Potentiating vascular-targeted photodynamic therapy through CSF-1R modulation of myeloid cells in a preclinical model of prostate cancer

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ABSTRACT
Vascular-targeted photodynamic therapy (VTP) induces rapid destruction of targeted tissues and is a promising therapy for prostate cancer. However, the resulting immune response, which may play an important role in either potentiating or blunting the effects of VTP, is still incompletely understood. Myeloid cells such as myeloid-derived suppressor cells (MDSCs) and macrophages are often found in tumors and are widely reported to be associated with cancer angiogenesis, tissue remodeling, and immunosuppression. These cells are also known to play a critical role in wound-healing, which is induced by rapid tissue destruction. In this study, we investigated the effects of VTP on the recruitment of tumor-infiltrating myeloid cells, specifically MDSCs and tumor-associated macrophages (TAMs), in the Myc-Cap and TRAMP C2 murine prostate cancer models. We report that VTP increased the infiltration of myeloid cells into the tumors, as well as their expression of CSF1R, a receptor required for myeloid differentiation, proliferation, and tumor migration. As anti-CSF1R treatment has previously been used to deplete these cells types in other murine models of prostate cancer, we hypothesized that combining anti-CSF1R with VTP therapy would lead to decreased tumor regrowth and improved survival. Importantly, we found that targeting myeloid cells using anti-CSF1R in combination with VTP therapy decreased the number of tumor MDSCs and TAMs, especially M2 macrophages, as well as increased CD8+ T cell infiltration, decreased tumor growth and improved overall survival. These results suggest that targeting myeloid cells via CSF1R targeting is a promising strategy to potentiate the anti-tumor effects of VTP.

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Introduction
Vascular-targeted photodynamic (VTP) therapy using padeliporfin (TOOKAD® Soluble (WST11)) is a promising therapeutic option currently being assessed for prostate cancer (PCA) treatment.1,2 VTP ablates targeted tissues by using WST11 as a photosensitizer which reacts with a near-infrared laser in the presence of oxygen. The photosensitizer is infused intravenously while the targeted prostate zone is illuminated by trans-perineal optical fibers.3 The photosensitizer absorbs light and transfers energy to oxygen molecules creating reactive oxygen species and inducing irreversible endothelial damage which is quickly followed by thrombosis, blood stasis, and vessel occlusion, ultimately leading to tumor necrosis.4,6 Results from multiple phase II clinical trials in prostate cancer patients treated by VTP hemiablation show that a high rate of patients have no detectable tumors years after therapy compared to the untreated population.2,7

Prostate tumors and other subsets of solid tumors have significant infiltration of immunosuppressive myeloid cells which promote tumor progression, metastasis, and therapeutic resistance.8–10 These include myeloid-derived suppressor cells (MDSCs),8 as well tumor-associated macrophages (TAMs), which can differentiate into either pro-inflammatory and anti-tumor M1 macrophages, or into immunosuppressive and pro-tumor M2 macrophages.11–13 Importantly, MDSCs and M2 macrophages are also recruited to sites of wound healing.14,15 In addition, they have been shown to generate a favorable microenvironment for tumors, at steady state and in therapeutic settings, by suppressing the adaptive immune response against cancer cells and by promoting tumor growth through angiogenesis or by producing tumorigenic growth factors.12,15,21 A study in a preclinical mouse model of colon cancer suggests that myeloid cells may be recruited to VTP-treated tumors.21 However, the effects of VTP on myeloid cell infiltration and the subsequent effects of these myeloid cells on tumor eradication require further in-depth investigation. The macrophage colony-stimulating factor (M-CSF or CSF1)
has a critical role in the differentiation, proliferation, and migration of myeloid cells to tumors via signaling through the CSF1 receptor (CSF1R). Studies in preclinical mouse models by our group and others show that blocking CSF1R potentiates the anti-tumor immune response, alone and in the context of immune checkpoint blockade. In this study, we sought to study the effect of myeloid cell modulation in the context of VTP-based treatment. We show that VTP increases the infiltration of immunosuppressive MDSCs and M2 macrophages, as well as their expression of CSF1R in the Myc-Cap and TRAMP C2 prostate cancer preclinical mouse models. We then demonstrate that targeting myeloid cells using anti-CSF1R treatment blocks the VTP-mediated influx of myeloid cells. The reduction in MDSCs correlated with an increase in tumor-infiltrating CD8+ T cells, as well as a decrease in tumor progression and improved survival. We therefore conclude that anti-CSF1R therapy potentiates the effects of VTP therapy.

