A Phase 2 Trial of Enhancing Immune Checkpoint Blockade by Stereotactic Radiation and *In Situ* Virus Gene Therapy in Metastatic Triple Negative Breast Cancer

Kai Sun[1], Yitian Xu[2], Licheng Zhang[2], Polly Niravath[1], Jorge Darcourt[1], Tejal Patel[1], Bin S. Teh[3], Andrew M. Farach[3], Carlo Guerrero[1], Sunil Mathur[1], Mark A. Sultenfuss[4], Nakul Gupta[4], Mary R. Schwartz[5], Susan L. Haley[5], Sindhu Nair[1] , Xiaoxian Li[6], Thi Truc Anh Nguyen[6], Joseph D. Butner[7], Joe Ensor[1], Jamie A. Mejia[8], Zhuyong Mei[9], E. Brian Butler[3], Shu-hsia Chen[2], Eric H. Bernicker[1], Jenny C. Chang[1]

[1] Houston Methodist Neal Cancer Center, 6445 Main Street, Houston, Texas 77030

[2]Houston Methodist Research Institute, Center for Immunotherapy Research, 6670 Bertner Ave, Houston, TX 77030

[3]Department of Radiation Oncology, Houston Methodist Hospital, 1130 Earle St. Houston, Texas 77030

[4]Department of Radiology, Houston Methodist Hospital, 6565 Fannin St. Houston, Texas 77030

[5]Department of Pathology and Genomic Medicine, Houston Methodist Hospital, 6565 Fannin St. Houston, Texas 77030

[6]Department of Pathology and Laboratory Medicine, Emory University, 201 Dowman Dr, Atlanta, GA 30322

[7]Mathematics in Medicine Program, Houston Methodist Research Institute, 6670 Bertner Ave, Houston, TX 77030

[8]Merck Research Laboratories

[9]Center for Gene and Cell therapy, Houston Methodist Hospital, 6565 Fannin St. Houston, Texas 77030

Corresponding author:
Jenny C. Chang, MD
Email: jcchang@houstonmethodist.org
Address: 6445 Main Street, OPC 24th floor, Houston, Texas 77030
Phone: 7134419948
Fax: 7134418791

Running title
Phase 2 study of ICI, SBRT and ADV/HSV-tk therapy in mTNBC

Clinical trial information
NCT03004183

Conflict of interest:

The authors declare no potential conflicts of interest.

Abstract

Purpose

A Phase 2 trial of stereotactic radiation therapy and *in situ* cytotoxic virus therapy in metastatic triple-negative breast cancer (mTNBC) patients followed by pembrolizumab (STOMP) was designed to evaluate dual approach of enhancing single-agent immune checkpoint blockade with ADV/HSV-tk plus valacyclovir gene therapy and SBRT in mTNBC patients.

Methods:
In this single-arm, open-label Phase 2 trial, mTNBC patients were treated with ADV/HSV-tk (5 x 10^{11} vp) intratumoral injection, followed by SBRT to the injected tumor site, then pembrolizumab (200mg, Q3w). The primary endpoint was clinical benefit rate (CBR, CR, PR or SD≥ 24 weeks per RECIST version1.1 at non-irradiated site). Secondary endpoints included duration on treatment (DoT), OS and safety. Exploratory endpoints included immune response to treatment assessed by correlative tissue and blood-based biomarkers.

Results:
28 patients were enrolled and treated. CBR was seen in 6 patients (21.4%) including two CR (7.1%), one PR (3.6%) and three SD (10.7%). Patients with clinical benefit had durable responses, with median DoT of 9.6 months and OS of 14.7 months. The median OS was 6.6 months in the total population. The combination was well tolerated. Correlative studies with CyTOF and IMC revealed a significant increase of CD8 T cells in responders and of myeloid cells in non-responders.

Conclusions:
The median OS increased by more than 2-fold in patients with clinical benefit. The therapy is a well-tolerated treatment in heavily pretreated mTNBC patients. Early detection of increased effector and effector memory CD8 T cells and myeloids correlate with response and non-response, respectively.

Introduction

Triple-negative breast cancer (TNBC) is an aggressive, difficult-to-treat disease. The median overall survival of locally advanced unresectable or metastatic TNBC (mTNBC) is 8 to 13 months[1,2]. Compared with other breast cancer types, TNBC has higher expression levels of programmed death-ligand 1 (PD-L1)[3] which makes immune checkpoint blockade (ICB) a potentially appealing treatment option. When checkpoint inhibitors as monotherapy were studied in mTNBC, durable responses were seen in those who responded. However, only 5.2% to 25.9% of patients benefited from monotherapy treatment and the responses were seen mostly in patients with high PD-L1 expression in earlier lines of treatment[4–7]. Thus, there is an unmet need of treatment strategies to enhance the antitumor activity of ICB.

Radiotherapy has traditionally been applied for local tumor control, based on its cytocidal activity through direct or indirect DNA damage. Recent studies have shown that radiotherapy can modify the tumor microenvironment to induce a systemic antitumor immune response through proinflammatory cytokines and engagement of the innate and adaptive immune systems[8,9] leading to abscopal effect and response to ICB in distant non-irradiated sites[10,11]. Abscopal effect was more often observed when fractionated instead of a single dose radiation was given[12,13]. The advent of stereotactic body radiotherapy (SBRT) allows for hypofractionated treatment with precise delivery of high radiotherapy doses, potentially achieving an ideal balance of tumor ablation and immune activation[14].

Even though abscopal effect has been reported, the overall occurrence rate is low[15]. Different strategies have been applied to enhance abscopal effect. Adenovirus-mediated expression of herpes-simplex-virus thymidine-kinase (ADV/HSV-tk) can catalyze the phosphorylation of acyclovir to a toxic form capable of inhibiting DNA synthesis and resulting in selective cytotoxicity to cells expressing HSV-tk[16]. ADV/HSV-tk gene transduction followed by acyclovir prodrug ganciclovir or valacyclovir therapy has shown both local and systemic antitumor activity in several cancer models[17–19]. It was found to induce a pronounced intratumoral infiltrate of macrophages, CD4+ and CD8+ T cells and a cytokine profile similar to Th1 immune response[20]. Combing ADV/HSV-tk plus ganciclovir gene therapy and radiotherapy appeared to result in increased CD4+ T cell tumor infiltration, additive killing activities and enhanced systemic antitumor activity in both prostate cancer and mammary tumor models[21,22].

