Lenalidomide-based maintenance therapy reduces TNF Receptor 2 on CD4 T cells and enhances immune effector function in acute myeloid leukemia patients

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A major limitation to improved outcomes in acute myelogenous leukemia (AML) is relapse resulting from leukemic cells that persist at clinical remission. Regulatory T cells (Tregs), which are increased in AML patients, can contribute to immune evasion by residual leukemic cells. Tumor necrosis factor (TNF), a pro-inflammatory cytokine present at high levels within patients, can induce TNF receptor-2 (TNFR2) expression on Tregs. We hypothesized that since TNFR2 is required for Treg stabilization and TNFR2+ Tregs are potent suppressors, targeting TNFR2+ Tregs may restore the effectiveness of immune-surveillance mechanisms. In this pilot study, we report AML patients in clinical remission have substantially increased levels of TNFR2+ T cells, including TNFR2+ Tregs and impaired effector CD4 T cell function with reduced IL-2 and IFNγ production. The immunomodulatory drug, lenalidomide, and the demethylating agent, azacitidine have been moderately successful in treating AML patients, but their combined effects on TNFR2+ T cells, including Tregs are currently unknown. Our data indicates that although treatment with lenalidomide and azacitidine increased cytokine production by effector T cells in all patients, durable clinical remissions may be observed in patients with a concomitant reduction in TNFR2+ T cells and TNFR2+ Tregs. In vitro studies further demonstrated that lenalidomide can reduce TNFR2 expression and can augment effector cytokine production by T cells, which can be further enhanced by azacitidine. These results indicate that reduction of TNFR2+ T cells in AML postremission phase may result from combined azacitidine/lenalidomide therapy and may contribute to an improved clinical outcome.


Introduction

Acute myeloid leukemia (AML) is an aggressive blood cancer of myeloid progenitors that results in the accumulation of bone marrow blasts and hematopoietic failure. Although intensive chemotherapy produces clinical remission in 60–80% of cases, the surviving blasts can result in AML relapse in a majority of patients [1,2]. The antileukemic effect of the immune system is demonstrated by reduced relapse rates in patients receiving allogeneic stem cells, which mediate a graft-versus-leukemia effect [3]. T cells play an important role in targeting residual leukemia, as depletion of T cells from the graft increases the risk of relapse, while reinfection of donor T cells may result in occasional remissions [4]. However, despite their potential beneficial effects, effector T cells are numerically and functionally defective in the peripheral blood of AML patients [5]. These defects can partly be attributed to the effect of regulatory T cells (Tregs), which suppresses the proliferation and function of effector T cells [6,7]. High frequencies of Tregs are observed in the peripheral blood of AML patients and are correlated with poor clinical outcomes in AML patients [8,9]. Leukemic blast cells directly promote Treg frequencies through a variety of mechanisms, creating an immunosuppressive niche [10–13]. Leukemic blast cells also produce high levels of the pro-inflammatory cytokine, tumor necrosis factor (TNF), which can induce Tregs by increasing the expression of TNF receptor-2 (TNFR2) and FOXP3 on T cells [14–18]. TNFR2+ Tregs are potent immune suppressors and express increased levels of a range of immunoregulatory molecules including CTLA4 and stGFβ when compared to TNFR2− Tregs [19–21]. Additionally, loss of TNF or TNFR2 expression on immune cells results in a reduced frequency of Tregs in a tumor bearing mouse model, and enables the immune system to control tumor growth and metastasis [22]. The above studies suggest that Tregs may aid residual leukemic cells in AML

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patients at clinical remission to evade immune responses and by reducing TNF or TNFR2 expression levels, the effectiveness of the host immune-surveillance mechanisms may be restored.