Results

VTP treatment increases total and CSF1R+ myeloid cell infiltration

To determine whether VTP induced myeloid cell infiltration in tumors, Myc-Cap tumor-bearing mice were treated with VTP and assessed ex vivo for myeloid cell infiltration of tumor by immunofluorescence (IF). We observe that VTP is associated with an increase in CD11b+ myeloid cells when compared to control tumors, including a trend towards more F4/80+ macrophages (Figure 1(a,b)). Furthermore, when we examined macrophage localization by Iba1 immunohistochemistry (IHC) staining, we found that macrophages were enriched in both the peritumoral and intratumoral areas (Figure 1(c,d)). Finally, we found elevated levels of CSF1R+ cell (Figure 1(a,b)), a receptor typically found on TAMs and MDSCs. These observations confirmed that myeloid cells were being recruited to Myc-Cap tumor in response to VTP.

Myeloid cell infiltration induced by VTP treatment express markers of immunosuppressive cells

We next wanted to determine whether the influx of CSF1R+ cells in response VTP was associated with immunosuppressive myeloid cells such as TAMs and MDSCs. To do this end, we profiled the myeloid cell populations by flow cytometry (Supplementary Figure 1) at multiple time points (3, 7 and 10 days) after VTP treatment. Consistent with the imaging results, we found a significant increase of CD11b+ myeloid cells in VTP-treated tumors over time as a percentage of total live cells (Figure 1(e,f)). When we examined subsets of CD11b+ cells, we found an increase of neutrophils as early as 3 days after therapy, which was maintained until day 10 (Figure 1(g)). This is likely due to the high level of necrosis induced by VTP, which is known to recruit neutrophils. Importantly, we found that CD11b+ myeloid cells expressing Ly6G+Ly6C+, generally considered MDSCs, were found in lower numbers early after treatment, likely as a direct consequence of VTP-mediated cell death, but accumulated subsequently over time compared to control tumors (Figure 1(h)). In addition, the same kinetics were observed for macrophages (Figure 1(i)). Since macrophages can differentiate into either pro-inflammatory M1 macrophages or immunosuppressive M2 macrophages, we profiled these macrophages for surface markers of M1 or M2 polarization (Supplementary Figure 1), which we have previously confirmed to be associated with the expression of known immunosuppressive factors. We observe that while both the anti-tumor M1 macrophages and the pro-tumor M2 macrophages broadly followed the same kinetics as MDSCs on a per cell basis, there was a consistent skewing toward more M2 macrophages in VTP-treated tumors (Supplementary Figure 2). Finally, we also showed that neutrophils, MDSCs, and macrophages had increased CSF1R expression in VTP-treated tumors compared to control tumor (Supplementary Figure 3). We therefore concluded that VTP therapy leads to an influx of myeloid cells, including neutrophils and immunosuppressive MDSCs and M2 macrophages.

CSF1R blockade enhances VTP treatment efficacy and improves survival in correlation with the number of macrophages and MDSCs in tumors

