Venetoclax Increases Intratumoral Effector T Cells and Antitumor Efficacy in Combination with Immune Checkpoint Blockade

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ABSTRACT

The antiapoptotic protein BCL2 plays critical roles in regulating lymphocyte development and immune responses, and has also been implicated in tumorigenesis and tumor survival. However, it is unknown whether BCL2 is critical for antitumor immune responses. We evaluated whether venetoclax, a selective small-molecule inhibitor of BCL2, would influence the antitumor activity of immune checkpoint inhibitors (ICI). We demonstrate in mouse syngeneic tumor models that venetoclax can augment the antitumor efficacy of ICIs accompanied by the increase of PD-1+ T effector memory cells. Venetoclax did not impair human T-cell function in response to antigen stimuli in vitro and did not antagonize T-cell activation induced by anti–PD-1. Furthermore, we demonstrate that the antiapoptotic family member BCL-XL provides a survival advantage in effector T cells following inhibition of BCL2. Taken together, these data provide evidence that venetoclax should be further explored in combination with ICIs for cancer therapy.

SIGNIFICANCE: The antiapoptotic oncoprotein BCL2 plays critical roles in tumorigenesis, tumor survival, lymphocyte development, and immune system regulation. Here we demonstrate that venetoclax, the first FDA/European Medicines Agency-approved BCL2 inhibitor, unexpectedly can be combined preclinically with immune checkpoint inhibitors to enhance anticancer immunotherapy, warranting clinical evaluation of these combinations.

INTRODUCTION

The regulation of programmed cell death is crucial for orchestrating the development of the immune system and the complex cellular dynamics of immune responses. B-cell lymphoma protein 2 (BCL2) family proteins, which regulate the intrinsic apoptosis pathway, play a key role in immune cell development, response, and homeostasis (1). The BCL2 family can be subdivided into antiapoptotic (prosurvival) and proapoptotic (prodeath) proteins, whose expression levels and dynamic interactions dictate whether a cell lives or dies. Cloned from the breakpoint of the t(14;18) translocation in human B-cell lymphoma, BCL2 was the first member of the family to be identified (2). Like its closely related family members BCL-XL and MCL1, BCL2 is a prosurvival protein and plays crucial roles during embryogenesis, hematopoiesis, and development of the immune system (3). Bcl2-deficient mice undergo normal embryonic development of the hematopoietic system, but develop lymphocytopenia by 3 to 4 weeks after birth as a result of cells of the thymus and spleen that...
undergo massive apoptosis (4, 5). Models of murine infection demonstrate that memory CD8+ T cells express higher levels of BCL2 than naive T cells, and that BCL2 is upregulated during antigen-induced stimulation (6, 7). In addition to BCL2, BCL-X, is induced in anti-CD3/CD28-activated T cells, which has been shown to enhance their survival in response to apoptosis-inducing agents (8). Interestingly, even though Bcl2-deficient mice developed lymphocytopenia, lymphocytes that survive in these mice responded normally to numerous stimuli, such as anti-CD3, phorbol 12-myristate 13-acetate plus ionomycin, lipopolysaccharide, and anti-CD3 antibody (9). Furthermore, BCL2 was not required to maintain memory T cells (10).

BCL2 has also been implicated in oncogenesis and maintaining the survival of numerous tumor types, making it an attractive therapeutic target (11). Venetoclax (ABT-199, Venclexta, Venclyxto) is a selective small-molecule inhibitor of BCL2 that can induce tumor cell apoptosis. Venetoclax is approved as a single agent and in combination with rituximab for patients with chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL), with or without 17p deletion, who have received at least one prior therapy, as well as in combination with obinutuzumab for previously untreated patients with CLL or SLL. It also received accelerated approval from the FDA in combination with azacitidine, decitabine, or low-dose cytarabine for the treatment of newly diagnosed acute myeloid leukemia (AML) in adults who are age 75 years or older, or who have comorbidities that preclude use of intensive induction chemotherapy. Signs of clinical activity have also been observed in a variety of other malignancies including mantle cell lymphoma, multiple myeloma, and breast cancer (12–15).

Tumor immunotherapy, especially targeting the immune checkpoint proteins PD-1 and its ligand PD-L1, has shown clinical promise in reinvigorating the immune system against cancer (16). Nivolumab/pembrolizumab/cemiplimab (anti–PD-1) and atezolizumab/durvalumab/avelumab (anti–PD-L1) are now approved for the treatment of various tumor types, including melanoma, Merkel cell carcinoma, lung cancer, renal cell carcinoma, head and neck cancer, triple-negative breast cancer, urothelial cancer, and unrespectable metastatic solid tumors with microsatellite instability or mismatch-repair deficiencies (16). Although anti–PD-1/PD-L1 therapies are promising, only subsets of patients in certain types of tumors respond. Thus, combining anti-PD-1/PD-L1 with other therapeutic modalities is of interest to improve the overall response rate and the durability of remission.

Although the importance of BCL2 has been established for both effector and memory T-cell responses during homeostasis or following infection (9, 10), little is known about the role of BCL2 during antitumor immune responses. Because venetoclax can cause reductions in T-cell numbers (17, 18), we hypothesized that combining it with immune checkpoint inhibitors would antagonize their antitumor activity.

