Multimodality analysis confers a prognostic benefit of a T-cell infiltrated tumor microenvironment and peripheral immune status in patients with melanoma


ABSTRACT

Background We previously reported results from a phase 1 study testing intratumoral recombinant poxvirus, learpolurev, in 12 melanoma patients. All 12 patients received anti-PD-1 systemic therapy before learpolurev, and 11 of these 12 patients also received anti-PD-1 after learpolurev. In preclinical models learpolurev induces intratumoral innate inflammation that engages antitumor T cells. In the current study, prelearpolurev and postlearpolurev tumor biopsies and blood were evaluated for biomarkers of response.

Methods The following analyses were performed on tumor tissue (n=11): (1) flow cytometric assessment of immune cell density, (2) NanoString Digital Spatial profiling of protein and the transcriptome, and (3) bulk RNA sequencing. Immune cell phenotypes and responsiveness to in vitro stimulation, including in vitro learpolurev challenge, were measured in peripheral blood (n=12).

Results Three patients who received anti-PD-1 therapy within 30 days of learpolurev have a current median progression-free survival (PFS) of 2.3 years and had higher CD8+ T cell infiltrates in prelearpolurev tumor biopsies relative to that of 7 patients with median PFS of 1.6 months and lower CD8+ T cell infiltrates in prelearpolurev tumor biopsies. In peripheral blood, four patients with PFS 2.3 years (including three that received anti-PD-1 therapy within 30 days before learpolurev and had higher pretreatment tumor CD8+ T cell infiltrates) had significantly higher effector memory (CD8+,CCR7−,CD45RA−) but lower CD8+PD-1+ and CD4+PD-1+ cells compared with eight patients with median PFS 1.6 months. In addition, pretreatment blood from the four patients with median PFS 2.3 years had more potent antiviral responses to in vitro learpolurev challenge compared with eight patients with median PFS 1.6 months.

Conclusion An inflamed pretreatment tumor microenvironment, possibly induced by prior anti-PD-1 therapy and a proficient peripheral blood pretreatment innate immune response (antiviral/interferon signaling) to learpolurev was associated with long term PFS after intratumoral learpolurev in a small cohort of patients.

These findings imply a link between intratumoral T cell inflammation and peripheral immune function.

Trial registration number NCT03712358.

INTRODUCTION

Both host-mediated antitumor immune responses and therapeutic strategies designed to revive antitumor host immune responses are subject to multiple tumor-driven immune suppressive mechanisms and protumorigenic signaling that occur in the tumor microenvironment (TME). Cellular and molecular pathway evaluation have shown that the TME can regulate tumor progression and that altering the TME can correlate with therapeutic benefits. In particular, pretreatment melanoma tumors with higher intratumoral CD8+ T cells and increased IFN-γ signaling within the TME, so-called ‘hot tumors’, have been associated with response to anti-programmed cell death protein 1 (PD-1) therapy. Anti-PD-1 therapy reinvigorates intratumoral inflammation by expanding stem-like effector T cell populations in the TME, thereby altering the composition within the TME. Alternately, ‘cold’ tumors are characterized by minimal or absent T cell infiltration, and are less responsive to anti-PD-1 therapy. Among ‘cold’ tumors, phenotypes can include a lack of any appreciable T cell infiltrate or the peritumoral accumulation of T cells which are unable to enter the tumor, likely as a result of immunosuppressive myeloid cells or stroma in the TME. In melanoma, anti-PD-1 therapies have become the cornerstone of treatment for patients with metastatic disease and yet the majority of patients fail to respond; treatment failure...
is thought to be related in part to a ‘cold’ tumor phenotype and failure to remodel the TME.11 One approach for melanoma patients lacking responses to anti-PD-1 therapy is intratumoral delivery of agents designed to promote inflammation in the TME, which could potentiate responses to anti-PD-1 therapy.12,13

Lerapolturev (formerly known as PVSRIPO) is the live attenuated, type 1 poliovirus (Sabin) vaccine modified with the internal ribosomal entry site of human rhinovirus type 2.14 In preclinical models, the antitumor effects of lerapolturev included tumor cell lysis and activation of antigen presenting cells in the TME, which can potentially promote a ‘hot’ tumor phenotype.15-17 Indeed, lerapolturev treatment of ex vivo patient tumor slice cultures culminated in robust induction of PD-L1 expression on myeloid cells; intratumor therapy of murine melanoma tumors resulted in increased tumor infiltration of PD-1-expressing CD4 and CD8 T cells and elicited functional antigenic tumor CD8 T cell responses.16,17 These studies indicated potential for lerapolturev to sensitize melanoma tumors to anti-PD-1 therapy. Recently, we reported results of a phase 1 trial of intratumoral PVSRIPO in 12 patients with unresectable melanoma.18 In the phase 1 trial, four patients achieved an objective response while eight patients had no response; the injections were well tolerated. To determine immunologic effects in the TME and in the periphery, we performed tumor biopsies and blood analysis before and after lerapolturev. Here, we describe evaluation of the TME and peripheral blood analysis from melanoma patients receiving lerapolturev including the relationship of the pretreatment TME with clinical outcome, differences in pretreatment versus post-treatment tumor, comparison of results among the independent techniques used to study the tumor, and the relationship between innate activation potential in the peripheral blood and clinical outcome.

