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

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Running title: Reduction of Tregs in AML via epigenetic therapies.

Key words: Regulatory T cells; AML; TNF Receptor 2; Panobinostat; Azacitidine

Financial support: We would like to acknowledge the Victorian Cancer Agency, Leukemia Foundation, Celgene and Novartis for funding support. MP is recipient of a Senior NHMRC Fellowship.

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Conflict of interest:

A.S and A.W are recipients of research funding from Celgene and Novartis.

The other authors declare no conflict of interest.

Test word count: 5250

Number of figures and tables: 6
Statement of translational relevance

Epigenetic therapies have proven to be effective when used in combination for the treatment of 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, which are key players in dampening effective anti-tumor immune responses. We demonstrate a marked and rapid effect of the combination therapy to selectively target and reduce Tregs, particularly the functional 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 immunological 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.
Abstract

**Purpose:** Acute myeloid leukemia (AML) provides an environment that enables immune suppression, resulting in functionally defective effector T cells; regulatory T cells (Tregs) are significant contributors to the impaired anti-tumor 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 fourteen AML patients 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 towards 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 IFNγ and 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 AML patients.
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 anti-leukemic 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 IFNγ and IL-2, which play a crucial role in anti-leukemic immunity (4, 5). However, T cells within AML patients 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) (7). Indeed, AML patients have abnormally high levels of Tregs within their peripheral blood (PB) and bone marrow (BM) compared to healthy donors (8). The presence of high Treg levels in AML patients correlate with poor clinical outcomes (9). This increase of Tregs has been attributed to AML blasts, which increase the frequency of Tregs through a variety of mechanisms including those linked to the effects of B7-H1 (10), indoleamine 2,3-dioxygenase (11) and CD200 (12). Depletion of Tregs in vivo using an interleukin-2 diphtheria toxin in a murine AML model resulted in an enhancement of the frequency of cytotoxic T lymphocytes and a reduced tumor burden (13). The importance of Tregs in dampening the anti-leukemic response is evident as their depletion results in an augmented immune response.

Tregs, however, are not a homogenous population as they contain cells with varying suppressive and migratory properties (14). Tregs can express chemokine receptors that are tissue specific, enabling them to localize within the tissue (15). The role of distinct Treg subsets in AML is currently not known, however, a functionally active Treg subset should have a high BM migratory capacity (16). TNF Receptor-2 (TNFR2) expression has recently been demonstrated to identify
highly functional Tregs in healthy donors (17, 18) and in various diseases like malaria (19), diabetes (20) and cancer (21). TNFR2+ Tregs have higher expression of regulatory markers including CTLA4 and proliferation markers like Ki-67 compared to TNFR2- Tregs (17, 22). These Tregs can be induced by either TCR ligation or by the presence of TNF (23), a cytokine detected at high levels in the serum and BM of AML patients (24, 25). Moreover, CXCL12, a chemokine secreted at high levels by marrow stromal cells and AML cells, has been demonstrated to promote TNFR2 expression on T cells (26, 27). We therefore, hypothesized that TNFR2+ Tregs may be readily induced and would identify the most disease relevant Treg population in AML patients.

As epigenetic mechanisms play a key role in the development and progression of AML, epigenetic-targeting agents such as the hypomethylating agent, azacitidine, have recently entered the therapeutic armamentarium for AML (28, 29). The epigenetic modifying effects of hypomethylating agents may be enhanced in combination with histone deacetylase inhibitors (HDACi) (30). Although azacitidine has been demonstrated to enhance overall survival in some AML patients (31), it can also increase FOXP3 expression and Treg levels, both in vivo (after stem cell transplantation) and in vitro (32, 33). In contrast, a recent review suggested that the HDACi, panobinostat at low doses can decrease FOXP3 expression and reduce Treg levels (34). The combined effect of azacitidine and panobinostat is currently being explored in clinical studies but the effect on Tregs, particularly on TNFR2+ Tregs fraction in AML is unknown.

