\), Andrew Wei\(^2\), Andrew Spencer\(^2\), and Magdalena Plebanski\(^1\)

Abstract

**Purpose:** Acute myeloid leukemia (AML) provides an environment that enables immune suppression, resulting in functionally defective effector T cells; regulatory T cells (Treg) are significant contributors to the impaired antitumor immune response. As TNF is present at high levels in AML and TNF receptor-2 (TNFR2)–expressing Tregs identify highly functional Tregs, we examine the hypothesis that TNFR2\(^+\) Tregs are a relevant Treg subset in this cancer. We also determine the effect of the novel combinatorial therapy of the demethylating agent, azacitidine with the histone deacetylase inhibitor, panobinostat on Tregs, particularly TNFR2\(^+\) Tregs.

**Experimental Design:** Thirty healthy donors and 14 patients with AML were enrolled in this study. Patients were treated with azacitidine and panobinostat for 28-day cycles. The frequency and functional relevance of TNFR2\(^+\) Tregs were analyzed subsequently.

**Results:** We report that TNFR2\(^+\) Tregs are increased in AML and have a high migration potential toward the bone marrow. Furthermore, we demonstrate that the level of TNFR2\(^+\) Tregs in the peripheral blood and the bone marrow of patients are decreased in vivo after exposure to panobinostat and azacitidine. Reductions in TNFR2\(^+\) Tregs were associated with increases in Interferon (IFN)-\(\gamma\) and interleukin (IL)-2 production by effector T cells within the bone marrow and beneficial clinical responses. In vitro mechanistic studies indicated panobinostat as the primary driver for the reduction of Tregs.

**Conclusions:** Our study provides for the first time, in vivo validation of the ability of panobinostat in combination with azacitidine to suppress prevalent TNFR2\(^+\) Tregs, resulting in clinical benefits within patients with AML. *Clin Cancer Res*; 20(3); 724–35. ©2013 AACR.

Introduction

Acute myeloid leukemia (AML) represents a molecularly diverse group of malignant hematopoietic cancers resulting in the accumulation of marrow blasts with arrested differentiation. Treatment strategies for AML include intensive chemotherapy, which in most cases is the only option for long-term survival; however, approximately 15% of elderly patients die due to treatment complications and only 50% achieve remission (1). Despite considerable research, AML therapy has remained static for almost four decades, with poor outcomes in the elderly (2). The importance of T cells for antileukemic function has been demonstrated in models of graft-versus-leukemia after allogeneic stem cell transplantation, whereby depletion of T cells abrogates this beneficial effect (3). T cells produce cytokines like IFN-\(\gamma\) and interleukin (IL)-2, which play a crucial role in antileukemic immunity (4, 5). However, T cells within patients with AML are found to be functionally defective as they are impaired numerically, phenotypically, and genetically (6). It has previously been demonstrated that these defective T cells may partly be due to the suppressive function of regulatory T cells (Tregs; ref. 7). Indeed, patients with AML have abnormally high levels of Tregs within their peripheral blood and bone marrow compared with healthy donors (8). The presence of high Treg levels in patients with AML correlate with poor clinical outcomes (9). This increase of Tregs has been attributed to AML blasts, which increase the frequency...
Translational Relevance

Epigenetic therapies have proven to be effective when used in combination for the treatment of acute myeloid leukemia (AML). We sought to determine whether the clinical benefit of the novel combination of epigenetic drugs, azacitidine and panobinostat, was associated with a reduction of regulatory T cells (Treg), which are key players in dampening effective antitumor immune responses. We demonstrate a marked and rapid effect of the combination therapy to selectively target and reduce Tregs, particularly the functional TNF receptor-2 (TNFR2+) Tregs in AML. This reduction of TNFR2+ Tregs is correlated with clinical responses in a population of patients unfit for intensive chemotherapy. In addition to providing new insights into novel and relevant immunologic parameters that can be targeted therapeutically, these findings provide for the first time, proof-of-concept in vivo validation of the ability of epigenetic therapies to suppress Tregs in AML.