The studies presented herein refute our hypothesis, indicating instead that the combinations of venetoclax with anti–PD-1 or anti–PD-L1 enhance tumor growth inhibition in syngeneic mouse models via an immune-dependent mechanism. Although venetoclax reduced lymphocytes, including T-cell numbers, it neither inhibited T-cell function in response to antigen nor antagonized the activity of immune checkpoint inhibitor to antigen stimuli. The reduction in T-cell numbers was attributed primarily to the sensitivity of naive T cells to venetoclax, whereas T effector cells were largely insensitive, likely due to increased reliance on BCL-XL. Tumor-bearing mice treated with venetoclax demonstrated an increase in CD8+PD-1+ T effector memory (TEM) cells within the tumors. Furthermore, venetoclax augmented anti–PD-1/PD-L1 activity in immunocompetent mouse tumor models without compromising the memory T-cell response. Moreover, administration of venetoclax in healthy human subjects showed that the proportion of effector cells increased following treatment, confirming our findings in murine models and human cells cultured ex vivo. Together, these data indicate that T cells critical for an antitumor immune response are unaffected by venetoclax and that combination of venetoclax with immune checkpoint blockade improves antitumor activity in preclinical models.

**RESULTS**

**Venetoclax Augments the Antitumor Activity of Anti–PD-1 and Anti–PD-L1 Antibodies In Vivo**

To investigate the effects of venetoclax on the antitumor activity of anti–PD-1/PD-L1 treatment, we performed tumor efficacy studies in immunocompetent C57BL/6 mice bearing MC38 tumors. Analysis from eight independent studies indicated that, as expected, anti–PD-1 led to significant tumor growth inhibition compared with isotype control (P = 0.0001), and this was, unexpectedly, further enhanced by cotreatment with venetoclax (venetoclax/anti–PD-1 cotreatment versus anti–PD-1 alone, P = 0.005; Fig. 1A; Supplementary Fig. S1A–S1I). Venetoclax also increased tumor growth inhibition when combined with anti–PD-L1 (Supplementary Fig. S2). We also observed increased tumor growth inhibition with venetoclax plus anti–PD-1 or anti–PD-L1 compared with anti–PD-1 or anti–PD-L1 alone in immunocompetent BALB/c mice bearing anti–PD-1/PD-L1–resistant CT26 tumors (Supplementary Fig. S3A and S3B).

These findings warranted exploration of whether the augmented checkpoint inhibitor activity in vivo may be the result of venetoclax-dependent tumor cell–intrinsic effects. When cultured in vitro, the MC38 colon cancer cell line was resistant to venetoclax-mediated killing up to concentrations as high as 3 μmol/L (Supplementary Fig. S4). We observed no decreases in viability or proliferation, and no signs of increased apoptosis over 3 days of treatment (Supplementary Fig. S4A–S4C). In addition, no increases in immunomodulatory markers including secreted cytokines and cell-surface MHC I or PD-L1 were observed following venetoclax treatment (Supplementary Fig. S4D and S4E). At the transcriptional level, we did not observe modulation of gene expression in MC38 cells following venetoclax treatment in vitro (data not shown). Treatment of MC38 tumor-bearing SCID mice with venetoclax showed minimal changes in gene expression compared with the vehicle-treated mice [only 62 genes significantly modulated (P < 0.05, and log fold change > 0.5), with no enrichment of distinct pathways; data not shown]. To determine whether the antitumor activity of venetoclax with anti–PD-1 or anti–PD-L1 was immune dependent, we next tested the efficacy of these combinations in SCID
A. CD8+ BCL2+, CD62L−, CD44+, PD-1+
B. CD8+ BCL2+, CD62L+, CD44−, PD-1−
C. B cells (CD19+)
D. CD4+
E. CD8+ T cells
F. CD8+ T cells
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mice transplanted with MC38 cells. As expected, treatments with either anti–PD-1 or anti–PD-L1 were not effective in this mouse strain. Consistent with the lack of in vitro activity against MC38 cells, venetoclax showed no antitumor activity in SCID mice, either as a single agent or in combination with the checkpoint inhibitors (Supplementary Fig. S5A and S5B). Furthermore, CD8 T-cell depletion was sufficient to abrogate the combination effect of venetoclax with anti–PD-1 (Supplementary Fig. S5C). These data suggest that cancer-cell-intrinsic inhibition of BCL2 in vitro or in vivo does not result in activation of cell death pathways and does not intrinsically enhance immunogenicity of MC38 tumor cells.

Following in vivo treatment in immunocompetent mice bearing MC38 or CT26 tumors, all mice achieving complete regression (CR) remained tumor-free for more than 3 months without evidence of tumor regrowth. Surviving CR mice were challenged by re inoculation with MC38 or CT26 tumor cells, respectively, to evaluate retention of tumor-specific memory T cells. Following rechallenge, none of the CR mice grew tumors, demonstrating that these mice had developed and retained immunologic memory. In addition, splenocytes isolated from these mice secreted IFNγ when cocultured with irradiated MC38 or CT26 cells, respectively, ex vivo (Supplementary Fig. S6), demonstrating tumor-specific immune memory. In total, these data demonstrate that the augmented efficacy of the combination treatment is T cell–driven and indicate that venetoclax improves anti–PD-1/PD-L1–driven antitumor immune responses.