Lenalidomide is an immunomodulatory agent with beneficial activity against a range of hematological malignancies including multiple myeloma, AML, myelodysplastic syndrome, and chronic lymphocytic leukemia [23–26]. The mechanisms of lenalidomide include direct cytotoxicity on leukemic blast cells as well as an enhancement of antitumor immunity [25]. Lenalidomide can activate T cells by acting as a CD28 agonist and can also inhibit TNF production, a cytokine that is present at high levels within the serum and bone marrow of AML patients and is involved in leukemogenesis [27–29]. Here, we investigated the immunomodulatory effect of maintenance lenalidomide treatment in a pilot cohort of AML patients in remission. We hypothesized that lenalidomide may inhibit TNF production thereby reducing TNFR2 expression and TNFR2+ Tregs within these patients. Furthermore, as the combination of lenalidomide and azacitidine is suggested to be clinically more effective [30], patients received azacitidine for 5 days followed by lenalidomide from Day 5 to Day 25, for each cycle. Our results indicate that TNFR2+ Treg levels remain high in AML patients despite achieving remission, with concordant defects in effector T cell function. Our results also indicate that treatment with azacitidine and lenalidomide can reduce TNFR2 expression on T cells as well as TNFR2+ Tregs in vivo, and this reduction may contribute to the maintenance of clinical remission.

### Methods

**Patients.** Samples for this study were obtained from patients providing written informed consent on a protocol approved by the Alfred Hospital Research and Ethics Unit. The study was registered with Australian and New Zealand Clinical Trials Registry (ANZCTR). ACTRN 1261100058147. A phase Ib dose escalation study enrolled patients with high risk AML in CR/CRi and high-risk features (age >60 years, adverse risk karyotype, FLT3-ITD+ or second complete remission). Ten patients in clinical remission (after induction chemotherapy) received azacitidine subcutaneously (50–75 mg/m²) on Days 1–5 of each cycle in combination with lenalidomide orally (5–10 mg) daily from Day 5 to Day 25 of each cycle until disease progression, while four patients received azacitidine only (50–75mg/m²) on Days 1–5 of each cycle. Blood sampling for mononuclear cell isolation was analyzed at screening (baseline) and on Day 25 of cycle 1 and Day 25 of cycle 3. At end of cycle 3 (EOC3), patients were categorized based on their disease outcome. The nonrelapse cohort of patients who remained at remission at EOC3 included three patients receiving azacitidine alone and three patients receiving azacitidine and lenalidomide. The relapse patient cohort consisted of three patients who received azacitidine and lenalidomide. 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. These cells were TCR stimulated with plate-bound anti-CD3 (2.5 μg/ml clone OKT, Biolegend) together with soluble anti-CD28 (1.25 μg/ml clone CD28.2, BD Pharmingen) in 96-well round bottom plates for 72 hr. Lenalidomide was added either alone or in combination with azacitidine at the indicated ratios on Day 0. TNF was added at a concentration of 20 ng/mL where indicated. Control wells had no drugs added to them. On Day 3, cells were washed and flow cytometry was performed to determine the proportion of TNFR2 expression within the PBMCs of healthy donors.

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

### Results

**Elevated levels of TNFR2+ CD4** T** cells during maintenance therapy is associated with disease response**

To determine TNFR2 expression levels on CD4 T cells, we performed flow cytometry on PBMCs isolated from healthy and AML donors. Live T cells were identified by first gating leukocytes based on CD45 expression, followed by CD3 and CD4 expression. Within CD3+CD4+ T cells, AML patients (mean +/- SEM = 8.8 +/- 1.3%) had a significantly higher percentage of cells expressing TNFR2 when compared to healthy donors (mean +/- SEM = 3 +/- 0.3%) (Fig. 1A) (P < 0.01). Levels of TNFR2 expression as reflected by the median expression intensity (MFI) in patients (average MFI = 165) were also significantly higher compared to healthy donors (average MFI = 97) (Fig. 1A) (P < 0.0001).

To determine whether the maintenance therapy using azacitidine and lenalidomide had an effect on TNFR2 expressing T cells, patient samples were obtained at EOC3 of treatment. We did not observe any significant changes in TNFR2 expression (as assessed by MFI) on CD4 T cells in AML patients during the course of treatment (data not shown). We further delineated our patients based on their disease outcome at EOC3 of treatment into relapse and nonrelapse cohorts. The fold change of both the TNFR2 MFI level and percentage at EOC3 to screening (dotted line) was found to be significantly (P < 0.05) lower on nonrelapse patients when compared to patients with disease relapse (Fig. 1B).