CSF1R signaling is required for the differentiation and survival of myeloid cells, including MDSCs and macrophages. We therefore hypothesized that CSF1R blockade would enhance the anti-tumor effects of VTP by targeting immunosuppressive myeloid cells. To test this, we treated two murine prostate tumor models, Myc-Cap and TRAMP C2, with VTP and anti-CSF1R antibody treatment. As hypothesized, we observed that the combination treatment led to significant tumor reduction and improved survival compared to control or mono-therapies alone in both Myc-Cap (Figure 2(a–c)), Supplementary Table 1 and 2) and TRAMP C2 (Figure 2(d–f), Supplementary Table 3 and 4) models. In order to test whether the resulting improved outcome from the combination therapy correlated with a reduction in myeloid cells, these cells were quantified in post-treatment tumors by immunostaining and flow cytometry. We observed that the CSF1R treatment reversed the myeloid cell increase associated with the VTP treatment. In the combination group, there was a notable reduction in CD11b+, F4/80+ and CSF1R+ myeloid cells by IF compared to VTP mono-therapy in Myc-Cap (Figure 3(a,b)) and TRAMP C2 (Supplementary Figure 4) tumors. The reduction of macrophages in the combination therapy group compared to VTP alone was also seen by Iba1 IHC staining in Myc-Cap (Figure 3(c,d)) and TRAMP C2 (Supplementary Figure 5) tumors, as well as by flow cytometry in Myc-Cap tumors (Figure 3(e)). Furthermore, when macrophages were further examined in Myc-Cap tumors for markers of polarization, we observed that anti-CSF1R alone or in combination with VTP led to an equal reduction of M1 and M2 macrophages (Supplementary Figure 6). We also observed a considerable reduction in the number of CD11b+ cells, including neutrophils and MDSCs in the tumor, when mice were given anti-CSF1R antibody alone or in combination with VTP (Figure 3(f–h)). Finally, our group has previously shown that VTP obliterates the tumor vasculature in PC-3 xenografts immediately post-VTP treatment and we wanted to determine if combining anti-CSF...
1R with VTP therapy had any effect on the vasculature at later time points. However, we found that while the vasculature in VTP-treated tumors mostly recovered by day 10 after therapy, combining anti-CSF1R to VTP therapy had modest to little effect, which contrasted with a greater increase in vasculature in the tumors only receiving anti-CSF1R (Supplementary Figure 7).

Together, our data demonstrate that while the anti-CSF1R therapy alone could deplete macrophages and MDSCs in the
Myc-Cap model, a reduction of tumor growth and an increase in overall survival was only seen when anti-CSF1R therapy was combined with VTP treatment. CSF1R blockade increases CD8^+ T cell infiltration in VTP treated tumors

MDSCs and M2 macrophages are known to inhibit T cell tumor infiltration and diminish their effector potential. We therefore hypothesized that anti-CSF1R-mediated MDSC and macrophage depletion in VTP-treated tumors would correlate with an increase in infiltrating T cells and effector function. To test this hypothesis, we examined T cell infiltration in Myc-Cap tumors collected 5 and 10 days post-treatment (VTP alone or VTP with anti-CSF1R). We found a significant increase in CD8^+ T cells at both time points in tumors treated with the combination therapy compared to tumors treated with VTP alone in the Myc-Cap (Figure 4(a,b) and Supplementary Figure 8a) and TRAMP C2 (Supplementary Figure 8b) models. Increased numbers of CD8^+ T cells, but not CD4^+ T cells, was further confirmed by flow cytometry (Figure 4(c)). This also correlated with a modest increase in granzyme B expression by CD8^+ T cells from mice treated with the combination of VTP and anti-CSF1R (Figure 4(d,e)). We conclude that when combined with VTP therapy, anti-CSF1R therapy decreases the number of MDSCs and macrophages and is associated with increased CD8^+

Figure 2. Combination anti-CSF1R and VTP therapy decreases tumor growth and improves survival. (a) Individual and (b) average Myc-Cap tumor growth in mice treated with control, VTP alone, anti-CSF1R alone or VTP with anti-CSF1R combination therapy. (c) Overall survival of Myc-Cap tumor-bearing mice from all treatment groups. (d) Individual and (d) average TRAMP C2 tumor growth in mice treated with control, VTP alone, anti-CSF1R alone or VTP with anti-CSF1R combination therapy. (f) Overall survival of TRAMP C2 tumor-bearing mice from all treatment groups. Statistical differences between tumor growth were measured by performing a t test of the area under the curve. Survival significance was performed by Log–Rank test. Multiple comparison test for A-D between all groups is shown in Supplementary Tables 1–4. Data shown are a single representative of experiments performed at least twice.
T cell infiltration and cytotoxic potential, and that this could be a potential mechanism contributing to the diminished tumor growth and improved survival.