Patients, Materials and Methods

Patients

Patients diagnosed with previously untreated myelodysplastic syndrome [MDS; International Prognosis Scoring System (IPSS; ref. 35): intermediate-2 or high risk] or AML (≥20% blasts), who were deemed ineligible for intensive chemotherapy, were enrolled in a phase Ib/II clinical trial investigating epigenetic therapy with the hypomethylating agent, azacitidine, and the HDACi, panobinostat. Patients received 28-day cycles of azacitidine 75 mg/m² daily by subcutaneous injection (days 1–5) and oral doses of panobinostat (10–30 mg), three times a week (Mon/Wed/Fri) starting on day 5. Further cycles of therapy were continued until disease progression, unacceptable toxicity, or patient choice to cease therapy. Responders were defined as patients who achieved complete remission (CR) or partial remission (PR) as per Cheson criteria for patients with AML or International Working Group (IWG) criteria for patients with MDS (36, 37). Patients who failed to achieve CR or PR were classified as nonresponders.

Ethical statement

The protocol was approved by the Alfred Hospital Research and Ethics Unit and patient consent was obtained. The study was registered with Australian and New Zealand Clinical Trials Registry (ANZCTR): study number, ACTRN12610000924055. For healthy blood samples, buffy coats were obtained from the Australian Red Cross Blood Bank Service.

Isolating mononuclear cells

Mononuclear cells were obtained from peripheral blood and bone marrow samples of patients with AML via...
Ficoll density gradient separation (Amersham Pharmacia Biotech). The isolated cells from both peripheral blood and bone marrow samples were frozen in a freeze mixture [10% DMSO (dimethyl sulfoxide; Sigma-Aldrich) and 90% fetal calf serum (JRH Bioscience)], stored in freezing containers (Nalgene) and transferred to vapor phase nitrogen until use. For use, cells were thawed in a 37°C water-bath and quickly resuspended using AIM-V (Invitrogen) with 5% human serum (Sigma).

Flow cytometry method and analysis

To determine the frequency, phenotype, migration, and regulatory capacities of TNFR2+ Tregs in healthy and patient volunteers, multicolor fluorescence-activated cell sorting analysis was performed using the following surface antibodies (BD Pharmingen): anti-CD3, anti-CD4, anti-CD8, anti-TNFFR2, anti-CD25, anti-CTLA4, anti-CD39, anti-CD73, and anti-CXCR4. For all samples, cells were also stained with a fixable dead cell stain (Invitrogen). Live leukocytes were identified with anti-CD45 (eBioscience) before analysis of Tregs levels. Intracellular levels of FOXP3 and Ki67 were determined by first using a fixation/permeabilization buffer kit (eBioscience) followed by staining with anti-FOX3 (eBioscience) and anti-Ki67 (BD Pharmingen). Flow cytometry data were acquired on a Becton Dickinson LSR II using Diva software, collecting a minimum of 150,000 events per sample. Isotype-matched antibodies were used as controls with all samples. Data were analyzed using FlowJo software (TreeStar).

Migration assays

CD4 T cells were isolated from healthy peripheral blood mononuclear cells (PBMC) by using the INFLUX flow cytometry-based cell sorter. Migration assays were performed as previously described (21). In brief, 2.5 × 10^5 cells in 100 µL of AIM-V media were added to the top chamber, whereas 600 µL of media or cell-free AML bone marrow fluid was added to the lower chamber of 5.0 µm pore size inserts in a 24-well plate (Corning). Cells were incubated at 37°C for 2 hours. Phenotypic analysis of CD45, CD25, and TNFR2 expression on migrated cells was carried out using flow cytometry and the percentages of TNFR2+ and TNFR2+ within the CD25hi T-cell population were determined. Migration indices were calculated by determining the ratio of the percentages of cells that migrated in response to cell-free bone marrow fluid when compared with media alone.

Intracellular cytokine analysis

Intracellular cytokines were analyzed by culturing cells at a ratio of 10^5 cells/50 µL with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (1 µg/mL) for 5 hours. Brefeldin A (BD Pharmingen) was added for the last 4 hours of the incubation. The cells were washed and the levels of intracellular cytokines were determined by flow cytometry. The cells were fixed using a fixation/permeabilization buffer kit before staining for the following cytokines: anti-IFN-γ (BD Pharmingen), anti-IL-2 (eBioscience), anti-IL-10 (eBioscience), and anti-TGF-β (R&D Systems).