Herein, we study the in vivo fate of Tregs, including TNFR2+ Tregs, following therapy with azacitidine and panobinostat in AML patients. We demonstrate for the first time that TNFR2+ Tregs are indeed the dominant Treg population within newly diagnosed AML patients. Furthermore, therapy with azacitidine and panobinostat selectively reduced TNFR2+ Treg proportions but not TNFR2- Tregs in AML, and patients showing such decreases in their blood and BM had associated clinical responses.
Patients, materials and methods

Patients

Patients diagnosed with previously untreated myelodysplastic syndrome (MDS) (International Prognosis Scoring System (IPSS) (35): intermediate-2 or high-risk) or AML (≥20% blasts), who were deemed ineligible for intensive chemotherapy were enrolled onto 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 75mg/m² daily by subcutaneous injection (days 1-5) and 7 oral doses of panobinostat (10-30mg), 3 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 or partial remission as per Cheson criteria for AML patients or International Working Group (IWG) criteria for MDS patients (36, 37). Patients who failed to achieve complete or partial remission were classified as non-responders.

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 PB and BM samples of AML patients via Ficoll density gradient separation (Amersham Pharmacia Biotech, Sweden). The isolated cells from both PB and BM samples were frozen in a freeze mixture (10% DMSO (Sigma-Aldrich, Australia) 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 re-suspended 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-TNFR2, 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) prior to analysis of Tregs levels. Intracellular levels of FOXP3 and Ki67 were determined using firstly a fixation/permeabilization buffer kit (eBioscience) followed by staining with anti-FOXP3 (eBioscience) and anti-Ki67 (BD Pharmingen). Flow cytometry data was 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 PBMCs using the INFLUX flow cytometry-based cell sorter. Migration assays were performed as previously described (21). In brief, 2.5 X 10^5 cells in 100μl of AIM-V media were added to the top chamber while 600μl of media or cell-free AML BM fluid was added to the lower chamber of 5.0μM 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 CD25^hi^ 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 BM fluid when compared to media alone.

**Intracellular cytokine analysis.** Intracellular cytokines were analyzed by culturing cells at a ratio of 10^5 cells/50μl with PMA (50ng/ml) and Ionomycin (1μg/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 prior to staining for the following cytokines: anti-IFN-γ (BD Pharmingen), anti-IL-2 (eBioscience), anti-IL-10 (eBioscience) and anti-TGF-β (RnD systems).

**CXCL12 detection.** ProcartaPlex Immunoassay Kit-Magnetic beads were to determine the level of CXCL12 present in the BM fluid and PB serum of AML patients. 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 while panobinostat was reconstituted with DMSO, at a 10mM 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 Peripheral Blood Mononuclear Cells (PBMCs) were cultured at 10^5 cells/50μl 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.

**Statistics.** 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 PB 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 firstly 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 to healthy donors. After treatment, CD4 T cell proportions were significantly enhanced at EOC3 when compared to 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 to screening (Fig. 1S). To investigate the proportion of Tregs within CD4 T cells, CD25$^{\text{hi}}$ T cells were gated on FOXP3 expression to identify Tregs. CD127 expression within the CD25$^{\text{hi}}$ T cells was also observed to be at low levels, confirming the identity of Tregs. The proportion of Tregs (CD25$^{\text{hi}}$FOXP3+) was compared between healthy donors and AML patients (Fig. 1C). Consistent with a previous study (8), we observed Treg proportions to be significantly higher in patients (mean = 5.5%) compared to healthy donors (mean = 0.7%). Strikingly, Treg proportions were halved after one month of azacitidine and panobinostat treatment (mean = 2.4%) and this was sustained at three months of treatment (mean = 2.5%) (Fig. 1D).