**Discussion**

Immunosuppressive myeloid cells such as MDSCs and M2 macrophages have been reported to inhibit the adaptive immune response against tumors and to promote tumor growth.\(^1\),\(^2\),\(^15\)–\(^20\) Notably, M2 macrophages, as opposed to M1 macrophages, are associated with less favorable outcomes in a wide variety of tumor types.\(^9\) In this study, we show that VTP induces the recruitment of myeloid cells in treated tumors, including MDSCs and M2 macrophages, which would generate an immunosuppressive microenvironment. In the case of VTP therapy, we hypothesized that when VTP fails to completely eradicate all tumor cells, the influx of immunosuppressive myeloid cells theoretically inhibits anti-tumor T cells from eliminating the remaining surviving tumors cells. As we observed that VTP therapy increases the expression of CSF1R on infiltrating myeloid cells, we predicted that anti-CSF1R-mediated myeloid cell targeting

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**Figure 3.** Anti-CSF1R treatment diminishes Myc-Cap tumor myeloid infiltration in control and VTP-treated mice.
could be a beneficial complementary treatment to delay the natural healing process after VTP treatment. Importantly, we confirmed that while CSF1R blockade inhibited myeloid tumor infiltration in all conditions, anti-CSF1R influenced tumor growth and survival only in combination with VTP therapy. The use of two murine prostate cancer models in this study attests to the reproducibility of this strategy. Taken together, our observations suggest that targeting pro-tumor myeloid cells such as MDSCs and M2 macrophages through CSF1R blockade is a promising approach for combination treatments for focal therapies such as VTP.

Interestingly, similar results using different therapeutic approaches have been reported. In a study by Xu et al.,29 where the authors showed that irradiation induced elevated levels of tumor-derived CSF1 secretion followed by enhanced TAM infiltration, and that this provided additional growth and survival factors to the tumor. However, when a selective CSF1R inhibitor was combined with the radiotherapy regimen, it resulted in suppressed tumor growth. In another study, androgen blockade therapy (ABT) was found to induce elevated levels of tumor-derived CSF1 and TAM infiltration in both human prostate cancer patients and preclinical mouse models of prostate cancer.37 However, when ABT was combined with anti-CSF1R treatment, the combination treatment led to reduced numbers of TAMs, diminished tumor progression and durable therapeutic benefit.37 Our work and the previous studies highlight the importance of CSF1/CSF1R signaling in the recruitment of tumor-infiltrating myeloid cells that can limit the efficacy of localized therapies for prostate cancers.

While we clearly show that targeting myeloid cells using anti-CSF1R potentiates VTP therapy, it is unclear whether this is due to removing a source of growth factors, releasing the inhibition on T cells, a combination of both or through other unexplored mechanisms. For example, other mechanisms of immunologic suppression were not addressed in this study, such as hypoxia, the presence of newly identified immunosuppressive cancer-associated fibroblasts, as well as other mechanisms.10,38,39 Nevertheless, it has been shown that CSF1R inhibition attenuates the turnover rate of TAMs while increasing the number of CD8+ T cells that infiltrate cervical and breast carcinomas,40 a correlation also observed in this study. Potentiating anti-tumor CD8+ T cells is quickly becoming a central focus when developing new therapies and we believe that this could be one of the main mechanisms driving the additive effect seen when anti-CSF1R is combined with VTP therapy. However, other pathways that were not addressed here could be targeted in future studies to determine if these could have an even greater effect on CSF1R-
targeted therapies in combination with VTP. For example, in a recent study examining a castration-resistant prostate cancer murine model, it was found that IL-23 production by MDSCs was driving androgen receptor pathway activity in prostate tumor cells in androgen-deprived conditions and these cells could not be targeted by anti-CSF1R therapy.\(^{41}\) It would therefore be interesting to see if targeting these cells could lead to even greater benefit compared to the VTP plus anti-CSF1R therapy.

In conclusion, combining VTP therapy with anti-CSF1R treatment improves survival and decreased tumor growth in two different murine models of prostate cancer. These effects correlated with the decrease of immunosuppressive myeloid cells and an increase in granzyme B\(^{+}\) CD8\(^{+}\) T cells in the tumor. Our observation constitutes the basis for the rational design of clinical trials combining CSF1R blockade with VTP therapy. In addition, the increase in T cell function in this setting warrants further combinations with immune checkpoint blockade.