Studies have shown the antitumor effect of ICB can be enhanced by both radiotherapy and ADV/HSV-tk plus ganciclovir gene therapy. Combining radiotherapy with ICB demonstrated enhanced systemic CD8+ T cell-dependent antitumor activity in several mouse cancer models[23,24], and improved responses in solid tumors[25–27]. In a study evaluating the combination of ADV/HSV-tk plus ganciclovir gene therapy and PD-L1 checkpoint inhibitor in glioblastoma, the combination therapy upregulated PD-L1 expression and improved long-term survival[28].

Here, we explored the approach of utilizing SBRT and ADV/HSV-tk plus valacyclovir gene therapy before pembrolizumab to enhance pembrolizumab efficacy in patients with mTNBC.

Methods

Patients

Patients with mTNBC who had relapsed on or were refractory to standard-of-care therapy were eligible for the study. Eligible participants were at least 18 years old, had histologically confirmed TNBC or low estrogen/progesterone receptor breast cancer (estrogen/progesterone receptor < 10%, and HER2 negative), an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, measurable disease based on Response Evaluation Criteria in Solid Tumors, version 1.1 (RECIST v1.1), a target lesion of at least 1 cm in diameter for SBRT and a measurable distant metastasis apart from the irradiated site of at least 1 cm to evaluate response. Patients with previously treated brain metastases were eligible for the study provided they were stable (without evidence of progression by imaging for at least 4 weeks prior to the first dose of trial treatment and any neurologic symptoms have returned to baseline), have no evidence of carcinomatous meningitis, or new/enlarging brain metastases, and are not using steroids for at least 7 days prior to trial treatment. Other required inclusion criteria included a willingness and ability to provide informed consent for the trial, a life expectancy of at least six months, and adequate organ functions.

Key exclusion criteria were bone metastasis only disease, prior treatment with immunotherapy including anti-PD-1, anti-PD-L1 or anti-PD-L2 agents, prior treatment with gene vector therapy, immunodeficiency or receipt of any form of systemic immunosuppressive therapy (including glucocorticoids) within seven days prior to the first dose of trial treatment, active autoimmune disease that required systemic treatment for the preceding two years, supplemental oxygen dependence, carcinomatous meningitis, known active hepatitis B or hepatitis C infection, or major surgery within four weeks prior to study enrollment.

Study oversight

Study design and subject consent forms were reviewed and approved by the hospital institutional review board. All patients provided written informed consent. The study was sponsored by Merck & Co., Inc. and was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Adverse events (AEs) were recorded regularly according to the protocol and were subject to mandatory reporting by the investigators. Any serious AEs were reported to Merck within 24 hours.

Trial Design

STOMP was designed as a single center Phase 2 study evaluating the approach of enhancing the abscopal effect of SBRT with ADV/HSV-tk plus valacyclovir gene therapy in augmenting the efficacy of pembrolizumab monotherapy in mTNBC patients. ADV/HSV-tk was created by using a backbone Ad5-dl309 with a Rous sarcoma virus promoter that drives the herpes simplex virus thymidine kinase gene insert (Center for Gene and Cell therapy, Houston Methodist Hospital) as previously reported[29]. ADV/HSV-tk (5×10^{11} vp) in a 2-mL total volume was injected

intratumorally to a single target lesion per patient on day 0 of the study using imaging guidance along with fiducial placement for SBRT. Valacyclovir was administered orally at a dose of 2 g three times a day for 14 days from day 1 to day 15. The injected target lesion was radiated. Metastatic target lesions were treated with SBRT (30 Gy; 6 Gy X 5 fractions) and primary breast/chest wall sites were treated with SBRT, IMRT, or 3DCRT (30 Gy; 6 Gy x 5 fractions, BED10 = 48 Gy or 33.6 Gy; 4.2 Gy x 8 fractions, BED10 = 47.71 Gy) at the treating physician's discretion. SBRT were administered over two weeks from day 2 to day 16 of the study. Pembrolizumab at a dose of 200 mg was administered intravenously every 21 days after radiation. Pembrolizumab monotherapy was continued until disease progression, development of unacceptable side effects, withdrawal of consent or up to 24 months (35 cycles) in patients without disease progression.

Peripheral blood for immune correlative analysis was collected on Day 1 (baseline), Day 17 and Day 38 and two panels of Cytometry by Time of Flight (CyTOF) on paired peripheral blood mononuclear cells (PBMCs) were performed. One panel of 35 myeloid markers (M-panel, Supplementary Table S1) and a second panel of 35 T cell markers (T-panel, Supplementary Table S2) were used on each sample. Barnes-Hut tSNE implementation in Rtsne package was used to plot tSEN. Data were 99th percentile normalized before the analysis, and we used the default tSNE parameters (initial dimensions, 110; perplexity, 30; and theta, 0.5).

Additionally, paired core biopsies were performed on Day 1 (baseline) and Day 17. Imaging mass cytometry (IMC) was performed after staining of these patient samples with a 35-marker panel of metal-tagged antibodies and ablated by Hyperion (Fluidigm Inc.) (Supplementary Table S3).

Assessment

PD-L1 expression was measured as the percentage of tumor area involved by PD-L1 positive tumor infiltrating immune cells as assessed by immunohistochemistry (IHC) using the Ventana SP142 antibody testing which was validated in the Immunohistochemistry section of our institutional Pathology laboratory. PD-L1 expression was also measured as a combined positive score (CPS) using the PD-L1 IHC 22C3 PharmDx kit (Neogenomics, Fort Meyers, FL performed the staining and results were interpreted by our institutional pathologists) or E1L3N antibody (Cell Signaling Technology, Danvers, MA, our laboratory performed the staining and results were interpreted by outside pathologists). CPS was defined as the ratio of PD-L1-positive cells out of the total number of tumor cells X 100.

Tumor assessments in non-irradiated sites by computed tomography scans or magnetic resonance imaging were performed at screening, every 8 weeks thereafter until completion of the protocol-specified study treatment and/or at the discretion of the treating physician, when clinically indicated, and 30 days after the last dose of pembrolizumab. Tumor response was assessed in these *non-irradiated metastatic lesions* by two independent radiologists according to RECIST v1.1. AEs were evaluated throughout the trial till 30 days after the end of treatment and were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE), version 4.03.