Venetoclax Increases the Number of Intratumoral CD8+ T Effector Memory Cells

To examine the effect of venetoclax on tumor-infiltrating lymphocytes (TIL), we performed flow cytometry analysis of excised MC38 tumors on day 14 following tumor inoculation (7 days after initial dosing). At day 14, the tumors across all treatment groups were of similar size (Supplementary Fig. S7A). We observed that venetoclax decreased the total number of B and T cells in the peripheral blood, as reported previously (18), and in the tumors of these mice (Supplementary Fig. S7B). t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis of tumor-infiltrating immune cells revealed two discrete populations of CD8+ BCL2+ T cells: Population 1 is a population of activated and effector-like cells that are CD62L−, CD44+, and PD-1+, and population 2 is a population of naïve-like cells that are CD62L+, CD44−, and PD-1− (Fig. 1B). In response to venetoclax treatment, the B-cell population was decreased (population 3; Fig. 1C) and no change was observed in the CD11b+,

F4/80+ macrophage population (Supplementary Fig. S7C). Interestingly, venetoclax treatment led to a decrease of the naïve-like CD8+ T cells (population 2, PD-1−) and an enrichment of the activated, TEM-like cells (population 1, PD-1+; Fig. 1C and D). This increase in TEM-like cells was further enhanced with the combination of anti–PD-1 and venetoclax (Fig. 1D). To depict the data highlighted from t-SNE analysis, we show, using bivariate scatter plots, the significant reduction in the naïve-like T cells (CD62L+, BCL2+, PD-1−) with venetoclax while enriching for activated effector-like T cells (Fig. 1E; Supplementary Fig. S7D). This phenomenon is the same regardless of combination treatment with anti–PD-L1 or anti–PD-1. The data presented show that venetoclax specifically targets naïve-like T cells. Previous studies have shown that activated T cells upregulate additional antiapoptotic molecules, including BCL-XL (8). Thus, we hypothesized that TEM-like cells infiltrating the tumor microenvironment (TME) might upregulate BCL-XL, which could render these cells insensitive to BCL2 inhibition. Indeed, we found that the CD8+ T cells remaining within the tumor after venetoclax treatment were enriched for BCL-XL expression (Fig. 1F; Supplementary Fig. S7E), providing a possible explanation for their resistance to venetoclax.

These data demonstrate that venetoclax treatment can augment the antitumor activity of anti–PD-1 or anti–PD-L1 antibodies. Moreover, venetoclax treatment not only spares TEM-like cells but also increases the absolute number of these effector T cells in the tumors of the MC38 syngeneic model. The number of TEM cells can be further increased by combining venetoclax with checkpoint inhibitors. Collectively, these data show that venetoclax and checkpoint inhibitors can work in concert to increase effector T cells in the TME and reduce tumor growth.

Venetoclax Treatment Differentially Affects Human T-cell Subsets In Vitro

We next explored the potential effects of venetoclax on lymphocyte subsets in cultured human peripheral blood mononuclear cells (PBMC). Treated samples exhibited a concentration-dependent decrease in the number of B cells and T cells (CD4+ and CD8+ T cells; Supplementary Fig. S8A). While B lymphocytes were the most sensitive to venetoclax, CD8+ T cells were more sensitive than CD4+ T cells (Fig. 2A; Supplementary Fig. S8A), confirming data reported previously (18). Natural killer cells and T regulatory cells (Tregs) were also less sensitive to venetoclax than CD8+ T cells (Supplementary Fig. S8A). With further characterization of the T-cell subsets, we found that naïve T cells (TN; CD62L+CD45RA−) were the most sensitive T-cell subset, and both their number and...
Figure 2. Venetoclax treatment differentially affects human T-cell subsets in vitro. A, Total CD8+ and CD4+ T-cell numbers following 24-hour treatment with increasing concentrations of venetoclax. B, Total cell numbers from CD8+ and CD4+ T-cell subsets following 24-hour treatment with venetoclax. TN, naïve T cells; TCM, central memory T cells; TEM, effector memory T cells; TEMRA, effector memory T cells expressing CD45RA, also known as terminally differentiated effector memory T cells. C, Venetoclax effect on the proportion of T-cell subsets [average of samples from nine donors examined in B]. D, BCL2 expression in CD8+ and CD4+ T-cell subsets (fluorescent intensity determined by flow cytometry). E, BCL2 and BCL-XL relative protein expression in resting and CD3/CD28-activated T cells (fluorescent intensity determined by flow cytometry). Paired t test was used for statistical analysis: ns, not significant (P > 0.05); *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 compared with DMSO control. MFI, mean fluorescence intensity.
proportion decreased with increasing venetoclax concentrations. In contrast, even though the total number of memory T cells decreased with venetoclax treatment, their relative proportion, specifically CD8+ TEM cells (CD62L-CD45RA+), increased (Fig. 2B and C; Supplementary Fig. S8B). CD8+ central memory T cells (TCM; CD62L-CD45RA) and terminally differentiated effector T cells/T effector memory RA cells (TEMRA; CD62L-CD45RA+) were also sensitive to venetoclax, but to a lesser extent than CD8+ naïve T cells, and their relative proportion increased with increasing venetoclax concentrations. Interestingly, anti-CD3/CD28-activated T cells were resistant to venetoclax treatment, and cytokine production by CD8+ T-cell subsets was not affected (Supplementary Fig. S8C and S8D). In resting human PBMCs, BCL2 expression was similar across all T-cell subsets (Fig. 2D). Upon anti-CD3/CD28 T-cell activation, a 2-fold increase in BCL2 expression and a 9-fold increase in BCL-XL expression were observed in all CD4+ and CD8+ subsets (Fig. 2E). These data suggest that BCL-XL likely accounts for the survival of activated T cells during BCL2 inhibition. Consistent with the results from the MC38 syngeneic mouse model, the in vitro data indicate that venetoclax spares TEM cells and activated T cells.