METHODS

All work was performed using Institutional Review Board approved protocols and patients signed written consent to participate. Trial details were published previously.18 Three patients received 1 injection of lerapolturev (patients 1–3), 3 patients received 2 total injections, each injection into a separate tumor (patients 4–6), 3 patients received 3 total injections each injection into a separate tumor (patients 7–9), and 3 patients received 3 total injections into the same tumor (patients 10–12).18 Tumor and peripheral blood were collected at multiple time points before and after lerapolturev. Pretreatment biopsies were collected 7–10 days prior to lerapolturev and post-treatment biopsies were performed 10 days after lerapolturev (for patients 1–9) and at median 30 days after last lerapolturev injection (for patients 11 and 12). In some cases, non-lerapolturev treated tumor was also biopsied. Flow cytometry analysis of tumor was conducted in real-time, shortly after sample collection with minimal processing to prevent exclusion or death of innate immune cells (eg, neutrophils, basophils, and eosinophils). A separate portion of each tumor was also preserved in paraffin. For each tumor specimen, a pathologist confirmed presence of viable tumor on H&E stains; if no viable tumor was noted, the sample was not used for analysis. Flow cytometry and in vitro stimulation assays were performed using banked frozen peripheral blood mononuclear cells (PBMCs) after isolation from blood.

Flow cytometry

Tumor tissue was collected and stored in MACS tissue storage solution at 4°C (Miltenyi). Storage time was 2–16 hours post-tumor collection. Tumor cells and tumor infiltrating immune cells were analyzed after tumors were processed using the Tumor Dissociation Kit and Gentle MACS mechanical dissociator (Miltenyi) following the manufacturer’s recommendations. The suspensions were then filtered three times through a 70 µm filter to remove undigested tissue and debris. Single cell suspensions were immediately incubated with the DuraClone IM Basic panel, which contains CD16-FITC, CD56-PE, CD19-APC, CD14-PC7, CD4-APC, CD8-AP700, CD3-AF750, and CD45-KO (Krome Orange) as previously described, to identify immune cells in tissue.19 In addition, propidium iodide (Sigma-Aldrich, St. Louis, MO) was added as a live-dead marker to single cell suspensions. Cells were incubated for 15–20 min in the dark and then washed twice in phosphate-buffered saline (PBS) prior to data acquisition. Samples were analyzed on a 13-color CytoFlex flow cytometer (Beckman Coulter). Data were analyzed using Kaluza Software (Beckman Coulter). Comparisons were made between two groups (responders and non-responders) using unpaired t-tests.

For analyses of PBMCs, frozen PBMCs were thawed in AIM-V media (Gibco) containing DNase I (1 µg/mL; Roche) and incubated for 30 min at 37°C. Cells were pelleted and resuspended in PBS (Gibco) containing Zombie-NIR stain (1:500; BioLegend) for 30 min. The cells were washed in PBS containing 2% Fetal bovine serum (FBS) (Sigma-Aldrich) and resuspended in PBS+2% FBS containing human Fc block (1:100, BioLegend). The following antibodies were added for staining at a 1:100 dilution: CD3-BUV737, CD8-BUV395 (from BD Biosciences); or CD4-BV510, CD25-PERCPc55.5, CD27-BV421, CD40L-PECF594, CD62L-FITC, PD1-BV711, CD127-PECy5, CD45RA-APC, CD45RO-BV786, CD28-PECy7, CD56-BV605, and CCR7-PE (all BioLegend). After 1 hour cells were washed in PBS+2% FBS and analyzed on a Fortessa X20 alongside fluorescence minus one controls for CD28, CD27, and PD1. Gating and analyses were performed using FlowJo V.10 (BD Biosciences).

In vitro PBMC stimulation assay

For in vitro stimulation of PBMCs, laboratory grade PVSRIPO was generated in HeLa cells (American Type Culture Collection, ATCC) as previously described.20 Frozen, pretreatment PBMCs were thawed in AIM-V media (Gibco), treated with DNaseI (1 µg/mL; Roche)
for 30 min, counted using a Countess II automated cell counter (Thermo-Fisher), and plated in 6 wells of a 24 well plate with PBMCs at a density of 5×10⁶ cells per well in RPMI-1640 (Gibco) containing 10% FBS (Sigma-Aldrich) with no other additives or added cytokines. The following stimulants were added to separate wells: mock (vehicle); PVSRIPO (1×10⁷ plaque forming units (pfu)); lipopolysaccharide (LPS) (100 ng/mL; Invivogen); Pam3CSK4 (1 µg/mL; Invivogen); R848 (1 nM; Invivogen); or combined anti-CD3 (1 µg/mL clone OKT3; BioLegend), anti-CD28 (1 µg/mL clone CD28.2; BioLegend), and goat anti-mouse IgG (4 µg/mL; Jackson Immunoresearch). Cultures were incubated for 24 hours in a 37°C and 5% CO₂ incubator, after which supernatant was harvested. Supernatant cytokines were measured using the Human Anti-Virus Response LegendPlex panel (BioLegend) on a Fortessa X20 (BD Biosciences), and the mean fluorescence intensity for each analyte was determined using the manufacturer’s LegendPlex data analysis software (BioLegend). Fold mock control values were determined for each PBMC sample for each analyte.