**Elevated levels of TNFR2+ CD4 T cells in AML patients are driven by an increase in TNFR2+ Tregs**

The observed increase in TNFR2+ T cells may have been driven by an increase in either regulatory or effector T cells in leukemic patients in remission. We therefore assessed both regulatory and effector T cell populations within the TNFR2 subset as per Fig. 2A. Tregs were characterized as CD25hiFOXP3+ cells while effector T cells were characterized by CD25low expression. Within the healthy donors, TNFR2+ T cells contained an average (mean +/- SEM) of 97 +/- 0.2% effector T cells and only 0.3 +/- 0.05% Tregs, while TNFR2+ T cells contained an average of 60 +/- 2.2% effector T cells and 29 +/- 1.7% Tregs (Fig. 2B). This result demonstrates that under normal conditions within healthy donors, TNFR2+ effector T
cells were the dominant effector T cell subset while TNFR2+ Tregs were the dominant Treg population. In AML patients, there was an overall increase in Tregs, which was most dramatic within the TNFR2+ population, as the frequency of TNFR2+ Tregs was 25-fold higher compared to TNFR2− Tregs (Fig. 2B) \((P < 0.0001)\). We also observed significant, although moderate decreases (1–1.5 fold) in the proportions of both TNFR2− and TNFR2+ effector T cells in AML patients compared to healthy donors \((P < 0.0001)\). As a consequence of the above, the ratio of Tregs to effector T cells was significantly higher in the TNFR2+ population compared to the TNFR2− CD4 T cell subset, and this difference was further exacerbated in AML patients compared to healthy donors \((P < 0.0001)\). Overall, our data shows that the increase seen in healthy TNFR2+ CD4 T cells within AML patients is due to an increase in TNFR2+ Tregs.

**Reduction of TNFR2 levels on CD4 T cells results in a concomitant reduction in TNFR2+ Treg proportions**

We have thus far demonstrated that albeit in clinical remission, AML patients have significantly higher levels of TNFR2+ Tregs within their peripheral blood compared to healthy donors. Patients who responded to maintenance therapy had significantly lower levels of TNFR2 on CD4 T cells compared to patients who had disease relapse. To determine whether this reduction led to a concomitant loss of TNFR2+ Tregs, we assessed the proportions of TNFR2+ Tregs within CD4 T cells at screening and end of cycle 3 of treatment. The fold change of the percentage of TNFR2+ Tregs at EOC3 to screening (dotted line) was found to be lower on nonrelapse patients \((P = 0.05)\) when compared to patients with disease relapse (Fig. 3). This change was confined to the TNFR2− subset as there were no changes observed with TNFR2− Tregs at EOC3. Additionally, the level of TNFR2+ effector T cells were lower on nonrelapsed patients compared to relapsed patients, but this was not significant (Supporting Information Fig. 1S). The above results demonstrate that the reduction of TNFR2 levels on nonrelapsed patients during maintenance therapy is associated with a concomitant reduction of TNFR2+ Tregs but not TNFR2− Tregs.

**CD4 effector T cells of AML patients have an impaired effector function**

As TNFR2+ Tregs have been previously demonstrated to impede cytokine production by effector T cells, the presence of high levels of TNFR2+ Tregs within the peripheral blood of AML patients at screening supports the hypothesis that AML patients may have low production levels of both IL-2 and IFNγ effector cytokines [31]. For our CD4 effector T cell analysis, we combined both TNFR2+ and TNFR2− subsets as it has previously been demonstrated that TNFR2+ Tregs are capable of dampening the function of both these effector subsets [19]. To assess intracellular cytokine production, we performed a 5-hr in vitro stimulation assay using PMA/Ionomycin in the presence of Breffeldin A for the final 4 hr. We tested the intracellular production of Th1 cytokines, IL-2, and IFNγ. AML patients had significantly lower percentages of IL-2+ \((P < 0.0001)\) and IFNγ+ \((P < 0.01)\) effector CD4 T cells compared to healthy donors (Fig. 4A,B).