CXCL12 detection

ProcartaPlex Immunoassay Kit-Magnetic beads were used to determine the level of CXCL12 present in the bone marrow fluid and peripheral blood serum of patients with AML. Quantification of CXCL12 was performed according to the manufacturer’s instructions using a magnetic plate holder (Affymetrix). Samples were acquired on a luminex instrument, collecting a minimum of 100 events. Results were analyzed using the luminex instrument.

In vitro drug experiments

To determine the direct effect of azacitidine and panobinostat on Tregs, in vitro induction assays were performed. Azacitidine and panobinostat were kindly provided by Celgene and Novartis, respectively. Azacitidine was reconstituted with saline, whereas panobinostat was reconstituted with DMSO at a 10 mmol/L concentration and aliquoted into smaller volumes to avoid multiple freeze-thaw cycles. The drugs were diluted to the indicated concentrations using AIM-V medium with 5% human serum. Healthy PBMCs were cultured at 10^5/50 µL of AIM-V medium with 5% human serum. These cells were TCR stimulated with plate-bound anti-CD3 together with soluble anti-CD28 in 96-well round bottom plates for 72 hours. Panobinostat was added either alone or in combination with azacitidine at the indicated ratios on day 0. Control wells had no drugs added to them. On day 3, cells were washed and flow cytometry was performed to determine the proportion of induced TNFR2+ Tregs within the PBMCs of healthy donors.

Statistical analysis

Statistical significance was determined by performing unpaired t tests between healthy and AML samples. Paired t tests were performed when comparing AML samples before and after treatment with azacitidine and panobinostat. P < 0.05 was considered to be significant. Statistical analyses were performed using GraphPad Prism.

Results

Elevated Treg levels are lowered following combined therapy of azacitidine and panobinostat in the peripheral blood of AML patients

To investigate the levels of T cells and Tregs, we performed flow cytometry on PBMCs isolated from healthy and AML donors. Patient samples were obtained at screening, at end of cycle 1 (EOC1) and at the end of cycle 3 (EOC3) of treatment. To identify T cells, we first gated leukocytes based on CD45 expression, followed by CD3 expression. The proportions of CD4 T cells within T cells were compared between healthy donors and patients, at screening (Fig. 1A). Patients had a lower percentage of CD4 T cells (P = 0.05) compared with healthy donors. After treatment, CD4 T-cell proportions were significantly enhanced at EOC3 when compared with screening, exceeding the normal range.
observed in healthy donors (Fig. 1B). The absolute number of CD4 T cells was also significantly higher ($P < 0.05$) at EOC3, when compared with screening (Supplementary Fig. S1). To investigate the proportion of Tregs within CD4 T cells, CD25hi T cells were gated on FOXP3 expression to identify Tregs. CD127 expression within the CD25hi T cells was also observed to be at low levels, confirming the identity of Tregs. The proportion of Tregs (CD25hiFOXP3$^+$) was compared between healthy donors ($n = 30$) and patients with AML ($n = 14$). D, Treg percentages within patients with AML were compared between Scr ($n = 14$) and EOC1 ($n = 12$), or Scr and EOC3 ($n = 8$) of treatment. Statistical analyses were performed by unpaired $t$ test (A and C) and paired $t$ tests (B and D). Data represent mean ± SEM. The healthy mean is represented by a straight line for (B) and (D). *, $P < 0.05$; ***, $P < 0.001$.

