BET inhibitors synergize with anti-PD1 by rescuing TCF1+ progenitor exhausted CD8+ T cells in Acute Myeloid Leukemia

Kyle A. Romine¹, Hyun J. Cho², Yoko Kosaka², Kaelan Byrd², Jesse L. Coy², Patrick A. Flynn², Matthew T. Newman³, Christopher Loo², Jaime Scott² and Evan F. Lind¹,²,⁴

¹Department of Cell, Developmental & Cancer Biology, Oregon Health & Science University, Portland, OR, USA

²Department of Molecular Microbiology and Immunology. Oregon Health & Science University, Portland, OR, USA

³School of Medicine, Oregon Health & Science University, Portland, OR, USA

⁴Knight Cancer Institute, Oregon Health & Science University, Portland, OR, USA
Abstract

Many AML patients exhibit hallmarks of immune exhaustion such as increased myeloid derived suppressor cells (MDSCs), suppressive regulatory T cells (Tregs), and exhausted/dysfunctional T cells. We developed an AML mouse model driven by FLT3-ITD and TET2 deficiency to evaluate the immune effects of small-molecule inhibitors (SMIs) and immune checkpoint blockade (ICB). This mouse model recapitulated immune-related features in AML patients, such as increased myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs). Moreover, we found that CD8+ T cells derived from these mice exhibit a terminally exhausted phenotype (TEx PD1+, TIM3+, TCF1-), which have been shown to be refractory to ICB monotherapy. Here we show that SMIs targeting Bromodomain and Extra-Terminal (BET) proteins, in addition to targeting tumor-intrinsic factors, rescue T cell exhaustion and ICB resistance. Ex vivo treatment of splenocytes from these mice with BET inhibitors (BETi) reversed CD8+ T cell exhaustion by restoring proliferative capacity and expanding the more functional precursor exhausted T cells (TPEx: PD1+, TCF1+, TIM3-). This reversal is even more pronounced with BETi in combination with anti-PD1. Finally, we show that BETi synergizes with anti-PD1 in vivo, resulting in the reduction of circulating leukemia cells, enrichment of CD8+ T cells in the bone marrow and increased TPEx CD8+ T cells. In total, we employ an AML mouse model that is characterized by leukemia-induced immune exhaustion to show the potential efficacy of combining BETi and ICB therapy in the treatment of AML.

Introduction

Acute Myeloid Leukemia (AML) is a myeloid lineage cancer with a poor prognosis and with limited therapeutic options for those who cannot withstand current frontline therapies1-3. For other difficult-to-treat cancers, such as metastatic melanoma, therapy with immune checkpoint blockade (ICB) has filled this void for many patients and has made meaningful increases in life expectancy4, 5. ICB functions to reinvigorate cancer-specific T cells via blockade of inhibitory immune checkpoint (IC) receptors which suppress T cell activity and function. Expression of IC receptors, which include CTLA-4, PD1, TIM3, LAG3, TIGIT, VISTA, and others, increases after initial antigen exposure and regulates T cell function through various signaling pathways. It has been hypothesized that these receptors are an evolutionary adaptation to chronic antigen exposure to prevent the development of autoimmunity after infection. PD-1 is thought to suppress TCR signaling, and thus T cell activity, through several direct and indirect pathways upon engagement by its ligands PD-L1 and PD-L26.
Dephosphorylation of ZAP70 and PI3K via recruitment of phosphatases SHP1 and SHP2 to PD-1's ITIM and ITSM domains. 2.) Regulation of CD28 co-stimulation via SHP1/SHP2 mediated dephosphorylation of CK2 which increases activity of PTEN and subsequently reduces PIP3 and PI3K activity. 3.) Cell cycle arrest at G0-G1 via accumulation of CDK inhibitor p27Kip1. 4.) CBL-b mediated internalization of the TCR complex, thus reducing TCR signaling. Recent work has identified a subset of CD8+ T cells that are generated during chronic antigen exposure and demarcated by expression of both PD1 and the transcription factor TCF1. Labeled as precursor exhausted T cells (TPEx), this population has been shown to expand in response to anti-PD1 therapy and retain anti-tumor capacity. This is in contrast to CD8+ T cells that express PD1 and TIM3 but lack TCF1, which have been shown to be “terminally exhausted” (TEx) and lack proliferative and functional capacity. Our lab and others have recently shown that a proportion of blood and bone marrow specimens from AML patients exhibit hallmarks of immune exhaustion, such as increased frequency of Tregs and MDSCs, decreased T-cell proliferation, elevated expression of immune checkpoint molecules and increased TEx vs. TPEx populations. Importantly, a subset of these patient samples containing dysfunctional T cells was able to be rescued by immune checkpoint blockade. This prompted us to further investigate potential combination treatment regimens by which we could reduce T cell exhaustion in AML.