**Tregs with high migratory capacity to the BM express TNFR2.**

TNFR2 expression on Tregs identifies a population of potent suppressor T cells in healthy conditions (17). Indeed, we also observed significant increases in the proliferation of effector T cells upon depletion of TNFR2$^{+}$ Tregs when compared to the non-depleted control wells, but not upon depletion of TNFR2$^{-}$ Tregs (Fig. 2S), confirming TNFR2$^{+}$ Tregs are potent suppressor cells. To characterize TNFR2$^{+}$ Tregs in AML, we assessed the levels and phenotype of TNFR2$^{+}$ and TNFR2$^{-}$ Tregs comparatively in healthy and AML donors. Tregs from patients had a higher expression level of TNFR2 when compared to healthy Tregs (p<0.01) (Fig. 2A). Additionally, patients had an almost 10-fold higher percentage of total TNFR2$^{+}$ Tregs when compared to healthy donors (Fig. 2A). While TNFR2$^{+}$ Tregs had higher levels of CTLA4 and CD73 compared to TNFR2$^{-}$ Tregs, there was no difference in CD39 expression level, which was high in both populations (Fig. 2B). CTLA4 dampens effector T cell function while CD39 and CD73 can generate adenosine, which has immunosuppressive properties (14, 38). The proportion of both CD39 and CD73 were also 9-fold
higher on TNFR2+ Tregs than on TNFR2- Tregs in healthy donors (data not shown). TNFR2+ Tregs also contained a higher proportion of proliferating cells, as demonstrated by increased proportion of cells with high intracellular Ki67 expression than the TNFR2- subset (p<0.0001) (Fig. 2B). This Treg subset produced significantly higher levels of the immunoregulatory cytokines, IL-10 and TGFβ when compared to TNFR2- Tregs (Fig. 2C). The above data supports a more potent regulatory phenotype for TNFR2+ than TNFR2- Tregs. In all of the above phenotypic analyses, we did not observe any significant differences between TNFR2+ Tregs isolated from healthy and AML donors.

To further test the potential relevance of TNFR2+ Tregs in patients, we determined the migratory potential of these Tregs to the BM by firstly assessing CXCR4 expression levels. As shown in figure 2D, TNFR2+ Tregs had significantly higher levels (6.8 fold higher) of CXCR4 compared to the TNFR2- fraction. Overall a positive correlation (p<0.0001) was observed between TNFR2 and CXCR4 expression levels on Tregs (Fig. 3S). Additionally, we performed migration assays to determine if TNFR2+ Tregs were capable of migrating towards the BM fluid of AML patients. We observed that the BM fluid was able to attract an increased number of CD25<sup>hi</sup> T cells when compared to media alone (p=0.06) (data not shown). Furthermore, within the CD25<sup>hi</sup> T cell subset, a significantly higher number of TNFR2+ cells migrated towards the AML BM fluid when compared to the TNFR2- subset (p<0.0001) (Fig. 2D).

**Azacitidine and panobinostat therapy selectively lowers TNFR2+ but not TNFR2- Tregs in the PB of AML patients.**

To determine if TNFR2+ Tregs were altered upon treatment, flow cytometry was performed at EOC1 and EOC3 of treatment. We observed a significant reduction of TNFR2 expression levels within Tregs as well as the percentage of total TNFR2+ Tregs at EOC1 when compared to 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+CD25<sup>hi</sup> and TNFR2-CD25<sup>hi</sup> 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+CD25\textsuperscript{hi} T cell subset (Fig. 3C) while no change was observed within the TNFR2-CD25\textsuperscript{hi} T cell subset (data not shown). Similarly, CTLA4 levels within the TNFR2+CD25\textsuperscript{hi} T cell subset were also reduced at EOC1 of treatment when compared to screening levels (Fig. 3C). These results demonstrate that treatment reduces the proportion as well as potentially alters the function of TNFR2+ Tregs within AML patients. As several recent papers have demonstrated the existence of a FOXP3+CD25\textsuperscript{low} Treg subset, we also determined the levels of CD25 expression within TNFR2+FOXP3+ T cells (40). Our results demonstrate TNFR2+FOXP3+ T cells have high expression levels of CD25 compared to the TNFR2- subset (Fig. 4S). Additionally, we demonstrate that the levels of TNFR2+FOXP3+ T cells are higher in the PB of patients compared to healthy donors and that these levels are reduced on treatment (Fig. 4S).

Response to epigenetic-targeted therapy is associated with a significant reduction in TNFR2+ Tregs within the BM.