**Figure 1.** Comparison of CD4 T cell and Treg proportions in healthy donors and patients with AML. CD4$^+$ T cells were identified by initially gating cells positive for CD3 expression followed by CD4 expression. The proportion of CD4 T cells was compared between healthy donors ($n = 30$) and AML (at screening) patients ($n = 14$; A). CD4 T-cell proportions within patients with AML were compared at screening (Scr; $n = 14$), EOC1 ($n = 12$), and EOC3 ($n = 8$) of treatment (B). Tregs were identified by high expression levels of CD25 followed by FOXP3$^+$ expression. CD127 levels were also determined on CD25hi T cells to further confirm the Treg identity. C, the percentage CD25hiFOXP3$^+$ T cells were compared between healthy donors ($n = 30$) and patients with AML ($n = 14$). D, Treg percentages within patients with AML were compared between Scr ($n = 14$) and EOC1 ($n = 12$), or Scr and EOC3 ($n = 8$) of treatment. Statistical analyses were performed by unpaired $t$ test (A and C) and paired $t$ tests (B and D). Data represent mean ± SEM. The healthy mean is represented by a straight line for (B) and (D). *, $P < 0.05$; ***, $P < 0.001$.
To further test the potential relevance of TNFR2⁺ Tregs in patients, we determined the migratory potential of these Tregs to the bone marrow by first assessing CXCR4 expression levels. As shown in Fig. 2D, TNFR2⁺ Tregs had significantly higher levels (6.8-fold higher) of CXCR4 compared with the TNFR2⁻ fraction. Overall, a positive correlation ($P < 0.0001$) was observed between TNFR2 and CXCR4 expression levels on Tregs (Supplementary Fig. S3). In addition, we performed migration assays to determine whether TNFR2⁻ Tregs were capable of migrating toward the bone marrow fluid of patients with AML. We observed that the bone marrow fluid was able to attract an increased number of CD25⁺ T cells when compared with media alone ($P = 0.06$; data not shown). Furthermore, within the CD25⁺ T-cell subset, a significantly higher number of TNFR2⁺ Tregs migrated toward the AML bone marrow fluid when compared with the TNFR2⁻ subset ($P < 0.0001$; Fig. 2D).
Azacitidine and panobinostat therapy selectively lowers TNFR2⁺ but not TNFR2⁻ Tregs in the peripheral blood of AML patients

To determine whether TNFR2⁺ Tregs were altered upon treatment, flow cytometry was performed to the EOC1 and EOC3 of treatment. We observed a significant reduction in TNFR2 expression levels within Tregs as well as the percentage of total TNFR2⁺ Tregs at EOC1 when compared with screening, and these levels remained low at EOC3 (Fig. 3A and B). Unexpectedly, this reduction was confined only to the TNFR2⁺ Treg subset, as there was no change observed in the levels of TNFR2⁻ Tregs upon treatment. Furthermore, to determine any potential functional changes upon treatment, we analyzed the expression levels of FOXP3 within TNFR2⁻CD25hi and TNFR2⁺CD25hi T cells, as FOXP3 is currently the most important marker for assessing Tregs and their suppressive capacity within human tumors (39). FOXP3 levels were significantly reduced on treatment within the TNFR2⁺CD25hi T-cell subset (Fig. 3C), whereas no change was observed within the TNFR2⁻CD25hi T-cell subset (data not shown). Similarly, CTLA4 levels within the TNFR2⁺CD25hi T-cell subset were also reduced at EOC1 of treatment when compared with screening levels (Fig. 3C). These results demonstrate that treatment reduces the proportion as well as potentially alters the function of TNFR2⁺ Tregs within patients with AML. As several recent articles have demonstrated the existence of a FOXP3⁺CD25low T-cell subset (40), our results demonstrate that TNFR2⁺FOXP3⁺ T cells have high expression levels of CD25 compared with the TNFR2⁻ subset (Supplementary Fig. S4). In addition, we demonstrate that the levels of TNFR2⁺FOXP3⁺ T cells are higher in the peripheral blood of patients as compared with healthy donors and that these levels are reduced on treatment (Supplementary Fig. S4).

Response to epigenetic-targeted therapy is associated with a significant reduction in TNFR2⁻ Tregs within the bone marrow