**NanoString digital spatial profiling**

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were analyzed using the NanoString GeoMx digital spatial profiling (DSP), a non-destructive method for high-plex spatial profiling of proteins and protein-coding genes. Detailed methods have been described. In brief, this method uses small photo-cleavable oligonucleotide ‘barcodes’ (PC oligos) covalently attached to in situ affinity reagents (antibodies and RNA probes) to provide unlimited multiplexing capability. The photo-cleavage light is projected onto the tissue slice using two-digital micromirror devices, containing 1 million semiconductor-based micromirrors allowing complete flexibility in the pattern of light utilized for high-plex digital profiling of the tissue. These spatial light patterns can be automatically configured to profile manually selected regions or molecularly defined compartments. Cell subset analysis can be done on a region with as low as 30 cells expressing the marker of interest. After a pathologist confirmed the presence of tumor on FFPE H&E stains, unstained slides were fluorescently stained to visualize the morphology for regions of interest (ROIs) selection and eventual proteomic or whole transcriptome analysis (WTA).

**Statistical analysis of NanoString DSP**

The protein panel included 59 proteins including internal-spike controls which were analyzed in selected ROIs. The raw data were normalized with a panel of three housekeeping proteins and three negative controls. The final step to control for variability in area and/or cellularity relies on determining the signal to noise ratio (SNR) which is calculated as the ratio of each probe over geometric mean of RNA sequencing

Fifty micron paraffin sections of tumor were used for RNA isolation using the RNeasy FFPE kit per manufacturer’s instructions by Agilent Technologies (formerly Genewiz). Ribosome depletion was used to enrich for mRNA. The resultant RNA quality and quantity were determined by TapedStation and Qubit assays and used for library preparation. Libraries were sequenced on an Illumina HiSeq (150 base pair (bp) paired end reads) instrument (Agilent Technologies). Genome alignment was performed using STAR (V.2.7.0). Differential expression analyses were performed using DEseq2 (V.3.14) and computed immune cell densities were determined using CIBERSORT.

**RESULTS**

**Clinical data**

Relevant clinical data are summarized in table 1; safety results have been previously reported. All 12 patients with advanced unresectable melanoma had received anti-PD-1 therapy prior to lerapoltuvec while 6 of the 12...
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*These patients received anti-PD-1 within 30 days of PVRSIP0, therefore, confirmation of disease progression on anti-PD-1 prior to study enrolment at 4 weeks did not occur. Patients in gray are responders to lerapoturev.
patients had received both anti-PD-1 and anti-CTLA-4 therapy. Eleven of 12 patients also received anti-PD-1 therapy after lerapolturev. Primary or secondary resistance was classified according to Society for Immunotherapy of Cancer taskforce consensus. Briefly, primary resistance is best response of progressive disease (PD) or stable disease (SD) <6 months after ≥6 weeks of drug exposure while secondary resistance is best response of complete response, partial response, or SD <6 months after ≥6 months of drug exposure. Both definitions also include confirmatory scans at least 4 weeks after initial scan shows disease progression. After lerapolturev treatment, patient 1 (primary resistance) received anti-PD-1 plus anti-CTLA-4 (no prior exposure to anti-CTLA-4) and continues without progression at 3.2 years. Two other patients with primary resistance died at 11 and 23 months after lerapolturev. Of patients with secondary resistance (n=5), patient 11 had objective response to lerapolturev and remains free of disease at 2.2 years, patient 5 restarted anti-PD-1 therapy after lerapolturev and had disease progression after 25 months, while patients 4, 7, and 10 died after lerapolturev. Finally, among patients (8, 9, and 12) with only prior anti-PD-1 therapy and who received anti-PD-1 therapy within 30 days before lerapolturev (n=3), median progression-free survival (PFS) and overall survival (OS) after lerapolturev is 2.3 years. Two of those patients restarted anti-PD-1 therapy (8, 9) and one received no additional treatment (12). As listed in table 1, all four patients with objective response to lerapolturev (patients 8, 9, 11, and 12) have no evidence of disease and have current median PFS and OS of 2.3 years.

**Flow cytometry and bulk RNA sequencing of tumors**

Table 1 lists the absolute number of CD5+ , CD8+, CD4+ cells in tumor specimens obtained before and after lerapolturev as determined using flow cytometry. Of 17 samples included in our analysis with confirmed viable tumor on matched H&E, 200,000 events were analyzed in 12 samples whereas in 5 samples the number of cells shown in table 1 was normalized to 200,000 events. In table 1, the absolute number of cells is listed to account for differences in the total amount of viable tumor and tumor associated melanophages in the specimens.