As AML originates within the BM, it is important to determine if the results obtained in the PB were comparable to that of the initiating tumor site. A previous study has demonstrated that Tregs are present at elevated levels within the BM of AML patients (9). Higher migration towards the AML BM fluid by TNFR2+ Tregs when compared to TNFR2- Tregs suggested that these cells would preferentially accumulate at high levels in the BM. In order to determine the levels of TNFR2+ Tregs in the BM 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 to TNFR2- Tregs in the BM at screening (data not shown). These BM TNFR2+ Treg proportions (mean; SEM=4.5%; 1.3) were similar to that found in the PB (mean; SEM =5.06%; 1.1) of patients. There was also a trend for reduced TNFR2+ Treg proportions in the BM at EOC1 when compared to screening (p=0.07) (Fig. 4A). When patients were categorized into clinical responders and non-responders at EOC1 based on hematologic reductions in blast cell counts (36, 37), a significant difference in TNFR2+ Treg proportions was observed. As shown in figure 4B, while both responders and non-responders had a reduction in TNFR2+ Tregs in the PB, only clinical responders
to treatment had a significant reduction ($p<0.05$) in TNFR2+ Tregs in their BM when compared to non-responders. Additionally, we observed no significant changes in the absolute cell counts of TNFR2+ Tregs in the PB at EOC1 between clinical responders and non-responders (data not shown). Although it would have been informative to perform absolute cell counts within the BM, this was not performed at the time of sample collection and this data is not available. Within the BM samples, the responder cohort consisted of 4 patients (2 MDS and 2 AML) at complete remission (CR), while the non-responder cohort consisted of 3 AML patients. Within the PB samples, the responder cohort consisted of 5 patients (3 MDS and 2 AML) at CR, 3 patients (1 MDS and 2 AML) at partial remission (PR), while the non-responder cohort had 4 patients (2 MDS and 2 AML). We also analyzed for any potential differences between MDS and AML patients 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 non-responders were performed at EOC1 due to the higher patient numbers compared to EOC3.

**Azacitidine and panobinostat treatment increases the low level of Th1 cytokines produced by CD4 effector T cells isolated from the BM.**

We determined if azacitidine and panobinostat had an effect on CD4 effector T cell function within patients. CD4 effector T cells capable of IFNγ and IL-2 production were found to be predominantly FOXP3- T cells. Figure 5A shows the gating strategy to identify intracellular IFNγ and IL-2 production by CD4 effector T cells. Generally, CD4 T cells from the BM of patients produced relatively low levels of both IFNγ and IL-2 (Fig. 5A). We also determined the effect of treatment on intracellular cytokine production on CD4 T cells from the PB and BM. There was no change in cytokine production by T cells isolated from the PB, but both IFNγ ($p=0.05$) and IL-2 ($p=0.07$) production by T cells from the BM of all patients was enhanced at the EOC1 when compared to screening samples (data not shown). Upon further delineation of patients into responders and non-responders to treatment, there were no significant differences in cytokine production between responders and non-responders in PB. However, responders to treatment showed significant increases in IFNγ (6-fold higher; $p=0.04$) and an increase for IL-2 (12-fold higher) production by
CD4 T cells from the BM at EOC1 when compared to non-responders (who had 1.1-fold higher for IFNγ and 1.4-fold higher for IL-2 production) (Fig. 5B). As some effector T cells also express TNFR2 expression, we determined if TNFR2+ effector T cells were reduced on treatment within patients. Firstly, we observed that majority of effector T cells were TNFR2-, and these cells were significantly lower within the PB of patients compared to healthy donors, while there was no significant change observed in the percentages of TNFR2+ effector T cells (Fig. 5S). On treatment, TNFR2- effector T cell levels were significantly enhanced while TNFR2+ effector T cells were significantly reduced within one month of treatment (Fig. 5S). Additionally, upon analyzing cytokine production, we observed no differences in IFNγ or IL-2 production by TNFR2- as well as TNFR2+ effector T cells within the PB samples between responders and non-responders, while within the BM, it was the TNFR2- effector T cells and not the TNFR2+ subset within the responder patients that produced significantly higher levels of IFNγ compared to the non-responder patients (data not shown).