As AML originates within the bone marrow, it is important to determine whether the results obtained in the peripheral blood were comparable with that of the initiating tumor site. A previous study has demonstrated that Tregs are present at elevated levels within the bone marrow of patients with AML (9). Higher migration toward the AML bone marrow fluid by TNFR2⁺ Tregs when compared with TNFR2⁻ Tregs suggested that these cells would preferentially accumulate at high levels in the bone marrow. To determine the levels of TNFR2⁺ Tregs in the bone marrow of patients, flow cytometry was performed on samples obtained at screening and at EOC1 of treatment. TNFR2⁺ Tregs were found to be the dominant Treg subset (16-fold higher; P < 0.01) when compared with TNFR2⁻ Tregs in the bone marrow at screening (data not shown). These bone marrow TNFR2⁺ Treg proportions (mean; SEM = 4.5%; 1.3) were similar to that found in the peripheral blood (mean; SEM = 5.06%/1.1) of patients. There was also a trend for reduced TNFR2⁺ Treg proportions in the bone marrow at EOC1 when compared with screening (P = 0.07; Fig. 4A). When patients were categorized into clinical responders and nonresponders at EOC1 based on hematologic reductions in blast cell counts (36, 37), a significant difference in TNFR2⁺ Treg proportions was observed. As shown in Fig. 4B, while both responders and nonresponders had a reduction in TNFR2⁺ Tregs in the peripheral blood, only clinical responders to treatment had a significant reduction (P < 0.05) in TNFR2⁺ Tregs in their bone marrow when compared with nonresponders. In addition, we observed no significant changes in the absolute cell counts of TNFR2⁺ Tregs in the peripheral blood at EOC1 between clinical responders and nonresponders (data not shown). Although it would have been informative to
perform absolute cell counts within the bone marrow, this was not performed at the time of sample collection and hence is not available. Within the bone marrow samples, the responder cohort consisted of 4 patients (2 MDS and 2 AML) at CR, while the nonresponder cohort consisted of 3 patients with AML. Within the peripheral blood samples, the responder cohort consisted of 5 patients (3 MDS and 2 AML) at CR, 3 patients (1 MDS and 2 AML) at PR, while the nonresponder cohort had 4 patients (2 MDS and 2 AML). We also analyzed for any potential differences between patients with MDS and AML as well as between patients at PR and CR, and found no significant differences in the proportion of TNFR2\(^+\) Tregs (data not shown). Patient characterization into clinical responders and nonresponders were performed at EOC1 due to the higher patient numbers compared with EOC3.

Azacitidine and panobinostat treatment increases the low level of Th1 cytokines produced by CD4 effector T cells isolated from the bone marrow

We determined whether azacitidine and panobinostat had an effect on CD4 effector T-cell function within patients. CD4 effector T cells capable of IFN-\(\gamma\) and IL-2 production were found to be predominantly FOXP3\(^-\) T cells. Figure 5A shows the gating strategy to identify intra-cellular IFN-\(\gamma\) and IL-2 production by CD4 effector T cells. Generally, CD4 T cells from the bone marrow of patients produced relatively low levels of both IFN-\(\gamma\) and IL-2 (Fig. 5A). We also determined the effect of treatment on intra-cellular cytokine production on CD4 T cells from the peripheral blood of patients with AML and disease correlation. A, the proportion of TNFR2\(^+\) Tregs within CD4 T cells from the peripheral blood and bone marrow of patients with AML were compared at Scr and EOC1 of treatment. Patients who responded to treatment are represented by tinted circles while nonresponder patients are represented as clear circles. B, the difference in the fold change of TNFR2\(^+\) Treg proportions at EOC1 when compared with Scr between responders and nonresponder patients, within the peripheral blood and bone marrow. The TNFR2\(^+\) Treg proportion at Scr is indicated by a straight line. Peripheral blood samples were analyzed between 5 responders and 5 nonresponders, whereas bone marrow samples were analyzed between 4 responders and 3 nonresponders. Statistical analyses were performed by paired t-tests (A) and unpaired t tests (B). Data represent mean \(\pm\) SEM. * \(P < 0.05\).
upon analyzing cytokine production, we observed no differences in IFN-γ or IL-2 production by TNFR2−/CD40− effector T cells within the peripheral blood samples between responders and nonresponders, whereas within the bone marrow, it was the TNFR2− effector T cells and not the TNFR2+ subset within the responder patients that produced significantly higher levels of IFN-γ compared with the nonresponder patients (data not shown).

**Panobinostat but not azacitidine lowers the frequency of TNFR2+ Tregs in vitro**

Our data, thus far, demonstrate that Tregs in the peripheral blood of patients with AML are decreased upon treatment with azacitidine and panobinostat and that this decrease is driven by a reduction in the TNFR2+ Treg subset. Moreover, a proportion of patients had this decrease within their bone marrow. To determine whether the decrease in TNFR2+ Treg percentages is due to the changes in TNF levels, we assessed TNF levels within the serum of the peripheral blood and bone marrow of patients. TNF levels were below detectable level for most patients at Scr as well as EOC1, and hence could not be further assessed (<6 pg/mL).