Statistical Analysis

The primary end point was clinical benefit rate [CBR; the proportion of patients with complete response (CR), partial response (PR) or stable disease (SD) for ≥ 24 weeks]. Secondary end points included overall survival (OS; the time from intratumoral viral injection to death or last date of contact), duration on treatment (DoT) and safety. Exploratory objectives included immune response to treatment, correlative tissue and blood-based biomarkers which included programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) expression, immune infiltrates, and cytokine expression (interleukin [IL]-1, IL-2, IL-6, IL-12, interferon [IFN]-c, tumor necrosis factor-α [TNF-α], and granulocyte macrophage colony-stimulating factor [GM-CSF]). Efficacy and safety were assessed in all patients who received intratumoral ADV/HSV-tk injection.

With the null and alternate hypotheses of 19% CBR and 39% CBR, respectively, an enrollment of 28 patients with TNBC was planned for the target response rate of 39%. Power study revealed that with a sample size of 28 patients if the observed number of responders is 11 (39.3%); the 95% CI estimate will have a margin of error of 18.1 percentage points (i.e., 21.2% to 57.4%) based on the normal approximation. Kaplan-Meier method was used to obtain estimates of 95% CIs for mean and median survival times. Pearson correlation analysis was used to assess the association of response with lactate dehydrogenase (LDH), disease-free interval (DFI), PD-L1 expression assessed by Ventana SP142 antibody testing, and CPS as assessed by IHC using the Dako 22C3 PharmDx kit.

Data Availability

The data generated in this study are stored in a secured drive and available upon request from the corresponding author.

Results

Patients

From June 7, 2017 to May 7, 2020, a total of 39 patients were screened and 28 patients with histologically confirmed mTNBC were enrolled (Table 1). Median age was 54 years (range, 34 to 78). The majority of patients were heavily pretreated, with a median of two (range, 0-7) previous lines of cytotoxic treatment in the metastatic setting, and eight patients (25.0%) had received ≥3 prior metastatic lines of chemotherapy. The median duration of response from the last line of treatment was two months (range, 1 to 7). Of note, three patients (10.7%) had treated brain metastasis at the time of enrollment. The majority of patients were PD-L1 negative; 18 (64.3%) patients had < 1% PD-L1 expression by Ventana SP142 antibody testing. Four patients (14.3%) had CPS combined score ⩾ 10 by PD-L1 IHC 22C3 PharmDx kit testing.

At the time of data cutoff on October 22, 2021, patients had received a median of three pembrolizumab doses (range, 0 to 23). Median duration of follow-up was 8.3 months (95% CI 3.0-10.1 months). One patient (3.6%) remained on treatment at the data cutoff date. Disease progression was the most common reason for treatment discontinuation (89.3%).

Efficacy

Of the 28 patients enrolled, six patients (21.4%) had clinical benefit, including two with complete response (7.1%), one with partial response (3.6%) and three with stable disease (10.7%) (Table 2). The median OS was 6.6 months (95% CI: 3.0 to 10.1 months) in the overall population, and 14.7 months (95%CI : 6.5 to 47.5 months) in the patients who had the clinical benefit (Figure 1A). The median DoT was 2.2 months (95% CI: 1.4 to 2.9 months) in the overall population, and 9.6 months (95% CI 4.8 to 23 months) in the patients who had clinical benefit (Figure 1B). In the overall population, 57.1% were alive at 6 months and 19.5% at twelve months; while in the responders, 100% were alive at 6 months and 66.7% at 12 months (Figure 1C).

Two patients who had clinical response were alive at the data cut-off date and one patient remained on treatment (Table 3). Patient 2 had biopsy-proven recurrent metastatic disease to the lungs and liver, with a 1.2 cm liver lesion diagnosed on PET scan which decreased in size on treatment. She then achieved CR but discontinued pembrolizumab after 18 doses due to Grade 3 pneumonitis. She remains in CR and has not received any systemic treatment for over 47 months. Patient 5 had recurrent biopsy-proven metastatic disease in the liver and has had SD on treatment for over 22 months.

We explored the potential predictive values of LDH, DFI and CPS. Clinical benefit was moderately associated with PD-L1 expression (r=0.46), but not with LDH, DFI or CPS.

Safety

Treatment-related AEs of any cause were reported in 22 patients (78.6%) (Table 4). The most common AEs were any grade constitutional (35.7%), hypothyroidism (21.4%), diarrhea (14.3%), nausea (10.7%), dyspnea on exertion (10.7%), alkaline phosphatase elevation (10.7%) and hyperthyroidism (10.7%). Grade 3 or higher treatment- related AEs were reported in 10 patients (35.7%). Immune-related AEs at any grade occurred in 13 patients (46.4%). The most common immune-related AEs were hypothyroidism (21.4%) and hyperthyroidism (10.7%). Grade 3 or higher immune-related AEs were reported in two patients (7.1%) including one pneumonitis (3.6%) and one transaminitis (3.6%). Two patients (7.1%) discontinued treatment due to Grade 3 pneumonitis and Grade 2 transaminitis. There were no treatment related deaths.

Immune profile analysis

To investigate the association between response and the immune populations in peripheral blood, CyTOF was performed on 25 patients (5 responders and 20 non-responders) on Day 1 and Day 17, and on 20 patients (4 responders and 16 non-responders) on Day 38. Cell populations were clustered, identified (Supplementary Table S4) and plotted on tSNE plots (Figure 2A). The log2 fold change between Day 17 or Day 38 versus Day 1 of responders and non-responders was calculated and illustrated as heatmaps (Figure 2B).

When the cell populations were compared between responders and non-responders at the same time points (Figure 2C), significantly more T cells (effector memory [Tem] CD4 T cells and

Tcm CD8 cells, unconventional T cells and NKT cells) were seen in the non-responders on Day 1. At Day 17, these T cell populations increased in the responders (Figure 2B&C), with Tem CD8 and the activated CD8 T cell (PD-1$^+$ CD8) showing the most profound increase. In the non-responders, a statistically significant increase of myeloid cells was observed in 14/20 (70%) patients. In those patients who had increased myeloid cells, 12/14 patients (85.7%) had a decrease of T cells. On Day 38, there was no continuous increase of CD8 T cells in responders except for one patient (Figure 2B). In comparison, continuous increase of myeloid cells was seen in the non-responders (10/16, 62.5%).