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

Our data, thus far, demonstrates Tregs in the PB of AML patients are decreased upon treatment with azacitidine and panobinostat and this decrease is driven by a reduction in the TNFR2+ Treg subset. Moreover, a proportion of patients had this decrease within their BM. To determine if the decrease in TNFR2+ Treg percentages is due to changes in TNF levels, we assessed TNF levels within the serum of the PB and BM 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 if an altered migration into the BM from the PB could account for the decrease in TNFR2+ Tregs within the BM of patients who responded to treatment, we firstly 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 to Scr between the two patient cohorts (Fig. 6A). Additionally, we assessed CXCL12 levels in the PB serum and BM fluid. CXCL12 levels at EOC1, when compared to screening were significantly higher in responder patients when compared to the non-responder patients (Fig. 6B). This increase was seen within the PB serum as...
well as the BM fluid of these patients. However, as the level of CXCL12 was similar in the PB serum and the BM 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 BM to the PB. 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 high-risk MDS patients 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 μM 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 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^hiFOXP3^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 CD4 T cells upon azacitidine treatment alone when compared to no treatment (Fig. 6C). Our findings thus indicate panobinostat can directly reduce the expression of Treg-associated markers induced by TCR activation including TNFR2 in vitro, while azacitidine 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 if panobinostat can reduce up-regulation of TNFR2, FOXP3 and CD25 on CD4 T cells when stimulated in the presence of TNF. As demonstrated by fig. 6S, there was a significant reduction in the up-regulation 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 anti-tumor immunity, it is crucial to identify and target the most active Treg population within AML patients. AML patients had significantly higher levels of TNFR2+ Tregs within their PB and BM when compared to 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 TGFβ than TNFR2- Tregs in both healthy and patient donors. CTLA4 blockade improves T cell responses towards AML blasts in vitro and also enhances patient survival with other cancers like melanoma (41, 42). CD39 and CD73 ecto-enzymes generate adenosine from extracellular nucleotides like ATP (43). This adenosine can dampen T cell proliferation and the production of cytokines including IFNγ and IL-2 via the A2A receptor present on activated effector T cells (38). Additionally, IL-10 and TGFβ are implicated in the down-regulation of anti-tumor 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 PB 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 AML patients. 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/TNFR2 and CXCL12/CXCR4 interactions exists, enabling both AML cells as well as TNFR2+ Tregs to flourish by creating an immunosuppressive niche within AML patients. This is intriguing, as CXCR4 expression has previously been associated with disease progression in AML patients (47).

Our results demonstrate that although there was a decrease in TNFR2+ Treg percentages within the PB of both the responder and non-responder patients on treatment, a reduction of TNFR2+
Tregs was observed only in the BM of the responder patients. This suggests that for patients to clinically respond, it may be essential to reduce Treg proportions within the BM as well as PB. Consequently, we observed increases in both IFNγ and IL-2 production by CD4 effector T cells isolated from the BM within one month of treatment of responders. We believe this increase in Th1 cytokines from patients who responded to treatment was a corollary effect of TNFR2+ Treg reduction within the BM and not a direct effect of the treatment, as both azacitidine and panobinostat failed to significantly enhance cytokine production in vitro (Fig. 6S). Although the precise mechanisms that result in a reduction of TNFR2+ Tregs within the BM of responder patients and the PB of all patients is currently unclear, we believe this may be due to several factors. Firstly, we observed that the responder patients had significantly higher levels of CXCL12 levels in both the BM fluid and PB serum at EOC1, compared to non-responder patients. Although, as previously mentioned, CXCL12 plays a key role in AML pathogenesis and recruiting TNFR2+ Tregs into the BM, a recent study demonstrates that once the AML BM survival niche is disrupted, CXCL12 may actually cause apoptosis of AML cells. This suggests that the treatment drugs may be disrupting the AML BM 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 BM 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 AML patients on azacitidine and panobinostat therapy. This suggests that azacitidine may not be responsible for reducing Tregs frequencies in vivo. Indeed, we found 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 up-regulate these markers upon *in vitro* stimulation, our *in vivo* results show that AML TNFR2+CD25^{hi} T cells had a reduction in expression of functional suppressor molecules, CTLA4 and FOXP3 within one 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 suggests that panobinostat may play a role in preventing an increase in TNFR2+ Treg frequencies within patients, perhaps by preventing their re-induction in AML patients. It has been demonstrated that low doses of pan-HDAC inhibitors, such as panobinostat reduce FOXP3 levels *in vitro* while high doses have the reverse effect (34). Panobinostat, at low doses inhibits HDAC1 and HDAC3, which in turn causes acetylation and activation of the Stat3 pathway, resulting in *Foxp3* down-regulation (34). In our study, the doses of panobinostat used *in vitro* ranged from 1-20nM, 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 PB of patients on treatment, this reduction was not significant. This is due to increases in other CD4 T cell populations. Additionally, as absolute cell counts were not performed within the BM of patients, it is difficult to determine whether the absolute cell counts within the BM reflect the changes observed with the percentages of TNFR2+ Tregs. Since 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 BM, the proportion of anti-leukemic immune effector cells is enhanced, potentially disrupting the immunosuppressive niche within the BM.