To determine whether an altered migration into the bone marrow from the peripheral blood could account for the decrease in TNFR2+ Tregs within the bone marrow of patients who responded to treatment, we first assessed any potential differences in CXCR4 expression levels on TNFR2+ Tregs. There was no significant difference observed within the CXCR4 levels on TNFR2+ Tregs at EOC1 when compared with Scr between the two patient cohorts (Fig. 6A). In addition, we assessed CXCL12 levels in the peripheral blood serum and bone marrow fluid. CXCL12 levels at EOC1, when compared with screening, were significantly higher in responder patients when compared with the nonresponder patients (Fig. 6B). This increase was seen within the peripheral blood serum as well as the bone marrow fluid of these patients. However, as the level of CXCL12 was similar in the peripheral blood serum and the bone marrow fluid within the responder patients, this suggests that the decrease in TNFR2+ Tregs may not be due to selective migration of these cells from the bone marrow to the peripheral blood. To further elucidate other potential mechanisms that can result in the reduction of TNFR2+ Tregs, we assessed whether the treatment drugs, azacitidine...
and panobinostat, can interfere with the induction of these cells in vitro. However, it has previously been demonstrated that an increase in Treg frequency was observed in vitro and also in vivo in patients with high-risk MDS when treated with azacitidine (32). To test whether azacitidine or panobinostat, or the combination was responsible for the Treg reductions, we performed in vitro functional assays. Azacitidine has been shown to be bioactive at 1 μmol/L in vitro (32) and a range of doses were tested for panobinostat (alone or with azacitidine), since there are currently no studies that have tested the effect of panobinostat on T cells in vitro. We observed a dose-dependent decrease in the median fluorescent intensity (MFI) levels of CD25 expression on CD4 T cells with increasing doses of panobinostat, both with and without azacitidine (Fig. 6C). This effect was also reflected in FOXP3 and TNFR2 expression by CD4 T cells. Hence, the induction of CD25^hi^FOXP3^+^TNFR2^+^ cells was decreased dose-dependently with increasing doses of panobinostat (Fig. 6D). However, consistent with a previous study (32), we also observed a significant increase in FOXP3 MFI within

Figure 6. The levels of CXCL12 within the bone marrow fluid and peripheral blood serum of patients with AML and the effect of azacitidine and panobinostat treatment on TNFR2^+^ Tregs in vitro. A, the difference in the levels of CXCR4 MFI on peripheral blood TNFR2^+^ Tregs between responder and nonresponder patient cohorts at EOC1 when compared with screening. B, the difference in CXCL12 levels within the peripheral blood serum and bone marrow fluid between responder (n = 2) and nonresponder patient cohorts (n = 7) at EOC1 when compared with screening. C, PBMCs were stimulated with anti-CD3/28 and cultured at a ratio of 10^5^ cells/50 μL. Cells were treated with 0 or 1 μmol/L azacitidine and with varying doses of panobinostat (as indicated), and cultured for 3 days (n = 8). On day 3, cells were stained with CD3, CD4, CD25, TNFR2, and intracellular FOXP3 and flow cytometry was performed. The expression levels of CD25, TNFR2, and intracellular FOXP3 within CD4 T cells were compared between the drug-treated samples (n = 8). D, the percentage of TNFR2^+^ Tregs upon varying doses of panobinostat in the presence or absence of azacitidine (n = 8). Statistical analyses were performed by paired t test. Data represent mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
the CD4 T cells upon azacitidine treatment alone when compared with no treatment (Fig. 6C). Our findings thus indicate that panobinostat can directly reduce the expression of Treg-associated markers induced by TCR activation including TNFR2 in vitro, whereas adenosine augments these markers. However, when used in combination, the effect of panobinostat was dominant. These in vitro findings provide a likely mechanism by which a major impairment in the induction of CD25, FOXP3, and TNFR2 expression on T cells would drive decreases in TNFR2+ Tregs upon combined azacitidine and panobinostat treatment in vivo. We also tested whether panobinostat can reduce upregulation of TNFR2, FOXP3, and CD25 on CD4 T cells when stimulated in the presence of TNF. As demonstrated by Supplementary Fig. S6, there was a significant reduction in the upregulation of these markers on CD4 T cells when stimulated in the presence of TNF and IL-2, suggesting that panobinostat can interfere with the induction of these cells even in the presence of TNF.