The tumor microenvironment was further assessed on paired tumor biopsies by IMC. A detailed description of IMC was previously described[30]. In responders, tumors contained myofibroblasts (SMA+) on Day 1 and became infiltrated with immune cells on Day 17 after treatment. In contrast, non-responder tumor cells were cohesive with few myofibroblasts present on Day 1 and limited immune cell infiltration was observed on Day 17 after treatment (Supplementary Figure S1A). In addition, more CD4 and CD8 T cells were found in responders with few Ki67$^+$ tumor cells, while more CD68$^+$ macrophages were found in the non-responders with more Ki67$^+$ tumor cells. The cell populations from three responders and 14 non-responders were then clustered and identified by unsupervised algorithms (Supplementary Figure S1B) and the log2 fold change between Day 1 versus Day 17 was plotted in a heatmap (Supplementary Figure S1C). Consistent with CyTOF findings, there was a significant increase of M2-like monocytes (CD163$^+$CD14$^+$) in non-responders (p=0.0012).

We then performed neighborhood analysis to assess the spatial relationship between the identified cell populations (Supplementary Figure S2). Comparing before and after treatment in responders, the epithelial cells or cancer cells (cluster 3) were seperated from most immune populations (cluster 17-24) at baseline, but became surrounded by these immune cells after treatment; CD8 T cells (cluster 23) were surrounded by immunosuppressive CD15$^+$ neutrophils (cluster 27) at baseline, but were freed from these neutrophils after treatment. Comparing responders and non-responders, the non-responders already had some immune populations surrounding epithelial cells or cancer cells (cluster 3) at baseline, which is consistent with our previous observation that non-responders had more CD4 T cells at baseline (Figure 1C). These results demonstrate distinct spatial patterns of tumor microenvironment and dynamic change with treatment in responders and non-responders.

Discussion

In this Phase 2 study to evaluate the efficacy of enhancing pembrolizumab monotherapy by engaging innate and adaptive immunity through SBRT and intratumoral ADV/HSV-tk gene therapy in mTNBC patients who had relapsed on or were refractory to standard-of-care therapy, the treatment demonstrated an acceptable safety profile. The incidence of grade 3 or higher any treatment-related AEs was 35.7% and immune-related AEs was 7.1%. The two grade 3 or higher immune-related AEs were managed with glucocorticoids and discontinuation of treatment. There were no treatment-related deaths. The safety profile of this trial was consistent with that seen in previous studies[5–7,31].

ICB monotherapy in mTNBC yields low ORR of 7.6% to 10% in second line and beyond metastatic setting[5,6], 2.6-5% in patients with low or negative PD-L1[6,7] and nearly no response in patients with liver metastases. Primary and acquired immune resistance involving antigen presenting machinery and T cell dysfunction play important roles in the low response rate to ICB in mTNBC[32]. Both radiotherapy and ADV/HSV-tk plus ganciclovir gene therapy can induce tumor antigen release, increase T cell infiltration and activation and PD-L1 expression[23,24,28]; adding ICB to either modality can potentially overcome PD-L1 inhibitory signals and enhance antitumor response. Indeed, many animal studies of combining radiotherapy or ADV/HSV-tk plus ganciclovir gene therapy with ICB have shown enhanced antitumor effect[12,23,24]. However, combining either SBRT or virus therapy with ICB only yielded moderate response in early phase clinical trials. In TONIC trial that explored different immune induction strategies with chemotherapy agents or radiation in mTNBC, the radiation arm had an ORR of only 8%[33]. In contrast to animal study showing radiation can induce T cell infiltration and activation[25], biopsy after treatment didn't show change of tumor infiltrating lymphocytes or CD8 cells in TONIC trial. Even though two Phase 2 studies combining SBRT with ICB in treated mTNBC[34,35] showed an ORR of 17.6 to 22%, the median duration of response was only 2.5 to 5 months and no response was observed in patients with liver metastases. Similarly, oncolytic virus type I herpes simplex virus Talimogene laherparepvec (TVEC, IMLYGIC®) in combination with ICB in mTNBC yielded only one partial response in a Phase 1 study[36].

Combining both SBRT and ADV/HSV-tk plus valacyclovir gene therapy with ICB may result in complementary mechanisms of DNA damage, alter and potentiate tumor- specific antigenicity and elicit a stronger immune response[22,37]. By combining three modalities, a CBR of 21.4% (two CR, one PR and three SD), a median DoT of 9.6 months and two long-term responders were observed. Although cross-trial comparisons could be confounded by different trial designs, patient populations and other factors, trimodality combination strategy improved ORR and DoT numerically compared to SBRT and ICB combination strategy[34,35]. Response observed was not associated with the line of treatment delivered in metastatic setting or PD-L1% or CPS. Among patients who had received chemotherapy in metastatic setting, 5/20 (25%) responded to the combination treatment, and 2/18 (11.1%) patients with negative PD-L1 had clinical benefit. Out of the six responders, three had CPS ≥ 10, two patients had CPS< 10, consistent with the observation that CPS is not predictive of response beyond first line metastatic setting[38]. Taken together, these data suggest that enhanced antitumor effect of SBRT, ADV/HSV-tk plus valacyclovir gene therapy and ICB combination therapy in pretreated patients and in patients with low or negative PD-L1.

We observed some atypical patterns of response in our patients, in particular in patients with liver metastases. Of the six responders in our study, two patients had liver metastases (Table 3). Patient 2 had biopsy-proven *lung metastases* and *one liver lesion* on PET scan which decreased in size with treatment. She then achieved CR but discontinued pembrolizumab after 18 doses due to Grade 3 pneumonitis. She remains in *complete remission without any systemic treatment* for over 47 months. Patient 5 had biopsy-proven relapsed metastatic disease in the

liver and has remained on treatment for over 22 months with stable disease. Visceral metastases, especially liver metastases, have been a poor prognostic factor in mTNBC[39,40]. Liver metastases have the lowest PD-L1 level among metastatic sites[41]. Historically, ICB monotherapy, ICB and SBRT combination therapy had very low response rates in those patients[5,34,36]. In contrast, previous studies of ADV/HSV-tk plus valacyclovir gene therapy demonstrated significant regression of liver metastases in breast cancer and lung cancer mouse models[42,43]. In the Phase 1 study of combining oncolytic type I herpes simplex virus Talimogene laherparepvec (TVEC, IMLYGIC®) with ICB in patients with mTNBC and liver metastases, one out of seven patients had a partial response[36]. The durable responses in these two patients in our study further suggest the role of ADV/HSV-tk plus valacyclovir gene therapy in re-priming T cells and potential added synergistic effect of ADV/HSV-tk plus valacyclovir gene therapy and SBRT in enhancing ICB in these with liver metastases, an observation that warrants further investigation.