It is clear that a subset of AML patients substantially benefited from this new combined therapy, and that this benefit was associated with immunological parameters. The rationale behind why some patients had decreased TNFR2+ Treg proportions in the BM after treatment while others did not currently remains unclear. For patients who did not have a reduction in their TNFR2+ Tregs
in the BM, 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 IFNγ and IL-2 production by T cells, may be beneficial to the non-responders still needs to be evaluated (49). However, due to the adverse side effects of a higher drug dosage or an additional drug to a combinatorial therapy, alternative options will need to be considered with caution.

Acknowledgements

We thank Geza Paukovics, Michael Thompson and Jeanne Le Masurier for their assistance with flow cytometry and Katie L. Flanagan for reviewing the manuscript. We thank the patient volunteers for their sample contribution.

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Figure legends

Figure 1. Comparison of CD4 T cell and regulatory T cell proportions in healthy and AML patients. 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 AML patients were compared at screening (Scr; n=14), End of Cycle 1 (EOC1; n=12) and End of Cycle 3 (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 CD25<sup>hi</sup> T cells to further confirm the Treg identity. (C) The percentage CD25<sup>hi</sup>FOXP3+ T cells were compared between healthy (30) and AML (14) patients. (D) Treg percentages within AML patients 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 2. Comparison of the levels and function of TNFR2- and TNFR2+ Tregs between healthy and AML patients at screening. Tregs were further gated on TNFR2 expression; clear histogram represents Tregs while tinted histogram represents CD25- T cells. The level of TNFR2 expression within Tregs and the percentage of TNFR2- and TNFR2+ Tregs were compared between healthy (n=43) and AML patients (n=14) at screening. Comparison of regulatory factors, CTLA4 MFI, CD39 MFI, CD73 MFI and intracellular Ki67 percentage (B), intracellular IL-10 and TGFβ production (C) (following PMA/ionomycin stimulation in the presence of Brefeldin A), and CXCR4 expression levels (D) on TNFR2- and TNFR2+ Tregs isolated from healthy donors and AML patients. (D) The migration capacity of TNFR2+ and TNFR2- cells within CD25<sup>hi</sup> T cells of healthy donors (n=4) towards the AML BM fluid was compared and migration indices were calculated by using media alone as controls. Statistical analyses between healthy and AML samples were performed by unpaired t-tests. Data represent mean +/- SEM. *p< 0.05. **p< 0.01. ***p<0.001. ****p<0.0001.
Figure 3. TNFR2- and TNFR2+ Treg proportions and function in AML patients during panobinostat and azacitidine treatment. The expression levels of TNFR2 within Tregs (A), TNFR2- and TNFR2+ Treg proportions (B) within AML patients were compared at Scr (n=14), EOC1 (n=12) and EOC3 (n=8) of treatment. (C) Comparison of the expression levels of regulatory factors, FOXP3 and CTLA4 on TNFR2+CD25hi Tregs isolated from AML patients at various time-points of treatment. The straight line in the graphs represents the value of the healthy mean. Statistical analyses were performed by paired t-tests. Data represent mean+/−SEM. The healthy mean is represented by a straight line for B. *p< 0.05. **p< 0.01.

Figure 4. TNFR2+ Tregs within bone marrow of AML patients and disease correlation. (A) The proportion of TNFR2+ Tregs within CD4 T cells from the peripheral blood and the bone marrow of AML patients were compared at Scr and EOC1 of treatment. Patients who responded to treatment are represented by tinted circles while non-responder patients are represented as clear circles. (B) The difference in the fold change of TNFR2+ Treg proportions at EOC1 when compared to Scr between responders and non-responder patients, within the peripheral blood and bone marrow. The TNFR2+ Treg proportion at Scr is indicated by a straight line. PB samples were analyzed between 5 responders and 5 non-responders while BM samples were analyzed between 4 responders and 3 non-responders. Statistical analyses were performed by paired t-tests (A) and unpaired t-tests (B). Data represent mean+/−SEM. *p< 0.05.