Discussion

The interplay between Tregs and AML blasts has been well documented. To enhance antitumor immunity, it is crucial to identify and target the most active Treg population within patients with AML. Patients with AML had significantly higher levels of TNFR2+ Tregs within their peripheral blood and bone marrow when compared with healthy donors. Tregs expressing TNFR2 are potent suppressor cells, as this Treg fraction expressed higher levels of the immunosuppressive factors, CTLA4, CD39, CD73, IL-10, and TGF-β than TNFR2- Tregs in both healthy and patient donors. CTLA4 blockade improves T-cell responses toward AML blasts in vitro and also enhances patient survival with other cancers such as melanoma (41, 42). CD39 and CD73 ectoenzymes generate adenosine from extracellular nucleotides like ATP (43). This adenosine can dampen T-cell proliferation and the production of cytokines including IFN-γ and IL-2 via the A2A receptor present on activated effector T cells (38). In addition, IL-10 and TGF-β are implicated in the downregulation of antitumor immunity (44). As TNFR2+ Tregs contain high levels of various suppressive factors, by reducing the frequency of these Tregs, we may potentially disrupt a range of regulatory circuits that ensures dampening of the immune response.

TNFR2+ Tregs within the peripheral blood of both healthy and AML donors were observed to have high CXCR4 expression levels, and TNFR2 expression was positively correlated with CXCR4 levels on Tregs. This suggests that in addition to TNF, CXCR4/CXCL12 interaction may play a role in the accumulation of TNFR2+ Tregs in patients with AML. Furthermore, it has been demonstrated that TNF can induce CXCR4 expression in a range of cells including ovarian cancer cells and human neuronal cells (45, 46), implying TNF may also play a role in the induction of CXCR4 expression on TNFR2+ Tregs. Our data along with these studies suggest that a complex network involving TNF/TNF2 and CXCL12/CXCR4 interactions exists, enabling both AML cells as well as TNFR2+ Tregs to flourish by creating an immunosuppressive niche within patients with AML. This is intriguing, as CXCR4 expression has previously been associated with disease progression in patients with AML (47).

Our results demonstrate that although there was a decrease in TNFR2+ Treg percentages within the peripheral blood of both the responder and nonresponder patients on treatment, a reduction of TNFR2+ Tregs was observed only in the bone marrow of the responder patients. This suggests that for patients to clinically respond, it may be essential to reduce Treg proportions within the bone marrow as well as peripheral blood. Consequently, we observed increases in both IFN-γ and IL-2 production by CD4 effector T cells isolated from the bone marrow within 1 month of treatment of responders. We believe that this increase in Th1 cytokines from patients who responded to treatment was a corollary effect of TNFR2+ Treg reduction within the bone marrow and not a direct effect of the treatment, as both azacitidine and panobinostat failed to significantly enhance cytokine production in vitro (Supplementary Fig. S6). Although the precise mechanisms that result in a reduction of TNFR2+ Tregs within the bone marrow of responder patients and the peripheral blood of all patients is currently unclear, we believe this may be due to several factors. First, we observed that the responder patients had significantly higher levels of CXCL12 levels in both the bone marrow fluid and peripheral blood serum at EOC1, compared with nonresponder patients. Although, as previously mentioned, CXCL12 plays a key role in AML pathogenesis and recruiting TNFR2+ Tregs into the bone marrow, a recent study demonstrates that once the AML bone marrow survival niche is disrupted, CXCL12 may actually cause apoptosis of AML cells. This suggests that the treatment drugs may be disrupting the AML bone marrow niche within the responder patients, which may not only reduce blast cells but also consequently prevent the induction of Tregs by blast cells, resulting in reduced TNFR2+ Treg levels. However, why only some patients have a reduction of blast cells and consequently TNFR2+ Tregs within their bone marrow remains unclear.