**Materials and methods**

**Reagents and antibodies**

Lyophilized WST11 was obtained from Steba Biotech (Cedex, France). Myc-Cap cells were cultured in DMEM supplemented with 10% fetal calf serum, 2-ME, pen/strep, L-glutamine, 5\(^{-}\)N2 Serum IV (BD Biosciences) HEPES, 2-ME pen/strep, supplemented with 5% fetal calf serum (FCS; Mediatech, Inc.), 5% Nu Serum IV (BD Biosciences) HEPES, 2-ME pen/strep, L-glutamine, 5 \(\mu\)g/mL insulin (Sigma), and 10 nmol/L DHT (Sigma). All cells were periodically authenticated by morphologic inspection and tested negative for mycoplasma contamination by PCR. Therapeutic anti-mouse CSF1R (clone AFS98) was obtained from BioXcell (NH, USA). The following antibodies and dilutions were used for flow cytometric analyses: CSF-1R PE (eBioscience; clone AFS98; 1:100), F4/80 PerCP-Cyanine5.5 (eBioscience; clone BM8; 1:100), CD45 Alexa Fluor 700 (eBioscience; clone 30-F11; 1:200), CD8a BV650 (eBioscience; clone 53-6-7; 1:200), CD11b PE-Texas Red (Invitrogen; clone M1/70.15; 1:200), CD11c-FITC (BD Pharmingen; clone HL3; 1:200), Ly6C-PE-Cy7 (BD Pharmingen; clone AL-21; 1:200), Ly6G v450 (BD Horizon; clone 1A8; 1:200), CD206 Alexa Fluor647 (Biolegend; clone C068C2; 1:200) and 1-A/1-E APC/Cy7 (Biolegend; clone M5/114.15.2; 1:200). The following antibodies and dilutions were used for IHC: CD8a (eBioscience; clone 4SM15; 1:100), CD31 (Dianova; clone SZ31; 1:250) and Iba1 (Abcam; polyclonal ab5076; 1:2500). The following antibodies and dilutions were used for IHC: CD8a (eBioscience; clone 30-F11; 1:200), CD8a BV650 (eBioscience; clone BM8; 1:100), CD45 Alexa Fluor 700 (eBioscience; clone 30-F11; 1:200), CD8a BV650 (eBioscience; clone 53-6-7; 1:200), CD11b PE-Texas Red (Invitrogen; clone M1/70.15; 1:200), CD11c-FITC (BD Pharmingen; clone HL3; 1:200), Ly6C-PE-Cy7 (BD Pharmingen; clone AL-21; 1:200), Ly6G v450 (BD Horizon; clone 1A8; 1:200), CD206 Alexa Fluor647 (Biolegend; clone C068C2; 1:200) and 1-A/1-E APC/Cy7 (Biolegend; clone M5/114.15.2; 1:200). The following antibodies and dilutions were used for IF: CD11b (Abcam; clone EPR1344; 1 \(\mu\)g/mL), F4/80 (Abcam; clone CI:A3-1; 2 \(\mu\)g/mL) and CSF1R (Santa Cruz, clone C-20; 0.5 \(\mu\)g/mL).

**Mice**

All animal work was performed in accordance with a protocol approved by the IACUC of Memorial Sloan Kettering Cancer Center. Myc-Cap prostate cancer cell tumors were established by injecting 4 \(\times\) 10\(^5\) cells into the hindlimb of male 6 to 8-week old FVB/6 mice (Taconic, Hudson, NY). TRAMP C2 prostate cancer cell tumors were established by injecting 7.5 \(\times\) 10\(^5\) cells into the hindlimb of male 6 to 8-week old C57BL/6 mice (Taconic, Hudson, NY). Tumor growth was monitored by caliper measurement weekly. When the volume of tumors reached approximately 100 mm\(^3\), the animals were randomly assigned to different cohorts for further experiments.

**Treatment**

For VTP treatment, an anesthetic cocktail of 150 mg/kg ketamine and 10 mg/kg xylazine was administered intraperitoneally prior to treatment and was supplemented with inhaled isoflu- rane. A single dose of carprofen (5 mg/kg) and 1 mL of subcutaneous warm saline was administered. WST11 was reconstituted in sterile 5% dextran in water at 2 mg/mL under light protected condition and the aliquots were stored at −20°C. On the day of VTP treatment, an aliquot was thawed and filtered through 0.2 \(\mu\)m disc syringe filter (Sartorius Stedim Biotech North America, Bohemia, NY). The mice were intravenously infused with WST11 (9 mg/kg) followed immediately by 10-min laser (Ceramoptec, Bonn, Germany) illumination (755 nm, 150 mW/cm\(^2\)) through a 1 mm frontal fiber (MedLight SA, Ecublens, Switzerland). The light field was arranged to cover the entire tumor area plus 1 mm rim using red-light aiming beam. For anti-CSF1R treatment, mice were injected with 250 \(\mu\)g intraperitoneally on days 1, 4, 7, 10, 13.