**Figure 1A,B** shows CD4+T cells and CD8+T cell densities in tumors before and after lerapolturev. Among seven patients with matched pre- and post-treatment tumors assessed via flow cytometry, two patients responded to lerapolturev and five patients had no response to lerapolturev. In three patients (non-responders), CD8+T cells decreased whereas in four patients (two non-responders and two responders) CD8+T cells increased, as listed in table 1 and shown in figure 1A. The mean increase in CD8+T cells across 7 patients was 13,946 (p=0.0822, paired t test). Across seven patients with matching prerlapolturev and postlerapolturev samples, the mean increase in CD4+T cells was 8998 (p=0.0522, paired t-test). The number of CD4+T cells increased in two responders, increased in two non-responders, and appeared to be similar among the other three patients (figure 1B).

Next, we compared the differences in CD8+ and CD4+ T cells in the tumor at baseline (7–10 days prerlapolturev) between three patients with median PFS (2.3 years) vs patients 7 with median PFS 1.6 months. In 3 patients who received anti-PD-1 within 30 days prior to lerapolturev and have current median PFS 2.3 years, the mean number of CD8+T cells in the pretreatment tumor was 28,414 (n=3) compared with a mean of 3519 in 7 patients with median PFS 1.6 months after lerapolturev (p=0.0109), shown in figure 1C. Although the pretreatment tumor sample for patient 11, who has PFS 2.2 years was not evaluable, the post treatment tumor had 13,075 CD8+T cells. Similarly, the mean pretreatment number of CD4+T cells in patients with median PFS 2.3 years (n=3) was 7789 compared with 2269 (n=7) in patients with median PFS 1.6 months (p=0.0261), shown in figure 1C. These results were corroborated by immune cell enrichment as determined by CIBERSORT analysis of prerlapolturev bulk RNA sequencing data of tumors (figure 1D, online supplemental figure S1). Patients 8, 9 and 12 (received anti-PD-1 within 30 days prior to lerapolturev with PFS (2.3 years)) had significantly higher enrichment of CD8+T cells (p=0.0134) and activated CD4+memory T cells (p=0.0167), compared with seven patients with median PFS 1.6 months after lerapolturev, shown in figure 1D. Patient 8 (PFS 2.5 years) had the highest number of CD8+T cells as assessed by both flow cytometry and bulk RNA sequencing with patient 9 (PFS 2.5 years) having the second highest number of CD8+T cell as assessed by both flow cytometry and RNA sequencing (online supplemental figure S1). Finally, bulk RNA sequencing revealed that patient 5 (PFS progressed in 25 months) had markedly higher levels of neutrophils in the TME compared with other patients (online supplemental figure S1).

**DSP of tumors**

To further examine the TME, we used NanoString DSP. Images after fluorescent staining (CD3, CD163, CD11c, DNA) and H&E stains for all prerlapolturev specimens are shown in figure 2. Each image also lists the number of CD3+T cells in the TME as determined by flow cytometry. The H&E for patient 11 (PFS 2.2 years) prerlapolturev sample shows necrotic tumor, and therefore, this sample was not included in all tumor analyses. This was thought to be a sampling error because the postlerapolturev biopsy for patient 11 did have viable tumor. As shown in figure 2 and similar to flow cytometry and bulk RNA sequencing results, patients 8, 9, and 12 (received anti-PD-1 within 30 days prior to lerapolturev with PFS 2.3 years) had markedly higher tumor infiltrating immune cells compared with patients 1–7 (median PFS 1.6 months).

**Figure 3** details patient 2 and patient 8 as examples of DSP. Patient 2 (primary resistance, OS 23 months) had minimal immune infiltrate and the few CD3 cells present were in the periphery of the tumor, consistent with an
immune excluded profile. There were minimal differences between the prelerapolturev and postlerapolturev samples in patient 2 as assessed by DSP and flow cytometry (figure 3). Patient 8 (prior therapy was 6 cycles of anti-PD-1 therapy, with last dose of anti-PD-1 within 30 days of lerapolturev, current OS 2.5 years) had more abundant immune cells compared with patient 2 (figure 3). There were no clear differences between the prelerapolturev and postlerapolturev biopsy in patient 8 (figure 3).

For all patients with tumor available, slides were also fluorescently stained (DNA, CD3, CD11c, and CD163) and ROIs were selected for DSP analysis as shown in figure 3; ROIs were selected based on geometric location of tumor, examples of tumor regions are shown in figure 3F. Protein values below background were excluded and geometric regions intended to represent tumor that lacked MART-1 (melanoma marker) expression below threshold were also excluded. A heatmap of protein expression (13 proteins) in prelerapolturev tissue relevant to the melanoma TME is shown in figure 3I. The values used for the heatmap are the mean SNR from at least two ROIs for each tumor sample. Online supplemental table 1 lists all SNR values used for comparison. Interestingly, patients 8, 9, and 12 (received anti-PD-1 within 30 days prior to

Figure 1  Flow cytometry and RNA sequencing analysis of tumor tissue. (A) Number of CD8+T cells and (B) number of CD4+T cells in tumor tissue as assessed by flow cytometry of fresh tumor tissue. (C) Number of CD8+T cells (left) and CD4+T cells (right) in pretreatment tumor tissue. (D) CIBERSORT predicted (bulk RNA-sequencing) enrichment of CD8+T cells and active memory CD4+T cells in pretreatment tumor tissue Please see online supplemental figure S1 for full heatmap of CIBERSORT enrichment scores for each patient. Patients in red have current median PFS 2.3 years compared with patients in black text with median PFS 1.6 months. PFS, progression-free survival.
lerapolturev with PFS 2.3 years) were markedly higher compared with mean pretreatment CD8+T cells from pretreatment tumor in 7 patients with median PFS 1.6 months (28414 vs 3519). Among 7 patients with matched prelerapolturev and postlerapolturev tumor available for DSP, there were no consistent patterns of change from prelerapolturev to postlerapolturev tumor in expression of the 13 proteins.