The Bromodomain and Extra-Terminal domain (BET) protein family is made up of bromodomain-containing proteins BRD2, BRD3, BRD4, and BRDT. BET proteins are epigenetic readers which bind acetylated histone residues via conserved BD1 and BD2 domains and mediate downstream functions, such as histone acetylation recognition, chromatin remodeling, and transcription regulation. BRD4 binds acetylated histone tail and recruits Positive Transcription Elongation Factor b (P-TEFb) to enhancer regions to mediate the phosphorylation of the c-terminal domain of RNA pol II, required for elongation of the nascent mRNA. RNAi screening studies identified BRD4 loss as a potent and selective inhibitor of leukemic growth and treatment with BETi induced leukemia cell death in vitro and in vivo. Clinically, however, BETi therapy was able to elicit a few complete remissions but ultimately failed as a monotherapy in the majority of patients. Interestingly, BETi have also been shown to positively affect CD8+ T cells, directly or indirectly, via reduction of PD-L1 expression on myeloid cells and inhibition of chronic TCR activation genes, such as BATF, which increased the persistence of stem-cell like memory CD8+ T cells. We therefore hypothesized that BETi may synergize with anti-PD1 therapy in AML through targeting of tumor-intrinsic factors, such as myc, and tumor-
extrinsic factors, such as promoting T cell stemness. Accordingly, we show that a FLT3-ITD/TET2 AML mouse model phenocopies the immune exhaustion and PD-1 refractoriness found in many AML patients. We found that these mice have a dramatically increased ratio of TEx:TPEx compared to wild-type mice (WTs) and that BETi can rescue immune exhaustion by promoting proliferation of TPEx CD8+ T cells. Finally, BETi treatment was found to have a synergistic effect with anti-PD1 ex vivo and in vivo in reducing leukemic tumor burden.

Results

**FLT3-ITD/TET2** mice exhibit hallmarks of immune exhaustion and decreased survival

We previously showed that our genetically-engineered mouse model, FLT3-ITD\(^{+/−}\) TET2\(^{lox/+}\) LysM-Cre\(^{+/−}\) displays a profound defect in T cell proliferative capacity\(^44\). To further characterize the tumor microenvironment and functional capacity of T cells derived from this mouse model, we performed multi-color flow cytometry to immunophenotype myeloid and lymphoid population and histology of multiple organ sites. We found dramatically increased levels of CD11b+ myeloid cells in the spleens and blood of AML mice compared to WT C57BL/6 mice (Fig 1a, b). In addition, we found significant evidence of myeloid tumor infiltration in the spleen—causing splenic follicle disruption, liver portal veins, and bone marrow (Fig 1c). Further, we found increased levels of GR1+ MDSCs and CD4+FOXP3+ Tregs, a frequent occurrence in AML patients\(^{24−26, 28, 45}\) (Fig S1a, b), but no difference in T cell frequency (Fig S1c). Characterization of the lymphoid populations identified significant increases in multiple exhaustion markers in both the CD4+ and CD8+ populations but most notably a significant decrease in T cell stem-like maintenance transcription factor TCF1 (Fig 1d,e). Taken together, we concluded that the AML mice replicated the tumor microenvironment found in AML patients who were unresponsive to ICB monotherapy.