In non-responders, immune profile analysis revealed high baseline T cells, however there was no increase of these T cells after treatment suggesting that these T cells may be tolerant to the tumor and are not proliferating. In contrast, in responders, the increase of T cells suggests that these T cells are newly proliferating in response to the treatment and can antigen-specifically target tumor cells. In addition, neighborhood analysis suggested these immune cells were actively engaged with cancer cells for tumor killing. Myeloid cells in the tumor can exhibit suppressive activity on T cells. In responders, CD8 T cells were distant from $CD15^+$ neutrophils after treatment, indicating that the immune populations were freed from immunosuppressive neutrophils. In contrast, myeloid cells increased in non-responders after treatment. These findings further support that myeloid cells play an important role in immunosuppression and treatment response. Six non-responders exhibited immune profiles similar to that of responders with increased Tem CD8 cells and PD-L1+ CD8 T cells on D17. Among these six patients, three had increased PD-L1+ monocytes at D17 and two exhibited delayed increase of myeloid cells on D38. Moderate upregulation of Tregs and exhausted CD4 T cells were also seen in one of these six non-responders. These observations are consistent with previously reported mechanisms of ICB resistance[44–46].

There are several limitations to this study: (1) As a single arm study, patients were not randomized and the exact contribution of single modality to the enhanced antitumor effect is unknown. Given that oncolytic virus alone[47] or in combination with ICB[36] had a low response rate of 0-12.5%, and ICB and SBRT combination only improved response with short duration of response in pre-treated patients without liver metastases[34], the results from our study suggest all three treatment modalities contributed to the observed enhanced antitumor effect. However, larger randomized studies are needed to elucidate the exact role of each modality and their additive effects on tumor microenvironment response. (2) Initial trial design defined PD-L1 expression by IHC using the Ventana SP142 antibody. CPS score as assessed by the PD-L1 IHC 22C3 PharmDx kit was later added as another measurement after FDA approval of pembrolizumab in combination with chemotherapy in untreated mTNBC based on CPS. This led

to the unavailability of CPS in almost one-third of patients. (3) Although the correlative biomarker analyses suggest that an increase of CD8 T cells and myeloid cells may serve as useful predictive markers for responders and non-responders, the results need to be interpreted with caution in view of the small sample size of this study. Larger studies are needed to validate these initial findings.

Conclusion

Even though the study did not meet its statistical significance, the durable responses in heavily pretreated mTNBC patients suggest that the efficacy of ICB monotherapy may be enhanced by harnessing abscopal effect of SBRT and intratumoral ADV/HSV-tk plus ganciclovir gene therapy, even in those with low PD-L1 level and liver metastases. Early detection of increased effector and effector memory CD8 T cells and myeloid cells correlates with response and non-response, respectively.

Acknowledgements

The clinical trial was supported by Merck Sharp & Dohme Corp.

The authors would like to acknowledge Dr. Mary and Ron Neal for their generous financial support; Dr. Helen Heslop and Dr. Malcom Brenner for their input in this research.

References

1. Kassam, F. *et al.* Survival outcomes for patients with metastatic triple-negative breast cancer: implications for clinical practice and trial design. *Clin Breast Cancer* **9**, 29–33 (2009).
2. den Brok, W. D. *et al.* Survival with metastatic breast cancer based on initial presentation, de novo versus relapsed. *Breast Cancer Res Treat* **161**, 549–556 (2017).
3. Mittendorf, E. A. *et al.* PD-L1 expression in triple-negative breast cancer. *Cancer Immunol Res* **2**, 361–370 (2014).
4. Nanda, R. *et al.* Pembrolizumab in Patients With Advanced Triple-Negative Breast Cancer: Phase Ib KEYNOTE-012 Study. *J Clin Oncol* **34**, 2460–2467 (2016).

5. Adams, S. *et al.* Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: cohort A of the phase II KEYNOTE-086 study. *Ann Oncol* **30**, 397–404 (2019).
6. Emens, L. A. *et al.* Long-term Clinical Outcomes and Biomarker Analyses of Atezolizumab Therapy for Patients With Metastatic Triple-Negative Breast Cancer: A Phase 1 Study. *JAMA Oncol* **5**, 74–82 (2019).
7. Dirix, L. Y. *et al.* Avelumab, an anti-PD-L1 antibody, in patients with locally advanced or metastatic breast cancer: a phase 1b JAVELIN Solid Tumor study. *Breast Cancer Res Treat* **167**, 671–686 (2018).
8. Lugade, A. A. *et al.* Radiation-induced IFN-gamma production within the tumor microenvironment influences antitumor immunity. *J Immunol* **180**, 3132–3139 (2008).
9. Matsumura, S. *et al.* Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. *J Immunol* **181**, 3099–3107 (2008).
10. Palma, D. A. *et al.* Stereotactic ablative radiotherapy versus standard of care palliative treatment in patients with oligometastatic cancers (SABR-COMET): a randomised, phase 2, open-label trial. *Lancet* **393**, 2051–2058 (2019).
11. Theelen, W. S. M. E. *et al.* Effect of Pembrolizumab After Stereotactic Body Radiotherapy vs Pembrolizumab Alone on Tumor Response in Patients With Advanced Non-Small Cell Lung Cancer: Results of the PEMBRO-RT Phase 2 Randomized Clinical Trial. *JAMA Oncol* **5**, 1276–1282 (2019).
12. Dewan, M. Z. *et al.* Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res* **15**, 5379–5388 (2009).

13. Vanpouille-Box, C., Formenti, S. C. & Demaria, S. TREX1 dictates the immune fate of irradiated cancer cells. *Oncoimmunology* **6**, e1339857 (2017).
14. Farach, A., Farach-Carson, M. C., Butler, E. B., Chang, J. C. & Teh, B. S. The role of combined radiation and immunotherapy in breast cancer treatment. *J Radiat Oncol* **4**, 347–354 (2015).
15. Liu, Y. *et al.* Abscopal effect of radiotherapy combined with immune checkpoint inhibitors. *J Hematol Oncol* **11**, 104 (2018).
16. Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* **46**, 5276–5281 (1986).
17. Hall, S. J., Mutchnik, S. E., Chen, S. H., Woo, S. L. & Thompson, T. C. Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. *Int J Cancer* **70**, 183–187 (1997).
18. Germano, I. M., Fable, J., Gultekin, S. H. & Silvers, A. Adenovirus/herpes simplex-thymidine kinase/ganciclovir complex: preliminary results of a phase I trial in patients with recurrent malignant gliomas. *J Neurooncol* **65**, 279–289 (2003).
19. Teh, B. S. *et al.* Phase I-II trial evaluating combined intensity-modulated radiotherapy and in situ gene therapy with or without hormonal therapy in treatment of prostate cancer-interim report on PSA response and biopsy data. *Int J Radiat Oncol Biol Phys* **58**, 1520–1529 (2004).
20. Vile, R. G. *et al.* Generation of an anti-tumour immune response in a non-immunogenic tumour: HSVtk killing in vivo stimulates a mononuclear cell infiltrate and a Th1-like profile of intratumoural cytokine expression. *Int J Cancer* **71**, 267–274 (1997).