**The initially low levels of IL-2 production by effector T cells from AML patients can be reversed by treatment in vivo and in vitro**

Given that TNFR2+ Tregs have been demonstrated to reduce cytokine production by effector T cells, it was tempting to speculate that a reduction in TNFR2+ Treg frequencies following treatment may also result in an increase in the production of these cytokines [31]. We therefore assessed the intracellular cytokine production of effector T cells during maintenance therapy. We observed an increase in intracellular IL-2 and IFNγ production by effector T cells on both relapsed and nonrelapsed patients (Fig. 5A), suggesting that a reduction in TNFR2+ Tregs may not be the only cause for increased production and that the treatment drugs may have a direct effect in IL-2 and IFNγ augmentation. To test whether the treatment drugs had an additional direct effect on enhancing intracellular IL-2 production by CD4 effector T cells, we performed in vitro assays. Azacitidine has previously been
demonstrated to be potent in vitro at a concentration of 1 µM, while lenalidomide has been used at 1 µM or 10 µM [32–34]. Healthy PBMCs were cultured for 3 days with anti-CD3 and anti-CD28 in the presence or absence of azacitidine along with varying doses on lenalidomide. On Day 3, cells were treated with PMA/Ionomycin for 5 hr in the presence of Brefeldin A for the final 4 hr. Figure 5B demonstrates that lenalidomide had a direct effect on increasing IL-2 production by effector T cells, as significant increases of IL-2 were observed in a dose-dependent manner. Furthermore, the presence of azacitidine further enhanced this IL-2 production when used in combination with 1 µM lenalidomide. Although there was no significant enhancement of IFNγ production upon lenalidomide treatment, the presence of azacitidine along with lenalidomide treatment enhanced IFNγ production by effector T cells when compared to azacitidine treatment alone. Our data suggests that maintenance therapy of lenalidomide can directly reverse the initial low levels of IL-2 cytokine production by CD4 effector T cells in AML patients at remission, and azacitidine augments this effect.

In vitro reduction of TNFR2 levels is an effect of lenalidomide and cannot be reversed by the addition of TNF

The above data demonstrates that patients who remained at remission at EOC3 of maintenance therapy had significantly lower levels of TNFR2 when compared to screening. This suggests a concomitant reduction of TNFR2 levels and an augmentation of effector cytokine production maybe required for a beneficial clinical outcome. We further sought to determine whether the reduction in TNFR2 levels was due to the direct effect of the treatment drugs or to an indirect effect of treatment, as the drugs can cause a reduction in any residual tumor cells resulting in lower TNF production, which may in turn reduce TNFR2 levels. Healthy PBMCs were cultured for 3 days with anti-CD3 and anti-CD28 in the presence or absence of azacitidine along with varying doses on lenalidomide. We first observed a significant increase in TNFR2 levels upon the addition of azacitidine alone when compared to controls (Fig. 6A) ($P < 0.05$). However, in the presence of lenalidomide (both 1 and 10 µM), the levels of TNFR2 were reduced when compared to control wells. This decrease was observed even in the presence of azacitidine, suggesting that the effect of lenalidomide on TNFR2 expression within CD4 T cell population was compared between healthy and AML donors. Statistical analyses were performed by unpaired student t-tests to compare healthy donors ($n = 13$) and AML patients ($n = 14$). Data represent mean ±/− SEM. **$P < 0.01$ ***$P < 0.001$ ****$P < 0.0001$.