Venetoclax Treatment Does Not Impair Human T-cell Function and Anti–PD-1 Activity In Vitro

We next examined whether venetoclax would affect antigen stimuli of T cells in vitro. We tested antigen-specific recall response of cytomegalovirus (CMV) CD8+ T cells from human CMV+ PBMCs. T2 cells loaded with CMV peptide were incubated with CMV+ PBMCs in the presence of increasing concentrations of venetoclax or the BCL-XL inhibitor A-1331852. CMV+ human PBMCs were also incubated with T2 cells without any peptide or loaded with MART1 peptide, serving as controls to assess antigen-specific response. Venetoclax treatment reduced overall CD8+ T-cell viability, whereas the BCL-XL inhibitor did not (Fig. 3A). Despite the reduction in cell number following venetoclax treatment, similar amounts of IFNγ were secreted compared with the control (Fig. 3B). In contrast, BCL-XL inhibitor treatment reduced the production of IFNγ. This suggests that venetoclax treatment does not restrict the response of antigen-specific memory T cells, which may mimic antitumor recall activity in patients with cancer.

We further evaluated the effect of venetoclax on human T-cell function in response to antigen stimulation with or without anti–PD-1 cotreatment in an allogenic mixed lymphocyte reaction (MLR). In this assay, we observed that venetoclax reduced total CD4+ T-cell viability in a concentration-dependent manner, but did not limit the proliferation of the surviving T cells (Supplementary Fig. S9A). Although the overall number of CD4+ T cells was reduced, there was no decrease in the amount of secreted IFNγ with or without PD-1 blockade (Fig. 3C). We hypothesized that the decrease in CD4+ T-cell number was the result of selective killing of nonactivated T cells and that the responding T cells remain unaffected by venetoclax treatment. Therefore, we next measured the proportion of T cells that produce IFNγ on the final day of the MLR. Venetoclax treatment resulted in a higher percentage of CD4+ T cells producing IFNγ regardless of PD-1 blockade (Fig. 3D; Supplementary Fig. S9B). Thus, venetoclax does not antagonize functional T-cell response and does not abrogate anti–PD-1 activity in the MLR. Next, we asked whether the activated T cells from the MLR upregulated BCL-XL and thus might be more resistant to venetoclax-mediated apoptosis. Indeed, both BCL2 and BCL-XL were upregulated as CD4+ T cells were activated in the MLR (Fig. 3E; Supplementary Fig. S9C). Although BCL2 upregulation was uniform, BCL-XL was bimodal, with one population showing expression similar to baseline and another with increased expression. However, when treated with venetoclax, the remaining CD4+ T cells in the MLR exhibited only high BCL-XL expression (Fig. 3E; Supplementary Fig. S9C). In contrast to venetoclax, the BCL-XL inhibitor did not affect CD4+ T-cell viability (Supplementary Fig. S9A), but reduced IFNγ production (reminiscent of the CD8+ CMV-recall assay) and diminished the effect of anti–PD-1 (Fig. 3D). Together with Fig. 2E, these data demonstrate that activated T cells express higher levels of BCL-XL than resting T cells, which may render them insensitive to venetoclax.

In conclusion, our data demonstrate that human naïve T cells are more sensitive to venetoclax than activated and memory T cells when treated in vitro. Venetoclax did not impair antigen recall or alloantigen-specific T-cell activation or function and did not antagonize the response of T cells to anti–PD-1.

Collectively, these data show that antigen-specific functional human T-cell responses are unaffected by venetoclax. Importantly for the potential clinical combination of venetoclax with immune checkpoint inhibitors, functional T-cell responses to anti–PD-1 are not inhibited by venetoclax.

Effect of Venetoclax on T-cell Subsets in Human Subjects

To confirm that effector T cells are more resistant to venetoclax than noneffector T cells, we analyzed PBMC samples from three healthy volunteers who received a single 100 mg dose of venetoclax under fasting conditions. T-cell subsets in peripheral blood were measured by flow cytometry one day before and 7 days after venetoclax administration. Peak venetoclax concentrations in these subjects ranged between 0.08 and 0.34 μg/mL, which is approximately 10% of the mean peak concentration at the approved venetoclax dose of 400 mg in CLL (19). Minimal changes were observed in the number of B cells and CD4+ and CD8+ T cells (Fig. 4A), as expected for a single dose of 100 mg. However, we did observe differences in the proportion of T-cell subsets, with the fraction of CD4+ and CD8+ effector memory cells (TEM and TEMRA) increased, and the proportion of noneffector cells (TN and TCM) decreased (Fig. 4B). Although preliminary and limited by the number of subjects, these data are consistent with our observations in syngeneic mice (Fig. 1C and D), as well as human PBMCs treated in vitro (Fig. 2C), and show that venetoclax primarily affects naïve T cells, leading to an increased proportion of effector memory cells.