**T cells derived from AML mice are dysfunctional and are phenotypically terminally exhausted**

We previously showed that T cells from the AML mice are dysfunctional and unresponsive to TCR stimulation\(^44\). We assessed the proliferative potential of CD4+ and CD8+ T cells derived from AML or WT mice by plating CFSE-labeled splenocytes on anti-CD3 coated plates, thus relying on naturally occurring antigen-presenting cells for co-stimulation. As shown previously, both CD4+ and CD8+ T cells derived from AML mice exhibited significantly reduced proliferation compared to WT mice in response to TCR stimulation (Fig 2a, b). We next asked whether the T cells were intrinsically dysfunctional or if the effect on proliferation occurred only when the T cells were in proximity to tumor cells. To do this, we isolated CD3+ T cells by negative selection...
magnetic bead sorting and assessed proliferation with anti-CD3 and anti-CD28 co-stimulation. The isolated T cells still lacked proliferative capacity compared to WT mice, indicating that the T cells are indeed intrinsically dysfunctional (Fig 2c). Finally, we asked whether the AML-derived CD8+ T cells were phenotypically terminally exhausted (TEx; PD1+, TIM3+, TCF1+) or precursor/progenitor exhausted (TPEx; PD1+, TCF1+, TIM3-), which have been shown to retain anti-tumor activity, predict clinical outcome, and expand with ICB16, 18, 46-49. Flow cytometric analysis of PD1+ CD8+ T cells derived from AML mice were primarily consistent with a TEx phenotype in contrast to WT mice, which were primarily TPEx (Fig 2d,e). Thus, the AML mice have an immunosuppressive microenvironment which drives CD8+ and CD4+ T cell exhaustion.

**Ex vivo treatment of splenocytes from AML mice with BETi rescues T cell dysfunction**

To identify rationally-derived combination treatment strategies which target tumor-intrinsic and extrinsic factors, we performed high-capacity drug screening as previously described50 on long-term cultures of primary cells derived bone marrow cells of the AML mice. Interestingly, we found that three of the top six most efficacious SMIs targeted BET proteins (JQ1, OTX-015, CPI-0610) (Fig 3a). Given previous work by Hirano et al. and Hogg et al. which established the role for BETi in maintaining stemness of CD8+ T cells via inhibition of TCR-activated transcription factor BATF43 and that BETi reduce expression of immunosuppressive ligand PD-L142, we asked whether BETi + anti-PD1 could impact the exhausted phenotype found in T cells from our AML mice.

To test whether BETi could rescue T cell dysfunction and rescue ICB therapy resistance, we performed proliferation assays as previously with whole splenocytes and anti-CD3 stimulation but also in the presence of 60nM BETi JQ1, 120nM JQ1, 60nM JQ1 + 10ug/mL anti-PD1, 120nM JQ1 + anti-PD1, or anti-PD1 alone. As expected, treatment with anti-PD1 alone had no effect on proliferation, as the T cells were shown to be primarily terminally exhausted (Fig 2d-g). However, treatment with JQ1 at 60nM, and more so with 120nM, significantly increased proliferation of AML-derived CD8+ T cells, whereas WT T cells were unaffected. Moreover, the combination of 120nM JQ1 and anti-PD1 had the largest effect, with a median three-fold increase in CD8+ T cell proliferation compared to anti-CD3 stimulation alone (Fig 3b, c). The benefit of adding BETi with anti-PD1 was also observed in CD4+ T cells, but was more variable between individual mice, with some only marginally benefitting from combined treatment while others were dramatically enhanced (Fig S2a). We next asked what phenotype demarcated these newly proliferating CD8+ T cells and found that they
expressed high levels of TCF1 and low levels of TIM3, indicating that BETi act mechanistically by increasing TPEx:TEx ratios (Fig 3d-f).

**In vivo**-treated AML mice have reduced tumor burden and increased T cell infiltration in the bone marrow

We next sought to determine the *in vivo* efficacy of BETi in combination with anti-PD1 and whether they modulate the tumor microenvironment. We treated AML or WT mice with Rat IgG (200ug/injection), JQ1 (50mg/kg), anti-PD1 (200ug/injection), or combined JQ1 and anti-PD1 (50mg/kg and 200ug/injection, respectively) for 14 days and measured blood white blood cell (WBC) count pre-treatment, mid-treatment, and at endpoint and characterized the tumor microenvironment by flow cytometry (Fig 4a). Analysis of WBC counts over time found that only the combination of JQ1 + anti-PD1 significantly reduced tumor burden and, as expected from the *ex vivo* proliferation assays, anti-PD1 alone had no effect (Fig 4b). Characterization of the tumor microenvironment showed no significant changes to the frequency of Tregs, MDSCs, or splenic CD3⁺ T cells (Fig S3a,b, 4c). However, we observed a significant increase in CD8⁺ T cells in the bone marrow in the JQ1 + anti-PD1 treatment mice, suggesting that this combination treatment is having a greater impact on CD8⁺ T cells specifically and not on other immunosuppressive cell types (Fig 4d). Finally, phenotypic characterization of the splenic T cells found that JQ1-treated mice had significantly increased TPEx CD8⁺ T cells (Fig 4e) and decreased TEx CD8⁺ T cells (4f). Together, these results highlight the potential of combining BETi and anti-PD1 to treat immunosuppressed T cells in AML, particularly in ICB-refractory cases.