The limitation to selecting geometric tumor region is that some tumors have a visual pattern of infiltrative immune cells (patient 8; figure 3F and H), precluding geometric separation of immune cells vs tumor, while visually other tumors had regions with tumor cells only or had immune cells only in scattered regions (Patient 2; figure 3B and D). A comparison of the protein expression on immune cells only may provide insight into important differences about the properties of tumor infiltrating immune cells. Accordingly, we also performed protein selection on only CD3+ cells from tumors to determine the protein expression of CD3+cells in the TME (figure 3J). Online supplemental table 1 includes SNR for all proteins. Figure 3J shows expression of 12 proteins involved in T cell signaling in prelerapolturev tumor tissue from CD3+cells only. It should be noted that expression of some markers such as MART-1 were present on CD3 cells (online supplemental table S1), suggesting there is some background protein expression not confined to CD3+cells. As expected, CD44 was high all for all patients since only CD3+cells were selected for analysis. Patient 8, 9, and 12 (received anti-PD-1 therapy within 30 days of lerapolturev with median PFS 2.3 years) grouped together based primarily on high expression of CD8 compared with other patients. Among 7 patients with matched prelerapolturev and postlerapolturev tumor available for DSP, there were no consistent patterns of change from prelerapolturev to postlerapolturev tumor in expression of the 12 proteins on CD3+T cells.

We noted that while patients 8, 9, 12 (median PFS 2.3 years) appeared to have a more inflamed tumor profile primarily driven by CD8+T cells, other patients namely 5 (PFS 25 months) and 7 (death at 7 months) also had immune cell infiltrates within the TME visualized by fluorescent staining (figure 2). To explore differences in these immune infiltrates such as the presence of T regulatory or other suppressor cells that may have led to the different clinical outcomes, we performed WTA on tumor from patients with immune infiltrates in tumor. FFPE tumor tissue sections from patients 8, 9, 12, (group 1) and patients 5 and 7 (group 2) were fluorescently stained for CD8 (teal), CD4 (red), CD11c (green), and DNA (blue, tumor) as shown in figure 4A,B. ROIs were then selected for areas enriched with CD8 and CD4 cells; WTA on cells only expressing the specific marker (either CD4 or CD8) was then performed as shown in figure 4A,B. At least two ROIs were selected for each cell type. Among 14442 human transcripts, we examined differences in
CD4+ and CD8+ cells between patients 8, 9, and 12 (group 1) and patients 5 and 7 (group 2). There were no significant differences in CD4+ transcripts between the two groups. Among CD8+ transcripts, five genes were significantly differentially expressed by more than twofold as shown in figure 4C, including higher HLA-DQA1, MIA (melanoma inhibitor y activity), SERPINA3 (Serpin Peptidase Inhibitor, clade A member 3), KRTAP5-8 (keratin-associated protein 5–8), and XAGE1A (human cancer/testis antigens X-antigen family member 1) in group 1. These differences may reflect distinct activation status of CD8+ T cells from patients 8, 9, and 12 (median PFS 2.3 years) compared with CD8+ T cells from patients 5 (progressed at 25 months) and 7 (death at 7 months). Although this methodology analyzes only cells expressing the selected fluorescent marker (CD4+ or CD8+) there may also be background expression from tumor cells.

Analysis of peripheral blood

Immune subsets in blood were also analyzed at multiple time points. Patients with PFS 2.3 years after lerapoltur ev (patients 8, 9, 11, 12), which includes the three patients who had received anti-PD-1 therapy within 30 days before lerapoltur ev (8, 9, and 12) had significantly higher numbers of CD8+CCR7+CD45RA- T effector memory cells, but significantly lower CD8+PD-1+, and CD4+PD-1+ T cells compared with 8 patients with median PFS 1.6 months after lerapoltur ev (figure 5 and online supplemental figure S2). Patients 8, 9, and 12 (median PFS 2.3 years) had received a mean of 6 cycles of prior anti-PD-1 therapy prior to trial entry with last dose within 30 days before lerapoltur ev. No other correlations with response or changes prelerapoltur ev to postlerapoltur ev were noted.