DISCUSSION

To date, clinical development of venetoclax has proceeded exclusively in hematologic malignancies and estrogen-positive
Figure 3. Venetoclax treatment does not impair human T-cell function in response to antigen stimuli. A, CMV+ PBMCs were stimulated by CMV peptide loaded on T2 cells in the presence of increasing concentrations of venetoclax or BCL-X<sub>L</sub> inhibitor (A-1331852) for 4 days and viability of CD8<sup>+</sup> T cells was assessed. As controls, CMV+ PBMCs were incubated with T2 cells without any peptide. B, Concentration of IFNγ secretion measured with the assay described in A. MART1 peptide was used as a control. Representative viability (A) and cytokine secretion (B) data shown from one donor with treatments in duplicate ± SD. C, IFNγ secretion in MLR with anti–PD-1 (10 μg/mL) and/or venetoclax (1 μmol/L) treatment for 5 days. Paired t test: ns, not significant (P > 0.05); *, P ≤ 0.05; **, P ≤ 0.01 compared with DMSO control. D, Percentage of CD3+ T cells producing IFNγ as measured by intracellular flow cytometry on the final day of the MLR in the presence of increasing concentrations of venetoclax or the BCL-X<sub>L</sub> inhibitor (A-1331852). E, BCL2 and BCL-X<sub>L</sub> protein expression as determined by flow cytometry prior to and after the MLR. MFI, mean fluorescence intensity.
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**Figure 4.** Venetoclax increases the proportion of effector memory cells in the blood of human subjects. **A,** Three healthy volunteers were administered a single 100 mg dose of venetoclax. The fold change of B cells and CD4+ and CD8+ T cells in the peripheral blood was determined by flow cytometry one day before (day −1) and seven days after (day 7) exposure to the drug. **B,** Assessment of the fraction of CD4+ and CD8+ T effector cells (TEM and TEMRA) in the blood of the human subjects following venetoclax administration.
subsets and evaluated whether venetoclax could be combined with anti–PD-1 or anti–PD-L1 antibodies for cancer immunotherapy. Clinically, venetoclax is viewed as a lymphodepleting agent, and thus our initial working hypothesis was that venetoclax-induced apoptosis of T cells and TILs would reduce the adaptive immune system’s ability to attack tumors and impair immune checkpoint inhibitors’ activity. Surprisingly, we found that even though venetoclax treatment led to a decrease in T cells, it was able to augment the activity of immune checkpoint inhibitors and did not interfere with the establishment of immune memory.

Although we observed improved efficacy for the combination of venetoclax and anti–PD-1/anti–PD-L1, we recognize that venetoclax does reduce overall T-cell number. Subsequent assessment of TILs revealed a depletion of naïve-like T cells, but an increase in the number and proportion of CD8\(^+\) PD-1\(^+\) TEM cells. Importantly, reinvigoration of antitumor immune responses with checkpoint inhibitors in patients’ malignancies is associated with intratumoral expansion of CD8\(^+\) memory T cells (20). Clinical responses have been correlated with increases in memory T cells (21), specifically CD8\(^+\) TEMs (20). PD-1 expression on CD8\(^+\) TILs also has been identified as a biomarker for the enrichment of tumor-specific T cells (22, 23). Moreover, reinvigoration of T cells was found to be accompanied by proliferation of CD8\(^+\) PD-1\(^+\) T cells in the peripheral blood that also express low levels of BCL2 (24). In fact, all responding CD8\(^+\) Ki-67\(^+\) PD-1\(^+\) T cells had low levels of BCL2 and high levels of T-cell activation and effector markers, suggesting that this population might be spared by venetoclax. The CD8\(^+\) T-cell subset reported to be responsible for the antitumor activity of immune checkpoint inhibitors was increased by venetoclax in our studies. Although venetoclax-mediated depletion of naïve T cells did not antagonize the activity of checkpoint inhibitors in our syngeneic tumor models, it is unclear what impact this might have on the depth and durability of clinical responses. Of potential relevance, the frequency of CD8\(^+\) naïve T cells in the peripheral blood of patients with lung cancer undergoing anti–PD-1 therapy was much lower than in healthy subjects (24), implying that this population of T cells may be less involved in antitumor immune response.

On the basis of the data presented here, a simple mechanistic hypothesis could focus on the ability of venetoclax to induce apoptosis of certain immune cell subsets dictated by the BCL2 family dependence profiles of the various immune cell populations. Venetoclax treatment may merely select for the most active, tumor-directed effector T cells and enable them to accumulate at their intended site of action. In support of this, we show that activated T effector cells and TEMs upregulate BCL-X\(_I\), both in vitro and in vivo and are resistant to venetoclax, whereas naïve T cells expressing low levels of BCL-X\(_I\) are depleted. This was observed in human PBMCs cultured ex vivo and confirmed in the MC38 syngeneic mouse model where PD-1\(^+\) CD8\(^+\) TILs expressed high levels of BCL-X\(_I\) and were enriched in tumors after venetoclax treatment. Any of these cells that are inhibited through PD-1–PD-L1 interactions could then be unleashed through the action of immune checkpoint inhibitors. Notably, Tregs and macrophages are resistant to venetoclax, indicating that the increased efficacy is not the result of depleting these cells in the TME. Of course, it is likely that the mechanism is more complex and could also involve venetoclax rendering cancer cells more susceptible to T cell–driven cytotoxicity. In a study using a MYC-dependent breast cancer model (WapMyc mouse), treatment with venetoclax and the diabetes drug metformin inhibited tumor growth and increased the intratumoral infiltration of PD-1\(^+\) T cells, indicative of an initial immune-mediated response followed by immune exhaustion (25). Subsequent experiments showed that adding neoadjuvant anti–PD-1 to this regimen led to significant improvements in durability of the antitumor response. Further understanding of the mechanism of venetoclax-mediated immune modulation and antitumor response remains an area of active investigation for our laboratories.