In Figure 2. Comparison of the proportions of Tregs and effector T cells within TNFR2+ and TNFR2− CD4 T cells between healthy and AML donors. TNFR2+ and TNFR2− CD4 T cells were further divided into effector T cells (Teff) and Tregs based on their expression of CD25 and FOXP3. (A) The gating strategy used to identify Teff and Tregs within the TNFR2+ and TNFR2− CD4 T cell population. Teff were identified as CD25high cells while Tregs were identified based on high expression levels of CD25 followed by FOXP3 expression. An isotype for FOXP3 is represented as the tinted histogram. (B) The percentages of Tregs and Teff within the TNFR2 T cell subsets were compared between healthy donors and AML patients. (C) The ratio of Tregs to Teff within the TNFR2+ T cell population was compared between healthy and AML donors. Statistical analyses were performed by unpaired student t-tests to compare healthy donors ($n = 13$) and AML patients ($n = 14$). Data represent mean ±/− SEM. **$P < 0.01$ ***$P < 0.001$ ****$P < 0.0001$.
the presence of lenalidomide had significantly lower levels of TNFR2 expression when compared to cells stimulated in the absence of lenalidomide (Fig. 6C) \((P < 0.01)\).

**Discussion**

Although a considerable number of AML patients achieve clinical remission upon induction chemotherapy of *de novo* disease, a majority of these patients will have disease relapse. The immune system, particularly CD4 T cells, plays a crucial role in antitumor immunity as demonstrated by the role of graft-versus-leukemia in reducing the risk of relapse after stem cell transplantation in AML patients [3]. The immune status of AML patients in remission and the immunological benefits of a maintenance therapy currently remain unclear. This pilot study indicates for the first time that AML patients in remission have high levels of CD4 T cells expressing TNFR2 and a concomitant increase in the level of TNFR2+ Tregs. These potent TNFR2+ Tregs have previously been demonstrated to express a range of suppressive factors including CTLA4, GARP, sTGFβ and the ecto-enzymes, CD39 and CD73 [21,31]. We have confirmed this phenotype on TNFR2+ Tregs from the peripheral blood of AML patients and have further shown that these Tregs are a highly active subset as demonstrated by high levels of Ki-67 expression (Supporting Information Fig. 2S). As TNFR2+ Tregs can impede effector cytokine production, we also observed a significant impairment of IL-2 and IFNγ production by effector T cells from AML patients in remission. Our data indicate that AML patients in remission have an imbalanced immune system with functionally impaired CD4 effector T cells and high levels of potent TNFR2+ Tregs, fostering an immunosuppressive environment.
This immune imbalance may potentially enable the growth of any minimal residual disease, thereby augmenting the propensity for relapse within these patients. By reducing the frequency of TNFR2+ Tregs, we may potentially disrupt the immunosuppressive environment that appears to persist within AML patients, even though in clinical remission. Our data suggest the need for maintenance therapy in the postremission phase to reverse this immunological imbalance.

Another novel finding from this study is the potential immunological benefits of a maintenance therapy consisting of azacitidine and lenalidomide for AML patients at clinical remission. Treatment resulted in a reduction of TNFR2 levels on CD4 T cells and an associated reduction in the level of TNFR2+ Tregs in patients who sustained their remission at 3 months of therapy. We believe the observed reduction of Tregs was due to the loss of TNFR2 expression, as it has been previously demonstrated that TNFR2 is required for Treg function and stabilization [36]. Surprisingly, azacitidine has previously been demonstrated to increase Treg levels in vivo and in vitro [32,37]. Consistently, we observed an increase in TNFR2 levels on CD4 T cells when PBMCs were treated with azacitidine alone in vitro. In contrast, lenalidomide has been demonstrated to decrease FOXP3 expression and inhibit IL-2 mediated Treg expansion in vitro [38]. Although we did not observe a reduction in FOXP3 expression on CD4 T cells in vitro (data not shown), nonrelapsed patients had lower levels of FOXP3 expression at EOC3 when compared to the relapsed patients (P = 0.07) (Supporting Information Fig. 3S). This suggests that the drugs may have also had a qualitative effect on Treg function within the nonrelapsed patients. Additionally, lenalidomide reduced TNFR2 levels, which is required for Treg stabilization. Lenalidomide can also inhibit TNF production and as TNF can increase TNFR2 expression levels on T cells, an obvious mechanistic hypothesis for the reduction of TNFR2 levels upon lenalidomide treatment is due to decreases in TNF levels [15,35,39]. However, we could not reverse the reduction of TNFR2 by lenalidomide via the addition of exogenous TNF, suggesting in this case, a mechanism not purely mediated by TNF. A previous study has demonstrated that thalidomide analogues including lenalidomide can reduce the surface expression of TNFR2 on T cells in vitro and this reduction was not due to a decrease in TNFR2 transcription or TNFR2 receptor shedding [40]. This study demonstrated that the total cellular TNFR2 levels remained constant upon lenalidomide treatment in vitro, and suggested that lenalidomide might either inhibit the transport of intracellular TNFR2 to the cell surface or result in an increased receptor endocytosis of the surface TNFR2 molecules [40]. We also did not observe a change in soluble TNFR2 (sTNFR2) levels within the serum of AML patients upon treatment, suggesting that an increase in TNFR2 receptor shedding may not be a likely mechanism of action by lenalidomide (Supporting Information Fig. 4S). Moreover, as TNFR2 receptor endocytosis has been demonstrated to occur at a faster rate in the presence of TNF, and we did not observe any further reduction of TNFR2 levels in the