21. Atkinson, G. & Hall, S. J. Prodrug activation gene therapy and external beam irradiation in the treatment of prostate cancer. *Urology* **54**, 1098–1104 (1999).
22. Vlachaki, M. T. *et al.* Enhanced therapeutic effect of multiple injections of HSV-TK + GCV gene therapy in combination with ionizing radiation in a mouse mammary tumor model. *Int J Radiat Oncol Biol Phys* **51**, 1008–1017 (2001).
23. Demaria, S. *et al.* Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. *Clin Cancer Res* **11**, 728–734 (2005).
24. Deng, L. *et al.* Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. *J Clin Invest* **124**, 687–695 (2014).
25. Twyman-Saint Victor, C. *et al.* Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature* **520**, 373–377 (2015).
26. Aboudaram, A. *et al.* Concurrent radiotherapy for patients with metastatic melanoma and receiving anti-programmed-death 1 therapy: a safe and effective combination. *Melanoma Res* **27**, 485–491 (2017).
27. Shaverdian, N. *et al.* Previous radiotherapy and the clinical activity and toxicity of pembrolizumab in the treatment of non-small-cell lung cancer: a secondary analysis of the KEYNOTE-001 phase 1 trial. *Lancet Oncol* **18**, 895–903 (2017).
28. Speranza, M.-C. *et al.* Preclinical investigation of combined gene-mediated cytotoxic immunotherapy and immune checkpoint blockade in glioblastoma. *Neuro Oncol* **20**, 225–235 (2018).

29. Chen, S. H., Shine, H. D., Goodman, J. C., Grossman, R. G. & Woo, S. L. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. *Proc Natl Acad Sci U S A* **91**, 3054–3057 (1994).
30. Liu, H.-C. *et al.* Potentiating Antitumor Efficacy Through Radiation and Sustained Intratumoral Delivery of Anti-CD40 and Anti-PDL1. *Int J Radiat Oncol Biol Phys* **110**, 492–506 (2021).
31. Cortés, J. *et al.* KEYNOTE-119: Phase III study of pembrolizumab (pembro) versus single-agent chemotherapy (chemo) for metastatic triple negative breast cancer (mTNBC). *Annals of Oncology* **30**, v859–v860 (2019).
32. Sharma, P., Hu-Lieskovan, S., Wargo, J. A. & Ribas, A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* **168**, 707–723 (2017).
33. Voorwerk, L. *et al.* Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. *Nat Med* **25**, 920–928 (2019).
34. Ho, A. Y. *et al.* A phase 2 clinical trial assessing the efficacy and safety of pembrolizumab and radiotherapy in patients with metastatic triple-negative breast cancer. *Cancer* **126**, 850–860 (2020).
35. David, S. *et al.* Abstract PD10-02: A randomised phase II trial of single fraction or multi-fraction SABR (stereotactic ablative body radiotherapy) with atezolizumab in patients with advanced triple negative breast cancer (AZTEC trial). *Cancer Research* **82**, PD10-02-PD10-02 (2022).
36. Hecht, J. R. *et al.* Abstract P3-09-19: Preliminary safety data of intrahepatic talimogene laherparepvec and intravenous atezolizumab in patients with triple negative breast cancer. in

Poster Session Abstracts P3-09-19-P3-09–19 (American Association for Cancer Research, 2020). doi:10.1158/1538-7445.SABCS19-P3-09-19.

37. Chhikara, M. *et al.* Enhanced Therapeutic Effect of HSV-tk+GCV Gene Therapy and Ionizing Radiation for Prostate Cancer. *Molecular Therapy* **3**, 536–542 (2001).
38. Adams, S. *et al.* Pembrolizumab monotherapy for previously untreated, PD-L1-positive, metastatic triple-negative breast cancer: cohort B of the phase II KEYNOTE-086 study. *Ann Oncol* **30**, 405–411 (2019).
39. Insa, A. *et al.* Prognostic factors predicting survival from first recurrence in patients with metastatic breast cancer: analysis of 439 patients. *Breast Cancer Res Treat* **56**, 67–78 (1999).
40. Wang, R. *et al.* The Clinicopathological features and survival outcomes of patients with different metastatic sites in stage IV breast cancer. *BMC Cancer* **19**, 1091 (2019).
41. Emens, L. A. *et al.* Atezolizumab and nab-Paclitaxel in Advanced Triple-Negative Breast Cancer: Biomarker Evaluation of the IMpassion130 Study. *J Natl Cancer Inst* **113**, 1005–1016 (2021).
42. Kwong, Y. L., Chen, S. H., Kosai, K., Finegold, M. J. & Woo, S. L. Adenoviral-mediated suicide gene therapy for hepatic metastases of breast cancer. *Cancer Gene Ther* **3**, 339–344 (1996).
43. Kwong, Y.-L., Chen, S.-H., Kosai, K., Finegold, M. & Woo, S. L. C. Combination Therapy With Suicide and Cytokine Genes for Hepatic Metastases of Lung Cancer. *Chest* **112**, 1332–1337 (1997).
44. Lainé, A. *et al.* Regulatory T cells promote cancer immune-escape through integrin αvβ8-mediated TGF-β activation. *Nat Commun* **12**, 6228 (2021).

45. Ustun, C., Miller, J. S., Munn, D. H., Weisdorf, D. J. & Blazar, B. R. Regulatory T cells in acute myelogenous leukemia: is it time for immunomodulation? *Blood* **118**, 5084–5095 (2011).

46. Mazzoni, A. *et al.* Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol* **168**, 689–695 (2002).

47. Kai, M. *et al.* A phase II study of talimogene laherparepvec for patients with inoperable locoregional recurrence of breast cancer. *Sci Rep* **11**, 22242 (2021).

Table 1. Patient characteristics at baseline.