We have provided the first data to suggest that venetoclax could contribute to antitumor activity through a distinct mechanism which is essentially immunomodulatory in nature. By enriching the number (and potentially the quality) of PD-1\(^+\) effector T cells within tumors, venetoclax may have the potential to augment the efficacy of immune checkpoint inhibitors. Furthermore, based on the absence of direct cancer cell–intrinsic effects of venetoclax in the syngeneic models presented here, our data support the notion that venetoclax requires an active antitumor immune response as is the case with anti–PD-1 reinvigoration of the T cells. These hypotheses are under active clinical investigation (NCT04274907, NCT03000257) and may inform the combination of venetoclax and immunotherapy. Of course, it may be attractive to leverage both the apoptosis-inducing and immune-related effects of venetoclax simultaneously for cancer therapy. Indeed, clinical studies combining it with the anti–PD-L1 antibody atezolizumab were initiated in lymphoma, CLL, SLL, and small-cell lung cancer (NCT03276468, NCT02846623, NCT04422210), where venetoclax has already demonstrated signs of clinical activity. The results of Haikala and colleagues (25) and those presented here suggest that venetoclax checkpoint inhibitor combinations merit exploration in cancers not previously expected to respond to venetoclax alone, including an array of solid-tumor malignancies.

**METHODS**

**Reagents and Cell Lines**

Venetoclax, BCL-X\(_I\), inhibitor (A-1331852), anti-human PD-1 (MDX-1106) AB426 [hu IgG1/k], anti-mouse PD-1 antibody (17D2[mu lgG2a/k]] DANA), anti–PD-L1 antibody (YW243.55.S70 [hu/mu IgG2a/k], and anti-mouse CD8 antibody used for depletion of CD8 T cells in vivo (PR-1928513) were synthesized at AbbVie. Antibodies used for flow cytometry are listed in Supplementary Table S1.

MC38 (mouse colon 38) cells were obtained from the National Cancer Institute (NIH, Rockville, MD) or from Kerafast. CT26 was obtained from ATCC. The cells were tested regularly for *Mycoplasma* using MycoAlert Detection Kit (Lonza), and authenticated via PCR using nine short tandem repeat (STR) markers (IDEXX BioResearch).

**Mouse MC38 Cell Line In Vitro Studies**

Cells were plated in 384-well plates in the presence of increasing doses of venetoclax or DMSO control. Viability was measured via CellTiter-Glo according to the manufacturer’s protocol (Promega). Confluence and apoptosis were assessed via IncuCyte Live-Cell Analysis and IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent, respectively.
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following equation: 

\[ \text{V} = \frac{L \times W^2}{2} \]

where \( V \) is the volume, \( L \) is the length, and \( W \) is the width of the tumor. The volume was calculated according to the equation above.

The volume was determined via measurements of the length (\( L \)) and width (\( W \)) of the tumor with electronic calipers and the volume was calculated according to the following equation: 

\[ V = \frac{L \times W^2}{2} \]

where \( V \) is the volume, \( L \) is the length, and \( W \) is the width of the tumor. The volume was calculated according to the equation above.

The analysis aggregated information from all studies to evaluate the fixed effect of treatment by time point (taken at baseline). The fixed effect includes time, treatment, and for each arm in each study. The dependent variable was the tumor size at each time point. The fixed effect comparison was based on data collected at the same study time point. Study log stats (AbbVie Inc.) were used for the statistical analysis and \( P \) values are derived from Student t test comparison (one-sided two-sample) of log-transformed data of treatment group versus control group.

Because of substantial variability in treatment responses caused by alternate sources of the MC38 cell line (NIH or Kerafast) and among studies, the antitumor growth efficacy data obtained from eight separate studies was evaluated using mixed-effect modeling applied with the R software.

The analysis aggregated information from all studies to evaluate the time trend of tumor growth and took into consideration the correlation between time points within each mouse and the correlation between mice within the same arm of the same study. The dependent variable of the model is the log of tumor fold change from the first time point (taken at baseline). The fixed effect includes time, treatment by time interaction, source by time interaction, and treatment by source by time interaction, where time is treated as a continuous variable. The random effect includes the time effect for each mouse and for each arm in each study. The fixed effect of treatment by source by time interaction could be removed from the model if it is discovered to be not significant.

Mouse Tumor Digestion and Flow Cytometry

Tumors were dissociated using a mouse tumor dissociation kit following the manufacturer’s protocol (Miltenyi Biotec). In brief, tumors were cut into 2-4 mm pieces and incubated with enzyme mix for 30 minutes in a 37°C shaker (200 rpm). Cells were strained with 100-μm and 70-μm strainers (Falcon/Corning) before washing twice with RPMI 1640 media containing 10% FBS, GlutaMax, penicillin, streptomycin, and gentamicin. Single-cell suspensions from tumors were counted and up to 2 × 10^6 live cells per tissue were stained with antibodies as indicated in Supplementary Table S1, adding Zombie UV reagent (BioLegend) to assess live versus dead cells, and analyzed by flow cytometry using a LSRFortessa X-20 instrument (BD Biosciences).