**Discussion**

**Immune checkpoint blockade in hematological malignancies**

ICB therapy in the blood cancer setting is relatively new and although dozens of clinical trials are ongoing and actively recruiting ⁵¹, no large scale study has been completed evaluating the efficacy of anti-PD1 therapies in AML. Our lab previously described the functional immune microenvironment of 50 newly-diagnosed AML patient bone marrow samples⁵⁶ and found that only 41% of samples showed T cell proliferation comparable to healthy donor. Further, we found that the AML patient samples with the most profound immunosuppression (37%) had severely dysfunctional T cells with significantly decreased effector cytokine production such as IFN-y, IL-2, and TNFa, and increased expression of inhibitory checkpoint markers such as CTLA-4. Of these non-proliferator AML samples, 6 of 18 samples were not rescued with ICB treatment in vitro, as measured by lack
of proliferation and production of effector molecules. Combination treatment regimens that potentially synergize and rescue TEx cells may be the key to greater success with ICB therapy clinically. Smaller clinical trials have evaluated the efficacy of anti-PD1 + hypomethylating agents (HMA) but only a small subset, roughly 33%, responded to therapy\textsuperscript{52}, which is consistent with our \textit{ex vivo} findings\textsuperscript{51}. Phenotypic profiling of these responsive patients before and after anti-PD1 +/- HMA identified pre-therapy bone marrow and peripheral blood CD3\textsuperscript{+} and CD8\textsuperscript{+} levels as predictive of response. In addition, we previously described the synergistic combination of combining MEKi and anti-PD1\textsuperscript{44}. We found that MEKi acted directly on both tumor cells and immune cells globally by reducing PD-L1 expression on patient samples and, at low doses, restored some proliferative function of AML-derived T cells. Thus, it is clear that treatment strategies that target both tumor-intrinsic factors as well as T cell suppression will be more effective. Here, we show the potential for BETi therapy to directly reprogram exhausted CD8\textsuperscript{+} T cells to a more stem-like, TCF1-expressing subtype which expands and proliferates in response to ICB in AML.

\textbf{Epigenetic regulation of T cell exhaustion}

T cell exhaustion is driven by chronic antigen exposure and is accompanied by vast changes in the epigenetic landscape\textsuperscript{22, 23, 53-55}. This T cell state is identifiable by three main traits: 1) increased expression of inhibitory receptors such as PD1, LAG3, TIGIT, and CTLA-4, 2) decreased secretion of effector cytokines such as IL-2, IFN-\textgamma, TNF\textalpha and 3) loss of proliferative capacity\textsuperscript{23, 56-59}. These epigenetic changes are largely driven by a pool of coordinating TCR-responsive transcription factors, such as BATF, IRF4, TOX, and NFAT\textsuperscript{15, 57-59}, chromatin remodeling complexes such as EZH2, and other polycomb repressive complex 2 proteins\textsuperscript{60-63}. Generation of TPEx cells relies on the de-repression of many critical pro-memory transcription factors such as TCF1, FOX01, and others as a consequence of targeted DNA methylation deposition acquired during early effector differentiation. Interestingly, the pro-exhaustion transcription factor TOX was found to also directly modulate histone acetylation via direct binding to histone acetyltransferase KAT7\textsuperscript{23, 53, 64}. This critical role for epigenetic regulation of T cell exhaustion has garnered much interest in investigating the potential of several epigenetic targeting SMI, whose original design was directed towards targeting tumor-intrinsic epigenetic dysregulation\textsuperscript{21}. Very recently, Milner \textit{et al.} showed that BRD4 regulates T cell differentiation by promoting super-enhancer activity at regions regulating key pro-exhaustion/differentiation genes such as \textit{Id2}, \textit{Cx3cr1}, and \textit{Runx1}\textsuperscript{65}. Using our unique mouse model of AML, we find that BET inhibition in combination with ICB therapy can lead to a shift
from predominantly non-responsive T cells with a TEx phenotype to TPEx and the production of functional
CD8+ T cells. These results form the basis for further study of such combination therapies in AML.