In preclinical studies, PVSRIPO (lerapoltur ev) mediated antitumor efficacy by inducing type-I IFN dominant innate inflammation in myeloid cells via the cytoplasmic pattern recognition receptor MDA5. Moreover, recent evidence indicates that patient-specific differences in peripheral blood responsiveness to type I interferon associates with outcome after anti-PD-1 therapy in patients.
We previously discovered that lerapolturev infects and induces cytokine responses in monocytes, but not T/B cells in PBMCs after 24 hours of treatment.\(^1\) Thus, we next asked if peripheral capacity to induce antiviral inflammation after lerapolturev infection associates with therapy outcome, possibly by reflecting the capacity of a patient’s innate immune system to respond to lerapolturev treatment or by reflecting the status of peripheral innate immunity at the time of treatment. To this end, we developed a peripheral immune function assay that measured pro-inflammatory cytokine responses of pretreatment PBMCs after in vitro challenge with lerapolturev or other innate stimuli to broadly probe innate immune status, including the TLR4 agonist LPS, the TLR1/2 agonist Pam3CSK4, and the TLR7/8 agonist R848 (figure 6; online supplemental figures S3 and S4). Since we observed higher levels of effector memory T cells in peripheral blood (figure 5) and tumor infiltrating T cells (figure 1) in patients living longer after lerapolturev therapy, we also compared responses to T cell stimulation using CD3/CD28 ligation. The fold increase in cytokine production after 24 hours of stimulation with lerapolturev over mock treatment is shown (figure 6; online supplemental figures S3 and S4). Patients with median PFS 2.3 years after lerapolturev (patients 8, 9, 11, 12) including the 3 patients with known prlerapolturev inflamed TME who received anti-PD-1 therapy within 30 days before lerapolturev (patients 8, 9 and 12), had significantly higher production of IFN-β, IFN-α2, IFN-γ, and GMCSF compared with eight patients with median PFS 1.6 months after lerapolturev. In addition, CXCL10 production was higher after LPS and anti-CD3/28 treatment in the four patients with PFS 2.3 years after lerapolturev compared with eight patients with median PFS 1.6 months (online supplemental figures S3). No difference in baseline cytokine secretion (Mock treatment) was observed between patients with PFS 2.3 years vs PFS 1.6 months, indicating the aforementioned superior poststimulation cytokine levels in patients with PFS 2.3 years were due to differential sensitivity to in vitro stimulation (online supplemental figures S4). Together, peripheral prlerapolturev immune responsiveness (figure 6) with the increased T cell inflammation in prlerapolturev tumors and higher levels of effector memory CD8+T cells in prlerapolturev periphery of patients with PFS 2.3 years, imply that an active and/or proficient

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**Figure 4** NanoString Digital Spatial Profiling (DSP) of tumor tissue. NanoString DSP images after fluorescent staining on pretreatment tissue, CD8=teal, CD4=red, CD11c=yellow, and DNA=blue. ‘Low power’ = 2 mm field of view, 100 × total magnification. Rectangles indicate ROIs selected based on enrichment for cell type or tumor. (A) Low power image patient 12. (B) Low power image patient 7. (C) Volcano plot comparing ROI on CD8+ cells only between patients 8, 9, 12, 5 and 7. Differentially expressed genes appear in blue. ROI, regions of interest.
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immune status at baseline in patients may result in favorable clinical outcomes.

DISCUSSION

The presence of tumor infiltrating lymphocytes (TILs) and an inflamed tumor in pre-checkpoint inhibitor therapy tumor (baseline) is associated with improved OS in melanoma and predictive of response to ICI therapy.\(^{30-32}\) Using three separate modalities—flow cytometry, DSP (protein and WTA), and RNA sequencing—we observed that three patients with advanced melanoma and a median PFS of 2.3 years had markedly elevated TILs in pre-lerapolturev tumor compared with seven patients with less abundant TILs in pretreatment tumors and median PFS 1.6 months. Although this observation aligns with previous findings that responses to immunotherapy preferentially occur in tumors with an ‘inflamed’ phenotype, our study is limited by a small sample size.\(^{31}\) Furthermore, in matching pre-lerapolturev blood (baseline) from the same three patients with an inflamed TME, median PFS 2.3 years and a fourth patient with PFS 2.2 years (no pre-lerapolturev tumor available in the fourth patient), a more pronounced peripheral innate immune response to in vitro lerapolturev challenge was observed compared with eight patients with median PFS 1.6 months (including seven patients with non-inflamed pre-lerapolturev tumor). Findings in peripheral blood are also limited by small sample size. Among the eight patients with PFS 1.6 months, six patients have died while current PFS and OS among the other four patients is 2.3 years.

Determining clinical and radiographic responses to anti-PD-1 therapy can be complicated by delayed responses and pseudoprogression.\(^{36}\) Guidelines that more precisely define anti-PD-1 failure predated our

Figure 5  Flow cytometry analysis of immune subsets in peripheral blood. PBMCs were analyzed for T cell memory populations and PD1 expression on CD8+ and CD4+ conventional T cells as shown at the pretreatment time point. Patients in red have median PFS of 2.3 years after lerapolturev and patients in black have median PFS 1.6 months after lerapolturev. Data brackets indicate mean ±SEM; p values are from unpaired t-test. PBMCs, peripheral blood mononuclear cells; PFS, progression-free survival.
Figure 6  Assessment of baseline peripheral immune cell function in response to lerapolturev. PBMCs from the pretreatment time point were challenged with laboratory grade PVSRIPPO in vitro for 24 hours. Supernatant was tested for proinflammatory cytokines. Patients in red have median PFS of 2.3 years after lerapolturev and patients in black have median PFS 1.6 months after lerapolturev. Data brackets indicate mean ± SEM; p values are from unpaired t-test. See online supplemental figure S3 for complete results of cytokines and stimuli tested; online supplemental figure S4 presents baseline raw MFI and concentration of cytokines for all stimuli. GMCSF, granulocyte-macrophage colony-stimulating factor; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; PFS, progression-free survival.