Additional mechanisms may include prevention of the induction of TNFR2+ Tregs by the treatment drugs. Several previous studies have demonstrated that azacitidine increases Tregs, both in vivo and in vitro (32, 33). Exposure to azacitidine results in the demethylation of the FOXP3 promoter and hence, enhanced FOXP3 expression (48). In contrast, we observed the percentage of total Tregs and TNFR2+ Tregs, but not the TNFR2+ fraction to be reduced in patients with AML on azacitidine and panobinostat therapy. This suggests that azacitidine may not be responsible for reducing Tregs frequencies in vivo. Indeed, we found that panobinostat reduced the proportion of TNFR2-expressing cells induced in vitro from PBMCs. Furthermore, we observed a dose-dependent decrease in the expression of the regulatory markers, CD25, FOXP3, and TNFR2 on CD4 T cells. Although effector T cells can also upregulate these markers upon in vitro stimulation, our in vivo results show that AML TNFR2+ CD25+ T cells had a...
reduction in expression of functional suppressor molecules, CTLA4 and FOXP3, within 1 month of treatment. In future studies, it would be of further interest to perform suppression assays to confirm the ability of panobinostat to alter the function of TNFR2⁺ Tregs. Nonetheless, the above findings suggest that panobinostat may play a role in preventing an increase in TNFR2⁺ Treg frequencies within patients, perhaps by preventing their reinduction in patients with AML.

It has been demonstrated that low doses of pan-HDAC inhibitors, such as panobinostat, reduce FOXP3 levels in vitro, whereas high doses have the reverse effect (34). Panobinostat, at low doses inhibits HDAC1 and HDAC3, which in turn causes activation and activation of the Stat3 pathway, resulting in Foxp3 downregulation (34). In our study, the doses of panobinostat used in vitro ranged from 1 to 20 nmol/L, which would potentially fall under the low-dose category. Although not performed in this study, it would be highly beneficial to additionally analyze epigenetic changes on Treg-related genes such as FOXP3 and TNFR2 on treatment with panobinostat.

Although we observed a reduction in the absolute cell counts of TNFR2⁺ Tregs within the peripheral blood of patients on treatment, this reduction was not significant. This is due to increases in other CD4 T-cell populations. In addition, as absolute cell counts were not performed within the bone marrow of patients, it is difficult to determine whether the absolute cell counts within the bone marrow reflect the changes observed with the percentages of TNFR2⁺ Tregs. Because several previous studies have demonstrated that the percentages of Tregs are positively correlated with poor cancer prognosis (8), it is likely that by reducing the percentages of TNFR2⁺ Tregs within the bone marrow, the proportion of antileukemic immune effector cells is enhanced, potentially disrupting the immunosuppressive niche within the bone marrow.

It is clear that a subset of patients with AML substantially benefited from this new combined therapy, whereas others did not currently remain unclear. For patients who did not have a reduction in their TNFR2⁺ Tregs in the bone marrow, whether increasing the concentration of the drugs given to these patients may alter their outcome remains to be determined. Moreover, whether the addition of other chemotherapeutic drugs like lenalidomide, which has been previously demonstrated to increase IFN-γ and IL-2 production by T cells, may be beneficial to the nonresponders still needs to be evaluated (49). However, due to the adverse side effects of a higher drug dosage or an additional drug to a combinational therapy, alternative options will need to be considered with caution.

Disclosure of Potential Conflicts of Interest

A. Spencer has honoraria from Speakers bureau and is a consultant/advisory board member of Celgene and Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Govindaraj, A. Spencer, M. Plebanski

Development of methodology: C. Govindaraj, M. Plebanski

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Govindaraj, P. Tan, P. Walker, A. Wei, A. Spencer, M. Plebanski

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Govindaraj, P. Tan, A. Wei, M. Plebanski

Writing, review, and/or revision of the manuscript: C. Govindaraj, P. Tan, P. Walker, A. Wei, A. Spencer, M. Plebanski

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Spencer, M. Plebanski

Study supervision: A. Spencer, M. Plebanski

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Chindu Govindaraj, Peter Tan, Patricia Walker, et al.


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