Methods

AML murine model

Original breeders were purchased from The Jackson Laboratory and bred in the Department of Comparative
Medicine animal facility at OHSU. Mice expressing FLT3-ITD under the endogenous FLT3 promotor (strain
B6.129-Flt3tm1Dgg/J, stock no. 011112) were crossed to mice with the Tet2 gene flanked by LoxP sites (strain
B6;129S-Tet2tm1.1Iaai/J, stock no. 017573). The FLT3-ITD/Tet2 flox mice were then crossed to mice
expressing CRE recombinase controlled by the LysM promotor (strain B6.129P2-Lyz2tm1(cre)Ifo/J, stock no.
004781). All mice used in these experiments were bred as heterozygous for FLT3-ITD, TET2flox, and
LysMCre. All mouse experiments were performed in accordance with Institutional Animal Care and Use
Committee protocol IP00000907.

Flow Cytometry Staining

Bone marrow, blood, or splenocytes were processed and subjected to red blood cell (RBC) lysis by ACK
before counting via hemacytometer. 5 million cells were resuspended in PBS and stained with Zombie Aqua
viability dye (Biolegend, Cat# 423102) for 15 minutes at room temperature, covered from light. The cells were
then washed with FACS buffer (PBS, 2% calf serum, 0.02% sodium azide) and resuspended in 25uL 1:50
mouse FC block (TruStain FcX, BioLegend Cat# 101320), and left on ice for 5 minutes. 25uL of a 2x cell
surface staining antibody cocktail was added directly on top of the cells (final FC block 1:100, 1x Ab
concentration) and stained on ice for 30 minutes. For intracellular staining, the cells were then washed with
FACS buffer, permeabilized and stained for intracellular targets according to manufacturer's protocol
(eBioscience FOXP3 Transcription Factor Staining Buffer Set, Cat# 00-5523-00), then resuspended in FACS
buffer before analyzing on either a BD LSRFortessa or Cytek Aurora flow cytometer. Data was analyzed using
FlowJo software.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Cat#</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbet</td>
<td>4B10</td>
<td>644823</td>
<td>Biolegend</td>
</tr>
<tr>
<td>LAG-3</td>
<td>C9B7W</td>
<td>125224</td>
<td>Biolegend</td>
</tr>
<tr>
<td>TIM3</td>
<td>RMT3-23</td>
<td>119723</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD44</td>
<td>IM7</td>
<td>125224</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD62L</td>
<td>MEL-14</td>
<td>104453</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>
Liver, spleen, and bone marrow snips were fixed and cut into paraffin blocks for H&E staining. The slides were then scanned using an Aperio AT2 scanner and analyzed with ImageScope.

**H&E Histology**

Bone marrow aspirates from three AML mice were combined and cultured in RPMI containing 20% fetal bovine serum (FBS), streptomycin/penicillin, 50µM 2-mercaptoethanol (RPMI-20), and supplemented with murine SCF (10ng/ml) and IL-3 (10ng/ml) (Peprotech or BioLegend). The cells were serially passaged for ~1 month.

Inhibitor library screening to evaluate drug sensitivity was performed as previously described. Briefly, cultured AML mouse-derived tumor cells were counted and seeded into four 384 well plates at a concentration of 2000 cells/well. The cells were then subjected to titrations of 188 unique small-molecule inhibitors in culture for 72 hours. MTS reagent (CellTiter96 AQueous One; Promega) was added and the optical density was read at 490 nm to assess viability.

**Ex vivo Proliferation Assays**

96-well round-bottom plates were coated in either 2.5ug/mL anti-CD3 (Biolegend, clone 145-2C11,Cat# 100359) or HlgG (Biolegend, clone HTK888, Cat#400959), at 4°C overnight, then washed with PBS.

Splenocytes were harvested from AML or WT mice and stained with 2uM CFSE (Life Technologies, Thermo Fisher) at 5x10^6/ml at 37°C in the dark. After 15 minutes, the CFSE was quenched with 10mL calf serum and washed with PBS. Cells were then plated at 5x10^5/well in RPMI with 10% FBS with BME supplementation and penicillin/streptomycin (RPMI-10). After a 72-hour incubation at 37°C the cells were stained for flow cytometric
analysis and analyzed as previously described. Anti-PD1 (Clone RMP1-14) and Rat IgG (2A3) were purchased from BioXCell. JQ1 was purchased from Cayman Chemical (#11187).