study design and as such five patients received anti-PD-1 therapy within 30 days of lerapolturev, although clinically these patients appeared to be progressing. Three of those patients (8, 9, and 12) had the highest number of CD8+T cells (mean 28,414) in prelerapolturev tumor (tumor biopsied within 30 days of anti-PD-1 therapy prelerapolturev) compared with mean value of 3509 (n=7) in other patients. This was consistent with bulk RNA sequencing and DSP analysis. In addition, patient 11 with PFS 2.2 years, had a higher number (13,075 CD8+T cells) in postlerapolturev tumor. Together, those four patients (8, 9, 11, 12) continue to have prolonged PFS (median 2.3 years vs 1.6 months median PFS for the other seven patients). Interestingly, patient 9’s (PFS 2.5 years) prelerapolturev non-treated lesion also had marked tumor inflammation while patient 1’s (SD after initiation of anti-CTLA-4 plus anti-PD-1) prelerapolturev non-treated lesion had little immune infiltrate (figure 3). Therefore, it is possible that anti-PD-1 therapy contributed to disease control for patient 8, 9, and 12, (median PFS 2.3 years) since the last dose of anti-PD-1 therapy was within 30 days and the TME of those patients had a more inflamed tumor profile prior to lerapolturev. However, lerapolturev may have provided additional support of inflammation and remodeling of the TME. Recent data suggest that there are T cell infiltrated tumors that are resistant to anti-PD-1 possibly due to lack of coexisting proinflammatory cytokines. Oncolytic viruses like lerapolturev may provide this type of inflammation which can lead to a clinical benefit as we observed in four patients on the study.

Use of spatial biology in our study in addition to flow cytometry and bulk RNA sequencing highlighted interesting aspects about studying the TME. Intratumoral heterogeneity was observed which is most apparent when viewing DSP images, which preserves spatial biology. Some patients had portions of tumor with abundant TILs, some had TILs only on the tumor periphery, and some patients had TILs arranged in an infiltrative pattern. Those relationships are not as easily captured using flow cytometry or bulk RNA sequencing. However, flow cytometry can provide more detailed information about live cells, and bulk RNA sequencing evaluates a larger spectrum of cell types. Although CD8+T cell infiltrated tumors are a known predictor of response to anti-PD-1 therapy, about 30% of patients with CD8+T cells in the tumor will still not respond to checkpoint therapy. WTA of CD8+T cells among two groups of patients, both with CD8+T cells present in tumor but with divergent outcomes revealed differences in the CD8+T cells that may in part explain the divergence. The higher HLA-DQA1 on CD8+T cells from patients with prolonged PFS suggests robust T cell activation. Spi2A, is encoded by SERPINA3. Spi2A enhances the initial development of memory CD8+T cells and SERPINA3 was markedly increased on CD8+T cells from patients with prolonged PFS.

Given the intratumoral heterogeneity and limitations of biopsies which do not evaluate the entire tumor, we also conducted blood analysis. Identification of a blood-based biomarker could also be a useful approach to evaluate therapy efficacy in larger clinical studies given that blood is easier to obtain, non-invasive, and facilitates longitudinal analysis of changes in the immune system. Previous reports indicate that anti-PD-1 therapy induces proliferation of CD8+PD-1+ and CD4+PD-1+ T cells in peripheral blood with the increase peaking at 1 week after initiating anti-PD-1 and then decreasing thereafter despite continuous anti-PD-1 treatment. Therefore, the finding that three of four patients with prolonged PFS (2.3 years) in our study had lower CD8+PD-1+ and CD4+PD-1+ T cells is not surprising since three of four patients with prolonged
PFS had received an average of six cycles of anti-PD-1 with last dose within 30 days prior to lerapoltuvec while only two of eight patients with poor PFS had received anti-PD-1 within 30 days. Since anti-PD-1 therapy works by expanding functional effector T cells in the TME and lerapoltuvec also recruits peripheral blood innate immune cells in the TME, we investigated if immune function in blood could predict presence of immune cells in the TME or ability to recruit peripheral cells into the tumor. Three patients who received anti-PD-1 within 30 days of lerapoltuvec, who had an inflamed prerapoltuvec TME as noted by flow cytometry, DSP, and RNA sequencing, and had PFS 2.3 years, appeared to elicit a robust response in peripheral blood as measured by cytokine production after in vitro stimulation with lerapoltuvec (and in some cases LPS and anti-CD3/CD28 stimulation) compared with eight patients with median PFS 1.6 months. A fourth patient (11) with no prerapoltuvec tumor available (but who had an inflamed post lerapoltuvec TME), with PFS 2.2 years also mounted a robust response in peripheral blood after in vitro stimulation with lerapoltuvec. Of note, all four patients with prolonged PFS (2.3 years) and robust innate immune response had only received anti-PD-1 therapy prior to lerapoltuvec while all other patients were more heavily pretreated (Table 1). This type of analysis may be useful in assessing peripheral innate immune responsiveness/competence and may predict which therapies can effectively recruit immune cells into the TME for an individual patient. Four patients with the most robust cytokine response had the highest levels of T cells in the TME.