	Total population (n= 28)	Responders (n= 6)	Non-responders (N= 22)
Median age (range)- years	54 (34-78)	62 (44-78)	50 (35-79)
Female sex – no. (%)	28 (100)	6 (100)	22 (100)
Race – no. (%)			
White	18 (64.2)	6 (100)	12 (54.5)
Black	5 (17.9)	0 (0)	5 (22.7)
Asian	5 (17.9)	0 (0)	5 (22.7)
Others	0 (0)	0 (0)	0 (0)
ECOG PS – no. (%)			
0	17 (60.7)	6 (100)	11 (50.0)
1	8 (28.6)	0 (0)	8 (36.4)
2	3 (10.7)	0 (0)	3 (13.6)
Germline BRCA1/2, no. (%)			
Mutation	0 (0)	0 (0)	0
Wild type	24 (85.7)	5 (83.3)	19 (86.4)
Unknown	4 (14.3)	1 (16.7)	3 (13.6)
Smoking status- no (%)			
Never smoker	24 (85.7)	4 (66.7)	20 (90.9)
Former smoker	4 (14.3)	2 (33.3)	2 (9.1)
Current smoker	0 (0)	0 (0)	0 (0)
Location of metastasis, no. (%)			
Lymph node only	6 (21.4)	2 (33.3)	4 (18.2)
Visceral metastasis	19 (79.2)	4 (66.7)	18 (81.8)
Brain metastasis	3 (10.7)	1 (16.7)	2 (9.1)
Previous lines of treatment in metastatic setting- no. (%)			
0	8 (28.6)	1 (16.7)	7 (31.8)
1	5 (17.9)	2 (33.3)	3 (13.6)
2	8 (28.6)	2 (33.3)	6 (27.3)
3	3 (10.7)	1 (16.7)	2 (9.1)
>= 4	4 (14.3)	0 (0)	4 (18.2)
Median lines of treatment	2 (0-7)	1.5 (0-3)	2 (0-7)
Median duration of response from last line of treatment (range), months	2 (1-7)	2 (1-6)	2 (1-7)
Prior chemotherapy received- no. (%)			
Anthracycline	24 (85.7)	5 (83.3)	19 (86.4)
Cyclophosphamide	24 (85.7)	5 (83.3)	19 (86.4)
Taxanes	27 (96.4)	5 (83.3)	22 (100)
Carboplatin	15 (53.6)	3 (50.0)	12 (54.5)
Microtubule inhibitors	7 (25.0)	1 (16.7)	6 (27.3)
Gemcitabine	9 (32.1)	0 (0)	9 (40.9)
Capecitabine	12 (42.9)	4 (66.7)	8 (36.4)
Anti-HER2 therapy	2 (7.1)	0 (0)	2 (9.1)
Anti-hormone therapy^	4 (14.3)	1 (16.7)	3 (13.6)
Others*	8 (28.6)	4 (66.7)	4 (18.2)
PD-L1%			
< 1%	18 (64.3)	2 (33.3)	16 (72.7)
≥ 1%	9 (32.1)	3 (50.0)	6 (27.3)
Unknown	1 (3.6)	1 (16.7)	0 (0)
CPS			
≥ 10	4 (14.3)	2 (33.3)	2 (9.1)
< 10	16 (57.1)	2 (33.3)	14 (63.6)
Unknown	8 (28.6)	2 (33.3)	6 (27.3)
Irradiated metastatic site			
Lymph nodes/skin/chest wall	13 (46.4)	3 (50.0)	10 (45.5)
Visceral	15 (53.6)	3 (50.0)	12 (54.5)
Baseline LDH			
normal	9 (32.1)	3 (50.0)	6 (27.3)
>1 ULN	15 (53.6)	3 (50.0)	12 (54.5)
>2 ULN	4 (14.3)	0 (0)	4 (18.2)

Abbreviations: ECOG PS: Eastern Cooperative Oncology Group Performance Status; CPS: combined positive score; LDH: lactate dehydrogenase.

^included aromatase inhibitors, CDK4/6 inhibitors, fulvestrant and everolimus.

*included pan-NOS inhibitor, PARPi with mTOR or AKT inhibitor, bevacizumab.

Table 2. Efficacy (RECIST 1.1) of non-irradiated metastatic lesion

	Breast (N= 28)
Best overall response – no. (%)	
Confirmed CR	2 (7.1)
Confirmed PR	1 (3.6)
Stable disease	3 (10.7)
Progressive disease	22 (78.6)
Clinical benefit (CR, PR and SD)	6 (21.4)

Abbreviations: CR: complete response; PR: partial response; SD: stable disease.

Table 3. Characteristics of patients with clinical benefit

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	44	62	58	67	77	60
ECOG PS	0	0	0	0	0	0
Location of metastasis	Chest wall, axillary and mammary LNs	Lung and liver	Lung, liver, brain, adrenal gland	Supraclavicular LN, pleura, bone	Liver	Mediastinal and axillary LN
Number of previous therapies for metastatic disease	2	1	2	1	0	3
Previous chemotherapy	Paclitaxel and bevacizumab, carboplatin	Docetaxel/pan-NOS inhibitor	Nab-paclitaxel/carbo, Docetaxel/pan-NOS inhibitor	Ixabepilone/ capecitabine	NA	Capecitabine, carboplatin/XRT, docetaxel/pan-NOS inhibitor
Duration of response on prior chemotherapy (months)	3	1	2	2	NA	6
DFI (month)	10.0	4.0	8.1	44.0	44.2	0.5
Irradiated site	Chest wall	Lung mass	Lung mass	Lymph node	Liver mass	Lymph node
LDH level at baseline	189	210	322	285	291	170
PD-L1 expression by Ventana SP142	2	1	Unknown	0	0	30
CPS by 22C3	10	80	Unknown	1	1	30
Best clinical response	CR	CR	PR	SD	SD	SD
DoT (month)	12.4	11.5	7.7	5.7	22.9	4.8
Overall survival (month)	17.7	47.5	11.7	8.3	22.9	6.5

Abbreviations: ECOG PS: Eastern Cooperative Oncology Group Performance Status; XRT: radiation; DFI: disease free interval; LDH: lactate dehydrogenase; DoT: duration on treatment.