**BETi + anti-PD1 treatment in vivo**

Age range of AML mice used in *in vivo* experiments was 25 to 54 weeks. WT and AML mice were given 7 doses of 200ug RlgG, 50mg/kg JQ1, 50mg/kg JQ1 + 200ug anti-PD1, or 200ug anti-PD1 via i.p injection over two weeks (3 times per week, harvesting 1 day after 7th/final injection). Drug solutions were prepared in a solvent of 10% cyclodextrin in PBS from a stock solution of JQ1 (50mg/mL) in DMSO, followed by sonication/heat batch to dissolve the JQ1. Assessment of tumor burden in the blood, as determined by WBC measurements from Hemavet (950FS, Drew Scientific), was monitored weekly pre-, mid-, and post-treatment. At endpoint, single cell suspensions of bone marrow, blood, and spleen tissues were stained for flow staining as previously described.

**Author’s Disclosure’s**

EFL has received research funding from Janssen, Celgene, Monojul, Ikena Oncology, Kronos Bio, Intellia Therapeutics and Amgen.

**Author Contributions**

**K.A. Romine**: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing. **H-J.C**: methodology, validation **Y.K**: Conceptualization, formal analysis, validation, investigation, writing-review and editing **K.B**: methodology **J.L.C**: methodology, writing-review and editing. **P.A.F**: methodology **M.T.N**: methodology **C.L**: methodology **J.S**: methodology **E.F.L**: Conceptualization, formal analysis, validation, investigation, writing-review and editing.

**Acknowledgments**

This work was funded by a grant NCI U54CA224019 and NCI U01 CA217862 to EFL. K.A.R was funded in part by a Program in Enhanced Research Training (PERT) T32 # 5T32GM071338-13. Flow Cytometry was performed at the OHSU Flow Cytometry Core Facility.

**References**


Figure 1: Mouse Model Characterization

A. WT vs AML Spleen Tumor Burden

B. WT vs AML Blood Tumor Burden

C. WT vs AML, Spleen, Liver, Bone Marrow

D. WT vs AML CD4 T cell CTLA-4 Expression, WT vs AML CD4 T cell TBET Expression, WT vs AML CD4 T cell CD44 Expression, WT vs AML CD4 T cell PD-1 Expression

E. WT vs AML CD8 CTLA-4 Expression, WT vs AML CD8 TBET Expression, WT vs AML CD8 CD44 Expression, WT vs AML CD8 TCF1 Expression, WT vs AML CD8 EOMES Expression, WT vs AML CD8 LAG3 Expression, WT vs AML CD8 TIGIT Expression

Figure 1: Mouse Model Characterization

A. WT vs AML Spleen Tumor Burden

B. WT vs AML Blood Tumor Burden

C. WT vs AML, Spleen, Liver, Bone Marrow

D. WT vs AML CD4 T cell CTLA-4 Expression, WT vs AML CD4 T cell TBET Expression, WT vs AML CD4 T cell CD44 Expression, WT vs AML CD4 T cell PD-1 Expression

E. WT vs AML CD8 CTLA-4 Expression, WT vs AML CD8 TBET Expression, WT vs AML CD8 CD44 Expression, WT vs AML CD8 TCF1 Expression, WT vs AML CD8 EOMES Expression, WT vs AML CD8 LAG3 Expression, WT vs AML CD8 TIGIT Expression

Figure 1: Mouse Model Characterization

A. WT vs AML Spleen Tumor Burden

B. WT vs AML Blood Tumor Burden

C. WT vs AML, Spleen, Liver, Bone Marrow

D. WT vs AML CD4 T cell CTLA-4 Expression, WT vs AML CD4 T cell TBET Expression, WT vs AML CD4 T cell CD44 Expression, WT vs AML CD4 T cell PD-1 Expression

E. WT vs AML CD8 CTLA-4 Expression, WT vs AML CD8 TBET Expression, WT vs AML CD8 CD44 Expression, WT vs AML CD8 TCF1 Expression, WT vs AML CD8 EOMES Expression, WT vs AML CD8 LAG3 Expression, WT vs AML CD8 TIGIT Expression
a.) Splenocytes isolated from FLT3-ITD^+/-, TET2^+/flox, LysCre^+/-(AML, red) or C57BL/6 (WT, black) mice were isolated and stained as described for live CD11b^+ cells and evaluated by flow cytometry. Significance determined by Mann-Whitney T-test.