Figure 5. The effect of azacitidine and lenalidomide on effector cytokine production by effector CD4 T cells in vivo and in vitro. (A) Representative plots of effector T cells producing IL-2 or IFNγ at screening and at EOC3 from both disease-relapsed and nonrelapsed patients. (B) PBMCs were stimulated with anti-CD3/28 and cultured at a ratio of 2 × 10^5 cells/100 μL. Cells were treated with 0 or 1 μM azacitidine and with the indicated doses of lenalidomide and cultured for 3 days (n = 4). On Day 3, cells were stimulated with PMA/ionomycin for 5 hr, with the addition of Brefeldin A for the last 4 hr. Flow cytometry was performed poststimulation to identify effector T cells. The collective data of IFNγ and IL-2 producing effector T cells was compared between the drug treated samples. Statistical analyses were performed by paired t-tests to compare drug treated samples in (B). Data represent mean ± SEM. *P < 0.05 **P < 0.01 ***P < 0.001.
causing an indirect reduction of TNFR2 may be further reduced by the maintenance treatment drugs and thus in patients at clinical remission, the relatively low number of blast cells CD4 T cells and Tregs, azacitidine may indirectly reduce TNF levels. As blast cells secrete TNF, which can induce TNFR2 levels on CD4 T cells and this was also treated with the indicated drugs and cultured for 3 days. On Day 3, flow cytometry was performed to determine TNFR2 expression. (C) Healthy PBMCs (n = 4) were stimulated with CD3/28 in the presence and absence of lenalidomide (1 µM) and were cultured for 3 days. TNF was added on Day 0, Day 1, and Day 2 as indicated. On Day 3, the levels of TNFR2 expression was determined by flow cytometry. Statistical analyses were performed by paired t-tests to compare drug treated samples. Data represent mean ± SEM. *P < 0.05 **P < 0.01 ***P < 0.001.

Figure 6. The effect of azacitidine and lenalidomide on TNFR2 expression on CD4 T cells in vitro. PBMCs were stimulated with anti-CD3/28 and cultured at a ratio of 2 × 10^5 cells/100 µL. Cells were treated with 0 or 1 µM azacitidine and with the indicated doses of lenalidomide, and cultured for 3 days (n = 4). On Day 3, cells were stained with CD3, CD4, and TNFR2, and flow cytometry was performed. (A) The level of TNFR2 percentage and MFI on CD4 T cells were compared between the drug treated samples. (B) PBMCs of healthy donors (n = 4) were stimulated with CD3 alone, CD4 and TNFR2 or CD3 and CD28 and cultured at a ratio of 2 × 10^5 cells/100 µL. These cells were also treated with the indicated drugs and cultured for 3 days. On Day 3, flow cytometry was performed to determine TNFR2 expression. (C) Healthy PBMCs (n = 4) were stimulated with CD3/28 in the presence and absence of lenalidomide (1 µM) and were cultured for 3 days. TNF was added on Day 0, Day 1, and Day 2 as indicated. On Day 3, the levels of TNFR2 expression was determined by flow cytometry. Statistical analyses were performed by paired t-tests to compare drug treated samples. Data represent mean ± SEM. *P < 0.05 **P < 0.01 ***P < 0.001.

presence of TNF in vitro, this may not be a likely mechanism of action by lenalidomide either [41]. This suggests that lenalidomide may inhibit TNFR2 expression on T cells by inhibiting the transport of intracellular TNFR2 to be expressed on the surface, but the rationale for how lenalidomide can specifically inhibit TNFR2 transport is likely to be mechanistically complex.