**Histology**

All tumor specimens were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), embedded in paraffin, sectioned at five-micron thickness, and stained with hematoxylin and eosin (H&E). IHC staining was performed on FFPE sections at the Laboratory of Comparative Pathology at MSKCC on a Leica Bond RX automated stainer (Leica Biosystems, Buffalo Grove, IL). Following HIER at pH 9.0, the primary antibodies followed by application of an anti-goat IgG secondary antibody and a polymer detection system (DS9800, Novocastra Bond Polymer Refine Detection, Leica Biosystems). The chromogen was 3,3-diaminobenzidine tetrachloride (DAB), and sections were counterstained with hematoxylin. Slides were scanned with Pannoramic Flash (3DHistech, Hungary) using 20x/0.8NA objective, and regions of interest were drawn using CaseViewer (3DHistech, Hungary). Image quantification for Iba1 and CD31 IHC were performed using QuPath 0.1.2 software. Positive area (%) was calculated as follows from the output of Positive pixel count analysis: Positive area (%) = (Positive pixel count (downsampled pixels) x Downsample factor2)/(Area (\(\mu\)m2)/\(\mu\)m2 per pixel). The immunofluorescent staining was performed at Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using Discovery XT processor (Ventana Medical Systems). The tissue sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with CC1 buffer (Ventana Medical Systems). Sections were blocked for 30 min with Background Buster solution (Innovex), followed by avidin-biotin blocking for 8 min (Ventana Medical Systems). Multiplex immunofluorescent stainings were performed as previously described (http://dx.doi.org/10.1038/srep09534). Sections were stained according
to the following steps: first, slides were incubated with anti-F4/80 (Abcam, cat#ab6640, 2 ug/ml) for 4 hr, followed by 60-min incubation with biotinylated goat anti-rat IgG (Vector Labs, cat# PK4004) at 1:200 dilution. The detection was performed with Streptavidin-HRP D (part of DABMap kit, Ventana Medical Systems), followed by incubation with Tyramide Alexa Fluor 488 (Invitrogen, cat# T20922) prepared according to manufacturer instruction with predetermined dilutions. Next, sections were incubated with anti-CSF1R (Santa Cruz, cat#sc-692, 0.5 ug/ml) for 5 hr, followed by 60-min incubation with biotinylated goat anti-rabbit IgG (Vector, cat# PK6101) at 1:200 dilution. The detection was performed with Streptavidin-HRP D (part of DABMap kit, Ventana Medical Systems), followed by incubation with Tyramide CF543 (Biotium, cat# 92174) prepared according to manufacturer instruction with predetermined dilutions. Next, sections were incubated with anti-CD8 (Cell Signaling, cat#98941, 2.4 ug/ml) for 5 hr, followed by 60-min incubation with biotinylated goat anti-rabbit IgG (Vector, cat# PK6101) at 1:200 dilution. The detection was performed with Streptavidin-HRP D (part of DABMap kit, Ventana Medical Systems), followed by incubation with Tyramide CF543 (Biotium, cat# 92172) prepared according to manufacturer instruction with predetermined dilutions. Finally, sections were incubated with anti-CD11b (Abcam, cat#ab133357, 1 ug/ml) for 5 hr, followed by 60-min incubation with biotinylated goat anti-rabbit IgG (Vector, cat# PK6101) at 1:200 dilution. The detection was performed with Streptavidin-HRP D (part of DABMap kit, Ventana Medical Systems), followed by incubation with Tyramide Alexa 647 (Invitrogen, cat# T20936) prepared according to manufacturer instruction with predetermined dilutions. After staining, slides were counterstained with DAPI (Sigma Aldrich, cat# D9542, 5 ug/ml) for 10 min and covered-slipped with Mowiol. Slides were scanned with Pannoramic Flash (3DHistech, Hungary) using 20x/0.8NA objective, and regions of interest were drawn using CaseViewer (3DHistech, Hungary). The images were then analyzed using ImageJ/FIJI (NIH) to count colocalized cells with F4/80, CSF1R, CD8, and CD11b. The DAPI channel was used to obtain the cell nuclear mask by thresholding and cell segmentation. The other channels were thresholded and checked to see co-localization and cell counts for the combination of channels were measured.