b.) Spleens were isolated from 15 WT (black) and 24 AML (red) mice and weighed. Median weights for each group displayed on right side of plot. Significance determined by Mann-Whitney T-test.

c.) Blood isolated from 24 WT (black) and 20 AML (red) mice and stained as described for live CD11b^+ cells and evaluated by flow cytometry. Significance determined by Mann-Whitney T-test.

d.) Representative H&E staining of WT and AML mice-derived spleen (Top row, 500um scale), liver (middle row, 200um scale), and bone marrow (bottom row, 100um scale).

e.) Briefly, splenocytes from untreated WT (black) and AML (red) mice were assessed for expression, via median fluorescence intensity, of markers of immune exhaustion on CD4^+ T cells (Live, CD11b^-, CD3^+, CD4^+). Top row (Left to right: CTLA-4, TBET, CD44, PD-1). Bottom Row (Left to right: EOMES, LAG3, TIGIT, TCF1). Significance determined by multiple Mann-Whitney T-tests. CD44, PD-1, LAG3 significantly increased in AML mice. CTLA-4, EOMES, and TCF1 significantly decreased in AML mice.

f.) Briefly, splenocytes from untreated WT (black) and AML (red) mice were assessed for expression, via median fluorescence intensity, of markers of immune exhaustion on CD8^+ T cells (Live, CD11b^-, CD3^+, CD8^+). Top row (Left to right: CTLA-4, TBET, CD44, PD-1). Bottom Row (Left to right: EOMES, LAG3, TIGIT, TCF1). Significance determined by multiple Mann-Whitney T-tests. CD44, EOMES, and TIGIT significantly increased in AML mice. TCF1 significantly decreased in AML mice.
Figure 2: AML mouse-derived CD8+ T cells are intrinsically dysfunctional and unresponsive to TCR stimulation

a, b.) WT (black) and AML (red) splenocytes were isolated, stained with proliferation dye (CFSE), and cultured for 72 hours with anti-CD3. Proliferation of a.) CD8+ T cells and b.) CD4+ T cells was then assessed by flow cytometry, staining with viability and markers to identify T cells. Proliferation displayed is percent CFSE diluted relative to unstimulated (HlgG) control for each cell type. Significance determined by Mann-Whitney T-tests.

c.) WT (black) and AML (red) T cells were isolated from splenocytes via CD3 negative isolation magnetic beads. The T cells were then stained with CFSE and plated for 72 hours with anti-CD3 and anti-CD28 stimulation. The cells were then harvested and assessed by flow cytometry. Significance determined by Mann-Whitney T-test.

d., e.) Splenocytes derived from WT (black) and AML (red) mice were stained for surface and intracellular markers of T cells exhaustion and evaluated by flow cytometry. d.) Terminally Exhausted CD8 T cells (TEx) are represented as %TIM3+/TCF1- of the CD8+/PD1+ T cells. e.) Progenitor Exhausted CD8 T cells (TPEx)
are represented as %TCF1+/TIM3- of the CD8+/PD1+ T cells. Significance determined by Mann-Whitney T-tests.
Figure 3: AML mice T cells are refractory to ICB therapy but partially rescued with BET inhibition ex vivo

a.) AML cells passaged for ~ 1 month were subjected to an inhibitor library panel, as previously described\textsuperscript{50}. Briefly, cells were counted and seeded into multiple 384 well plates containing titrations of 188 inhibitors, incubated for 72 hours, and viability assessed by MTS assay. Plot represents each Areas Under the Curve (AUC) for every inhibitor on the panel. BET inhibitors JQ1, OTX-015, and CPI-0610 are highlighted in red.

b., c.) Splenocytes were isolated from b.) 7 AML or c.) 6 WT mice, stained with CFSE, and cultured for 72 hours without TCR stimulation (HlgG), anti-CD3 alone, anti-CD3 with anti-PD1 alone, and titrations of anti-CD3 with JQ1 or anti-CD3 with both JQ1 and anti-PD1. Cells were then harvested and stained for assessment by flow cytometry. Plots represent the fold change in proliferation in CD8 T cells, as measured by percent CFSE diluted relative to anti-CD3 stimulated alone. Significance determined by Kruskal-Wallis multiple comparisons T-tests.