FC5 3.1 data files were exported from FACSDiVa and analyzed using FlowJo v10.4.1. Briefly, compensation was performed using single-stained bead controls and applied to all samples. Instrument acquisition anomalies were removed using a time-based histogram gate. Dead cells, cell aggregates, and debris were removed from analysis utilizing Zombie UV intensity, pulse processing channels, and scatter, respectively. Lymphocyte subsets were gated according to a hierarchy to identify various T-cell subsets as well as characterize their phenotype. An example layout of the gating hierarchy is found in Supplementary Fig. S10.

Absolute cell counts were normalized to tumor volume using the following method: digested whole tumors were counted by flow cytometry. Analyses of nucleated and live/dead cell yielded the initial count. Up to 2 × 10^6 live cells were stained per sample, and the number of live cells detected during final acquisition was tabulated as the final count. The ratio of the initial count to final count provided the cell number normalization factor to calculate the total number of cells per tumor in any given gated population. A tumor size normalization factor was created by dividing all tumor sizes by 50 mm^3. A normalized cell count could finally be plotted for any given population by first multiplying by the cell number normalization factor and then dividing by the tumor size factor to yield the cell number per 50 mm^3 tumor volume metric for each sample.

For t-SNE analysis, data files were passed through a preprocessing pipeline that included cleanup for viability, cell aggregates, and instrument acquisition anomalies using a combination of manual gating and the flowAI plug-in (FlowJo Exchange). Files were down-sampled to a fixed number of lymphocytes after gating for Live/Singlet/CD45^−/CD3^+ or CD19^+ events per sample. Down-sampled events were concatenated into a single file and the t-SNE algorithm was applied using all antibodies in the panel as parameter input values. t-SNE X and Y parameters were plotted for the fully concatenated file (Fig. 1B) or the deconvolved, individual treatment group files (Fig. 1C) to assess global changes in population frequencies. A third parameter was displayed by marker heat map that revealed major immune cell subsets as well as marker expression within these subsets (Fig. 1E and F). Viable populations based on the t-SNE plot were manually gated to explore relevant subsets and expression of additional markers on these subsets.

Human PBMC In Vitro Studies

Frozen viable human PBMCs were thawed and cultured overnight in 30 U/mL of IL2 (BD Biosciences), then washed once with RPMI 1640 complete media supplemented with 10% FBS, GlutaMax, and penicillin/streptomycin (Gibco/Thermo Fisher Scientific). Cells were treated with increasing concentrations of venetoclax for 24 hours and then harvested, counted, and stained with antibody cocktail containing anti-CD19, anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, and anti-CD62L (Supplementary Table S1) and viability dye and analyzed by flow cytometry using a BD LSRII instrument (BD Biosciences). FCS 3.0 data files were exported from FACSDiVa and analyzed using FlowJo v10.4.1. Each subset of cell numbers was calculated taking into account the viable cells followed by the percentage of CD19^+ B cells and CD3^+ T cells. T cells were further analyzed to determine each immune cell subset (TN, TCM, TEM, TEMRA) and plotted using GraphPad Prism software.

For 48-hour viability experiments, fresh PBMCs from healthy donors were plated at 2 × 10^6 cell per well in 96-well plates. Venetoclax was added at indicated concentrations. After 2 days, cells were collected and stained with anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD127, anti-CD56, anti-CD19, and 7-AAD (BD Biosciences). Live CD3^+CD4^+ T, CD3^+CD8^+ T, CD19^+ B, CD56^− NK, and CD3^+CD4^+CD25^−CD127^− Treg cell numbers were quantified by flow cytometry LSRFortessa X-20 instrument (BD Biosciences).
For activation of T cells, cells were plated in anti-CD3-coated wells (2.5 μg/mL; Thermo Fisher Scientific, clone OKT3) and soluble anti-CD28 was added (1 μg/mL; Thermo Fisher Scientific, clone CD28.2).

**Cytokine Analysis in C8 T-cell Subsets**

CD8+ T cells were enriched from PBMCs utilizing RosetteSep Human CD8+ T Cell Enrichment Cocktail (StemCell Technologies). CD8+ T cells were stained for CCR7 and CD45RA, and naive (CD45RA-/CCR7+), effector (CD45RA-/CCR7-), effecter memory (CD45RA+/CCR7+), and central memory (CD45RA+/CCR7-) subsets were sorted on a BD FACS Aria Fusion (BD Biosciences). Cells were resuspended in RPMI 1640 media supplemented with 10% FBS, 1-glutamine, and penicillin/streptomycin with or without 400 nmol/L venetoclax, activated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies; 1 bead:2 cells) and incubated at 37°C for 18 hours. Supernatant was harvested and analyzed by Luminex. Cytokines and chemokines were quantified using a Milliplex human multiplex bead-based 30-plex Luminex assay (Millipore) according to the manufacturer’s protocol. Data were acquired on a verified and calibrated FlexMap3D system (Luminex, Inc.) and analyzed with Bio-Plex Manager 6.0 software (Bio-Rad).