Table 4. Adverse events in all patients

Events	Total population (N=28)	
	Any grade	Grade 3 or 4
Treatment-related AEs	22 (78.6)	10 (35.7)
AEs leading to discontinuation	2 (7.1)	2 (7.1)
Treatment related death	0 (0)	0 (0)
GI		
Mucositis	1 (3.6)	0 (0)
Nausea	3 (10.7)	0 (0)
Vomiting	0 (0)	0 (0)
Colitis	1 (3.6)	1 (3.6)
Diarrhea	4 (14.3)	0 (0)
Constipation	1 (3.6)	0 (0)
ALT elevation	2 (7.1)	0 (0)
AST elevation	2 (7.1)	1 (3.6)
ALP elevation	3 (10.7)	1 (3.6)
Abdominal pain	1 (3.6)	1 (3.6)
Decreased appetite	1 (3.6)	0 (0)
Hematologic toxicity		
Anemia	2 (7.1)	2 (7.1)
Leukocytosis	1 (3.6)	0 (0)
Pulmonary toxicity		
Pneumonitis	2 (7.1)	1 (3.6)
SOB	2 (7.1)	0 (0)
DOE	3 (10.7)	0 (0)
Cough	2 (7.1)	0 (0)
Endocrine		
Hypothyroidism	6 (21.4)	0 (0)
Hyperthyroidism	3 (10.7)	0 (0)
Infection		
Pneumonia	1 (3.6)	0 (0)
Shingles	1 (3.6)	1 (3.6)
Sepsis	1 (3.6)	1 (3.6)
Oral thrush	1 (3.6)	0 (0)
Skin	0 (0)	0 (0)
constitutional	10 (35.7)	2 (7.1)
Immune related AEs		
Hypothyroidism	6 (21.4)	0 (0)
Hyperthyroidism	3 (10.7)	0 (0)
ALT elevation	2 (7.1)	0 (0)
AST elevation	2 (7.1)	1 (3.6)
Pneumonitis	2 (7.1)	1 (3.6)

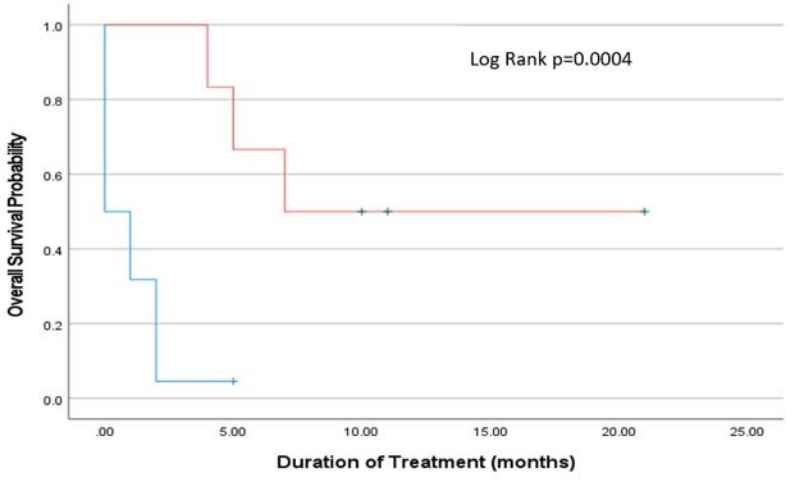
Abbreviations: AEs: adverse events; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; SOB: shortness of breast; DOE: dyspnea on exertion.

Legends

Fig. 1. Kaplan-Meier curve of overall survival and duration of treatment in responders and non-responders. (1A) Overall survival in responders (red line) and non-responders (blue line). The comparison of survival curves shows that patients who responded to the treatment had a significantly higher probability of survival (p=0.007). (1B) Kaplan-Meier curve of duration on treatment in responders (red line) and non-responders (blue line) showing significantly longer duration of treatment in responders vs non-responders (p=0.0004). (1C) Swimmer plot of patient survival by response. X axis represents survival in months and Y axis represents patients. Bars are colored by RECIST v1.1 response with red representing responders and blue representing non-responders. For patients who were responders (red bar), a green triangle represents complete response, a dark blue dot represents partial response, and a black square represents stable disease. For non-responders, a yellow diamond represents progression. A horizontal arrow at the end of a bar represents patient alive status**.** Responders: patients who had clinical benefit including these that had CR, PR or SD $\geqslant$ 24 weeks per RECIST v1.1 at non-irradiated metastatic sites.

Fig. 2. CyTOF analysis of paired PMBC on Day 1, Day 17 and Day 38. (2A) tSNE plots showing cell population clusters. (2B) Heatmap of log2 fold change between Day 17 versus Day 1 of responders and non-responders (upper figure) and log2 fold change between D38 versus Day 1 of responders and non-responders (lower figure). (2C) Comparison of different cell populations between responders (red box) and non-responders (green box) at Day 1, Day 17 and D38. Tem: effector memory T cell. Tcm: central memory T cells. P value was calculated by unpaired t test with Welch's correction for responder and non-responder comparison, and paired t test for fold change comparison at different days in responders.

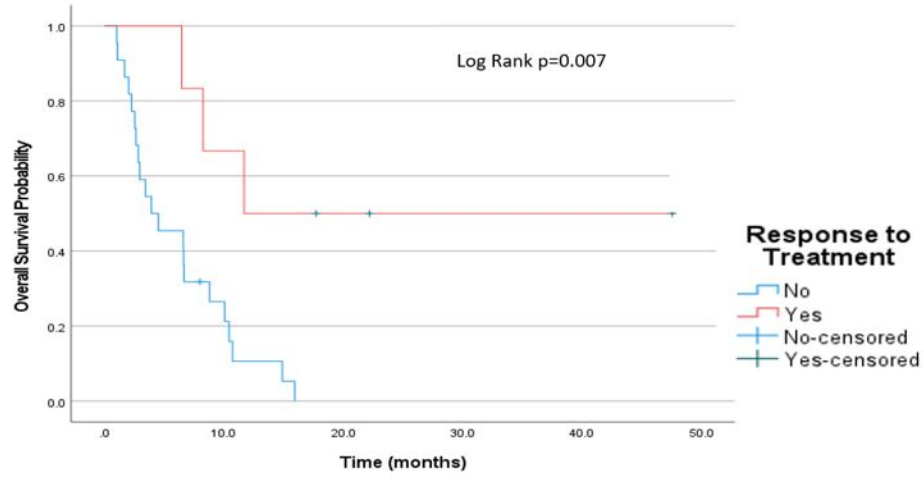
1A



Numbers at risk

Non-responders	22	1			
Responders	6	5	3	1	1

1B



Numbers at risk

Non-responders	22	5	0			
Responders	6	4	2	1	1	0

1C