d., e.) Effect of BETi, aPD1, or BETi + anti-PD1 treatment on the percent of d.) TPEx CD8\textsuperscript{+} T cells and e.) TEx CD8\textsuperscript{+} T cells after culturing for 72 hours as previously in b-c. Significance determined by Kruskal-Wallis multiple comparisons T-tests.

f.) Splenocytes were isolated from 4 AML mice, stained with CFSE, and plated for 72 hours without TCR stimulation (HlgG), anti-CD3 alone, anti-CD3 with anti-PD1 alone, and titrations of anti-CD3 with JQ1 or anti-CD3 with both JQ1 and anti-PD1. Proliferation of specifically TPEx (black) and TEx (red) CD8\textsuperscript{+} T cells in AML mice was assessed by flow cytometry. Significance determined by Kruskal-Wallis multiple comparisons T-tests.
comparisons T-tests. Representative flow dot plot (right) shows CFSE (x-axis) vs TCF1 (y-axis) on CD8+ T cells.
Figure 4: *in vivo* treatment with BETi and anti-PD1 synergizes in reducing tumor burden and enhances CD8 T cell activity

**A.** Schematic detailing *in vivo* BETi + anti-PD1 treatment strategy and functional readouts of efficacy.

**B.** Mice treated with RIgG, JQ1, JQ1 + anti-PD1, or anti-PD1 alone were bled periodically over a two-week period and white-blood cell count assessed by hemavet. Data displays fold change WBC (k/uL) normalized per mouse (ie mid-point = Mouse A day 7 WBC/Mouse A Day 0 WBC, etc.). Significance determined by one-way anova. X-axis indicates time point (Pre-Bleed = day 0, Mid-bleed = day 7, Endpoint = day 14). Y-axis indicates fold change WBC (k/uL).

**C.** Bone marrow cells were isolated from treated AML and WT mice and assessed by flow cytometry. Graph denotes c.) %CD3+ T cells in the bone marrow vs treatment and d.) %CD8+ T cells in the bone marrow as a percent of all T cells. Significance derived from combining two experimental replicates and determined by one-way anova.

**D.** Splenocytes derived from AML mice treated with RIgG, JQ1, JQ1 + anti-PD1, or anti-PD1 were assessed by flow cytometry to determine e.) TPEX CD8+ T cells (%TCF1+/TIM3- of CD8/PD1+) and f.) TEX CD8+ T cells (%TIM3+/TCF1- of CD8/PD1+). Significance determined by one-way ANOVA.
Supplemental Figures

**Supplemental Figure 1:** Treg, MDSC, and total T cell frequencies in AML and WT mice

a-c.) Splenocytes isolated from *FLT3-ITD* <sup>-/-</sup>, *TET2* <sup>+/-flox</sup>, *LysCre* <sup>+/-</sup> (AML, red) or C57BL/6 (WT, black) mice were isolated and stained as described for a.) Treg cells – CD4<sup>+</sup>, FOXP3<sup>+</sup>, b.) MDSCs – CD11b<sup>+</sup>, GR1<sup>+</sup>, and c.) All T cells – CD3<sup>+</sup>, and evaluated by flow cytometry. Significances determined by Mann-Whitney T-tests.
Supplemental Figure 2: CD4+ T cell ex vivo proliferation vs BETi + anti-PD1

a.) Splenocytes were isolated from b.) 6 AML mice stained with CFSE, and cultured for 72 hours without TCR stimulation (HlgG), anti-CD3 alone, anti-CD3 with anti-PD1 alone, and titrations of anti-CD3 with JQ1 or anti-CD3 with both JQ1 and anti-PD1. Cells were then harvested and stained for assessment by flow cytometry. Plots represent the fold change in proliferation in CD4 T cells, as measured by percent CFSE diluted relative to anti-CD3 stimulated alone. Significance determined by Kruskal-Wallis multiple comparisons T-tests.
Supplemental Figure 3: Treg and MDSCs vs \textit{in vivo} BETi + anti-PD1 treatment

A. MDSCs vs BETi + aPD1

B. Tregs vs BETi + aPD1

a., b.) Splenocytes derived from AML mice treated with RlgG, JQ1, JQ1 + anti-PD1, or anti-PD1 were assessed by flow cytometry to determine a.) \%MDSCs (CD11b\textsuperscript{+}/GR1\textsuperscript{+}) and b.) \%Tregs (CD4\textsuperscript{+}/FOXP3\textsuperscript{+}). No significance was observed between treatments in AML or WT mice independently.