**CMV Recall Assay**

HLA-A*0201-restricted cytotoxic T-cell peptide from the CMV protein pp65 (NLVPMAVT) was used to stimulate CMV+ CD8+ T cells from donor PBMCs (both from Astarte Biologics). CMV pp65 peptide (2 μg/mL) was loaded on T2 cells (ATCC) in RPMI 1640 media supplemented with 10% FBS, 1% antibiotics, and Brefeldin-A (BD Biosciences). After 3-hour loading, T2 cells were irradiated (30 Gy) and washed with AIM V media (InVitrogen). Irradiated T2 cells loaded with CMV peptide were then incubated with CMV+ donor PBMCs (n = 3 separate donors) in AIM V media at 37°C in a 5% CO2 incubator. These cells were treated with venetoclax or the BCL-2 inhibitor for the duration of the 4-day incubation period. Cells were stained with CD8 antibody and Zombie green fixable viability dye (BioLegend) and analyzed using a LSRFortessa X-20 instrument (BD Biosciences). Secreted IFNγ was measured by MSD ELISA (Meso Scale Diagnostics).

**Mixed Lymphocyte Reaction**

Monocyte-derived dendritic cells (MoDC) were generated from fresh human blood. Briefly, human PBMCs were isolated using a Ficol gradient and allowed to adhere to the plate for 2 hours, after which cells in suspension were removed. Fresh AIM V medium (Thermo Fisher Scientific) supplemented with 80 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech) and 50 ng/mL IL4 (R&D Systems) were added to the culture. After 5 days, the MoDCs were stimulated with IL2 and TNFα (0.2 μg/mL each, PeproTech) for 48 hours to increase expression of MHCII molecules. Activated MoDCs were then cocultured with viable thawed CD4+ T cells (Biological Spe-. Scale Diagnostics). After 3-hour loading, T2 cells were irradiated (30 Gy) and treated with control IgG (Isotype) or anti–PD-1 antibody (10 μg/mL along with venetoclax). The MLR was cultured for 5 days, after which the cells were analyzed by flow cytometry using an LSRFortessa X-20 instrument (BD Biosciences) to determine cell number and functional cytokine (IFNγ) responses.

Secreted IFNγ was analyzed using a human IFNγ AlphaLISA Detection Kit as per manufacturer’s recommendation (Perkin Elmer).

**Venetoclax Study in Healthy Subjects**

Three female volunteers, ages 20, 47, and 58 years, received one 100 mg commercial venetoclax tablet orally. Venetoclax plasma concentrations were evaluated prior to oral dosing and at 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hours post-dose. A liquid–liquid extraction and LC/MS-MS detection method was used to determine venetoclax plasma concentrations. The effect of venetoclax on T cells was assessed as described above for in vitro experiments.

**Statistical Analyses**

Unless otherwise specified, GraphPad Prism was used for statistical analyses using t test statistical calculations.

**Disclosure of Potential Conflicts of Interest**

F.J. Kohlhapp reports a patent for ABV12428WWO1/PCT application, no. PCT/US2019/018241 pending. D. Haribhai reports a patent for WO 2019/161221 A2 pending. R. Mathew reports a patent for WO 2019/161221 A2 pending. E. Lasater is an employee of Genentech, Inc. Y. Li reports other (employee) from Genentech/Roche outside the submitted work. A. Raval reports other from Roche Pharma (employee) outside the submitted work. M. Merchant reports personal fees from Genentech/Roche (employee) during the conduct of the study and outside the submitted work; in addition, M. Merchant has a patent pending (for Genentech/Roche, AbbVie). A.H. Salem reports other from AbbVie (employment and stocks) outside the submitted work. K.M. Hamel is a full-time employee of AbbVie, the maker of venetoclax. J.D. Levenson reports a patent for WO 2019/161221 A2 pending; and is an employee and shareholder in AbbVie, Inc. W.N. Pappano reports a patent for WO 2019/161221 A2 pending. T. Uziel reports a patent for WO 2019/161221 pending. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

F.J. Kohlhapp: Conceptualization, investigation, visualization, methodology, writing-original draft. D. Haribhai: Conceptualization, investigation, visualization, writing-review and editing. R. Mathew: Conceptualization, investigation, visualization, methodology, writing-original draft. R. Duggan: Formal analysis, investigation, visualization, methodology, writing-review and editing. P.A. Ellis: Investigation, visualization, methodology, writing-review and editing. R. Wang: Conceptualization, investigation, visualization, writing-review and editing. E.A. Lasater: Conceptualization, supervision, investigation, visualization, writing-review and editing. Y. Shi: Investigation, visualization, writing-review and editing. N. Dave: Investigation, writing-review and editing. J.J. Riehm: Investigation, writing-review and editing. V.A. Robinson: Investigation, visualization, writing-review and editing. A.D. Do: Investigation, visualization, writing-review and editing. Y. Li: Investigation, visualization, writing-review and editing. C.J. Orr: Investigation, visualization, writing-review and editing. D. Sampath: Conceptualization, supervision, writing-review and editing. A. Raval: Investigation, visualization, writing-review and editing. M. Merchant: Investigation, visualization, writing-review and editing. A. Bhathena: Supervision, writing-review and editing. A.H. Salem: Investigation, writing-review and editing. K.M. Hamel: Investigation, visualization, writing-review and editing. J.D. Levenson: Conceptualization, supervision, writing-review and editing. W.N. Pappano: Conceptualization, supervision, investigation, visualization, writing-original draft, project administration. T. Uziel: Conceptualization, supervision, investigation, visualization, writing-original draft, project administration.

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