Although lenalidomide reduced TNFR2 levels on CD4 T cells in vitro and azacitidine did not, both these drugs were able to reduce TNFR2 levels on CD4 T cells as well as TNFR2+ Tregs in vivo. As in vitro studies show azacitidine to increase TNFR2 levels and lenalidomide to decrease these levels, it would be expected that patients treated with azacitidine alone to have higher levels of TNFR2 when compared to patients treated with both azacitidine and lenalidomide. However, our clinical results suggest that patients who were administered azacitidine alone had a reduction in TNFR2 levels on CD4 T cells and this was associated with a better disease outcome. This potentially contradictory finding can be explained by several reasons. First, azacitidine may be targeting other factors that aid in preventing relapse. One such factor may be the reduction in proliferation of residual blast cells by azacitidine. As blast cells secrete TNF, which can induce TNFR2 levels on CD4 T cells and Tregs, azacitidine may indirectly reduce TNFR2 levels. In patients at clinical remission, the relatively low number of blast cells may be further reduced by the maintenance treatment drugs and thus causing an indirect reduction of TNFR2+ Tregs.

Patients also had an increase in the initially low levels of IL-2 and IFNγ production by CD4 effector T cells within 3 months of treatment, regardless of their relapse status. Lenalidomide increased effector cytokine production by CD4 T cells, as others and we have demonstrated that lenalidomide can increase IL-2 and IFNγ production by T cells in vitro and in vivo [25,42]. The increase in cytokines like IL-2 has been attributed to the ability of lenalidomide to act via the costimulatory molecule, CD28, and can induce tyrosine phosphorylation of the CD28 molecule [43]. This in turn results in an increase in transcription factors like AP-1 and NFAT-2 that are involved in the classical T cell activation pathway thereby causing higher production of effector cytokines like IL-2 [44,45]. Moreover, azacitidine when used alone was not sufficient to enhance effector cytokine production by CD4 T cells in vitro. However, when used in combination with lenalidomide, azacitidine was able to further augment the ability of lenalidomide to enhance cytokine production by CD4 T cells in vitro.

Although the practice of a maintenance therapy is not standard for the treatment of AML, our data provides evidence of immunological benefits obtained with azacitidine and lenalidomide. This combination resulted in a reduction of TNFR2 levels due to the dominant effect of lenalidomide and both these drugs worked synergistically to enhance effector cytokine production by CD4 T cells in vitro. Patients had an increase in cytokine production by CD4 effector T cells, however only a portion of these patients maintained their remission status at 3 months of therapy. Additionally, these nonrelapsed patients had significantly lower levels of TNFR2 expression at 3 months of therapy compared to the relapsed patient cohort, and this reduction was reflected by a decrease in the levels of TNFR2+ Tregs. Our pilot data suggests that despite the relatively small patient numbers in this phase Ib/II clinical trial, a concomitant reduction of TNFR2 levels and enhancement of effector cytokine production may be beneficial for AML patients to maintain their remission status. The rationale behind why some patients...
had a decrease in their TNFR2 levels and others did not, remains to be investigated. It will also be important to explore whether the patients who relapsed within 3 months of therapy may have benefited if the concentration of the drugs given was increased. Additionally, given the exploratory nature of this pilot study and due to the small patient numbers, we were not able to compare the treatment differences between the combination therapy and azacitidine alone. We hope the results of this pilot Phase I/IIb study will encourage validation in larger clinical trials to confirm the potential key role of immune modulation in preventing leukemic progression.

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