Flow cytometry

Single cell suspensions were collected from tumors by physically disrupting tumors through a 70 µm nylon mesh in PBS. Cell suspensions were incubated in Fc block (2.4G2; MSKCC antibody and bioresource core) for 10 min on ice in 0.5% bovine serum albumin and 2 mM EDTA in PBS, followed by antibodies at previously noted dilutions plus Fixable viability dye eFluor 506 (eBioscience; cat# 65–0866-14) for 30 min on ice. For CD8+ T cell staining, single cell suspensions were first stained, without any additional restimulation, with αCD8 and αCD45 as described above, followed by fixation and permeabilization using the eBioscience™ FixP3/Transcription Factor Staining Bugger Set (Invitrogen) according to manufacturer’s instructions and then stained αGZMB for 30 min on ice in 1X permeabilization buffer provided by the commercial kit. Data were acquired using the LSRII Flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar). Gating strategy is reported in Supplementary Figure 1.

Statistical analysis

Data are represented as means ± SEM and differences between groups were calculated using the unpaired two-tailed Student t-test. Differences in tumor growth were calculated by measuring the area under the curve and followed by an unpaired two-tailed Student t-test. Log Rank tests were used to compare differences in overall survival. Statistical analysis was performed with the Prism 7.0.2 software (GraphPad Software, Inc.).

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Disclosure of Potential Conflicts of Interest

AR. Azzouzi is proctor for Steba Biotech.
K. Kim, A. Scherz, and J.A. Coleman are listed as co-inventors on a provisional patent application on VTP and combination therapy that is owned by Memorial Sloan Kettering Cancer Center and the Weizmann Institute of Science.
A. Scherz is the inventor of WST11 that has been licensed by “Yeda”, the commercial branch of Weizmann Institute to Steba Biotech with royalties agreement.
T. Merghoub is a consultant for: Immunos Therapeutics and Pfizer; is co-founder and has equity in: IMVaq therapeutics; has research support from: Bristol-Myers Squibb; Surface Oncology; Kyn Therapeutics; Infinity Pharmaceuticals, Inc., Peregrine Pharmaceuticals, Inc., Adaptive Biotechnologies, Leap Therapeutics, Inc., and Aprea; is an inventor on patent applications related to work on Oncolytic Viral therapy, Alpha Virus Based Vaccine, Neo Antigen Modeling, CD40, GITR, OX40, PD-1 and CTLA-4.
Wolchok is consultant for: Adaptive Biotech; Advaxis; Amgen; Apricity
Array BioPharma; Ascenta Pharma;Astellas; Bayer; Beigene; Bristol Myers Squibb; Celgene; Chugai; Elucida; Eli Lilly; F Star; Genentech; Imvq; Janssen; Kleo Pharma; Lineaeus; MedImmune; Merck; Neon Therapeutics; Oneo; Polaris Pharma; Polynoma; Psioxus; Puretech; Recepta; Trieza; Sellas Life Sciences; Serametrix; Surface Oncology; Syndax. Research support: Bristol Myers Squibb; MedImmune; Merck Pharmaceuticals; Genentech. Equity in: Potenza Therapeutics; Tizona Pharmaceuticals; Adaptive Biotechnologies; Elucida; Imvq. Beigene; Trieza; Lineaeus. Honorarium: Esanex. Patents: Xenogeneic DNA Vaccines; ALPHAVIRUS REPLICON PARTICLES EXPRESSING TRP2; Myeloid-derived suppressor cell (MDSC) assay; NEWCASTLE DISEASE VIRUSES FOR CANCER THERAPY; Genomic Signature to Identify Responders to Ipilimumab in Melanoma; Engineered Vaccinia Viruses for Cancer Immunotherapy; Anti-CD40 agonist mAb fused to Monophosphoryl Lipid A (MPL) for cancer therapy; CAR+ T cells targeting differentiation antigens as means to treat cancer; Anti-PD1 Antibody; Anti-CTLA4 antibodies; Anti-GITR antibodies and methods of use there of.

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