Figure 5. Comparison of effector T cell function between healthy and AML patients. Mononuclear cells from PB and BM of healthy donors and AML patients were cultured at a ratio of 10^5 cells/50μL and were stimulated with PMA/Ionomycin for 5 hours, with the addition of Brefeldin A for the last 4 hours. Flow cytometry was performed post-stimulation to identify effector cytokine producing T cells (CD3+CD4+FOXP3−). (A) The gating strategy employed to identify IFN-γ and IL-2 producing CD4 effector T cells within healthy donors. The percentage of IFN-γ and IL-2 producing cells within the PB of healthy donors (n=7), PB of AML patients (n=7) and bone marrow (BM) of AML patients (n=7). (B) The percentage of IFN-γ and IL-2 producing effector T cells within PB and BM of AML patients were compared at Scr and EOC1, between responders and non-
responders. PB samples were analyzed between 3 responders and 4 non-responders while BM samples were analyzed between 4 responders and 3 non-responders. The cytokine production at Scr is indicated by a straight line. Statistical analyses were performed by one-way ANOVA (A) and unpaired t-tests (B). Data represent mean+/-SEM. *p< 0.05. **p<0.01.

Figure 6. The levels of CXCL12 within the BM fluid and PB serum of AML patients and the effect of Azacitidine and panobinostat treatment on TNFR2+ Tregs in vitro. (A) The difference in the levels of CXCR4 MFI on PB TNFR2+ Tregs between responder and non-responder patient cohorts at EOC1 when compared to screening. (B) The difference in CXCL12 levels within the PB serum and BM fluid between responder (n=2) and non-responder patient cohorts (n=7) at EOC1 when compared to screening. (C) PBMCs were stimulated with anti-CD3/28 and cultured at a ratio of $10^5$ cells/50μL. Cells were treated 0μM or 1μM 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.
Figure 1

A

![Graph showing % CD4 for Healthy and AML groups with p=0.05.]

B

![Graph showing % CD4 for Scr, EOC1, and EOC3 groups with asterisks indicating statistical significance.]

C

![Graph showing CD25^hiFOXP3^+ % for Healthy and AML groups with *** indicating statistical significance.]

D

![Graph showing CD25^hiFOXP3^+ % for Scr, EOC1, and EOC3 groups with asterisks indicating statistical significance and 0.07 as the p-value.]
Figure 3

A

TNFR2 MFI (within Tregs)

Scr  EOC1  EOC3

**

B

% TNFR2+ Tregs

Scr  EOC1  EOC3

*

C

FOXP3 MFI

Scr  EOC1  EOC3

0.05

CTLA4 MFI

Scr  EOC1  EOC3

**

Healthy mean
Figure 5

**A**

IFN-γ

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<th>AML BM</th>
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**B**

Peripheral Blood

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Bone Marrow

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IL-2

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**B**

Peripheral Blood

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Figure 6

A

CXCR4 MFI (PB TNFR2+ Tregs) (Fold change)

Responders Non responders

B

Bone Marrow

Peripheral blood

CXCL12 (pg/mL) (Fold change)

Responders Non responders

Responders Non responders

C

CD25 MFI within CD4+ (%)

0 1 5 10 15 20 0 1 5 10 15 20 0 1 5 10 15 20

LBH (nM) alone LBH (nM) + Aza (1μM)

LBH (nM) alone LBH (nM) + Aza (1μM)

LBH (nM) alone LBH (nM) + Aza (1μM)

D

CD25^+FOXP3+TNFR2+ within CD4+ (%)

0 1 5 10 15 20

LBH (nM) alone LBH (nM) + Aza (1μM)

LBH – Panobinostat
Aza - Azacitidine

0nM was used as a control for statistical analyses.
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, Peter Tan, Patricia Walker, et al.

Clin Cancer Res  Published OnlineFirst December 2, 2013.

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