A current trial, LUMINOS-102 (lerapoltuvec with or without immune checkpoint blockade) in patients with advanced PD-1 refractory melanoma (NCT04577807) is ongoing. Patients will have confirmed progression on anti-PD-1 therapy before enrollment. In treatment naive patients, final results of MASTER-KEY-265 suggested that the intratumoral oncolytic virus, talimogene laherparepvec plus pembrolizumab did not significantly improve PFS or OS compared with placebo plus pembrolizumab in a large phase III randomized study. Therefore, intratumoral therapies may be more relevant for selected patients not initially responding to checkpoint therapy as is being explored in LUMINOS-102. In addition, the mechanisms of lerapoltuvec are unique and efficacy may be different.

In conclusion, patients with a prerapoltuvec inflamed TME and increased sensitivity to in vitro lerapoltuvec challenge in prerapoltuvec peripheral blood had prolonged median PFS (2.3 years) after treatment with lerapoltuvec compared with patients with median PFS of 1.6 months, who had prerapoltuvec ‘cold’ tumors and lower cytokine response in prerapoltuvec peripheral blood. All four patients with prolonged PFS had also received anti-PD-1 therapy (three of 4 within 30 days of lerapoltuvec) and no additional systemic therapies. Given this observation in our pilot study in 12 patients, future studies will test if immune response measured in the periphery (blood) may be important in predicting ability to recruit immune cells into the TME in a larger cohort of patients.

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Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study involves human participants and was approved by Duke Cancer Center (no ID)Duke IRB Pro00090774 Western IRB1 IBCL-17-0046-01. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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REFERENCES


Multimodality analysis confers a prognostic benefit of a T-cell infiltrated tumor microenvironment and peripheral immune status in patients with melanoma

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Running Head: Immune status is linked with viral immunotherapy response
Supplementary Table 1

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**Supplementary Table 1.** Signal to noise ratios for represented proteins in regions of interest chosen by CD3+

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**Supplementary Table 2:** Signal to noise ratios for represented proteins in regions of interest chosen by location of tumor
Figure S1. CIBERSORT results of bulk RNA sequencing data from pre-treatment tumor tissue.
Patient number is indicated on X-axis. Patients in red have median PFS of 2.3 years after lerapolturerv and patients in black have median PFS 1.6 months after lerapolturerv.
**Supplementary Figure 2 (S2)**

![Flow cytometry gating strategy](image-url)

**Figure S2.** Flow cytometry gating strategy for detection of memory T cell populations and PD1 expression on CD8+ and CD4+ conventional T cells in Figure 5.
Supplementary Figure 3 (S3)

**Figure S3. Assessment of baseline peripheral immune cell function in response to lerapoltagev.**
PBMCs from the pre-treatment time point were challenged with laboratory grade PVSRIPO in vitro for 24 hours. Supernatant was tested for pro-inflammatory cytokines. Patients in red have median PFS of 2.3 years after lerapoltagev and patients in black have median PFS 1.6 months after lerapoltagev.
Supplementary Figure 4A (S4A)

A

- median PFS 2.3 years post-therapy
- median PFS 1.6 months post-therapy

Mock

PVSRIPO (virus)

Pam3CSK4 (TLR1/2 agonist)

LPS (TLR4 agonist)

R848 (TLR 7/8 agonist)

anti-CD3/CD28 (T cell agonist)
Supplementary Figure 4B (S4B)

- red circle: median PFS 2.3 years post-therapy
- black circle: median PFS 1.6 months post-therapy

B

Mock

Log (pg/ml)

PVSRIP0 (virus)

Log (pg/ml)

Pam3CSK4 (TLR1/2 agonist)

Log (pg/ml)

LPS (TLR4 agonist)

Log (pg/ml)

R848 (TLR 7/8 agonist)

Log (pg/ml)

anti-CD3/CD28 (T cell agonist)

Log (pg/ml)
Figure S4. Peripheral immune cell function at baseline.
Raw MFI (A) or concentration (pg/ml, B) of analytes after each treatment corresponding to data presented in Figure 6. (B) For values below or above the maximum standard concentration threshold, values were set to the minimum or maximum threshold, respectively. Patients in red have median PFS of 2.3 years after lerapoltuerv and patients in black have median PFS 1.6 months after lerapoltuerv.
A Phase I trial of PVSRIPO for Patients with Unresectable Melanoma

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# Summary of Protocol Amendment Changes

<table>
<thead>
<tr>
<th>Version Dates</th>
<th>Summary of Major Changes by Section</th>
</tr>
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</table>
| 12/18/2017 to 06/29/2018 | • Section 5.3 clarification of regional chemotherapy definition  
• Table 2 – Injection volume was updated to 0.5 ml for all lesions  
• Section 9.2 Clarification on biopsy  
• Section 10.1.4 – PVSRIPO stability information was updated for consistency  
• Section 12 - Schedule of Study tests/ procedures and text were updated to include footnotes for Cohort 1 and Cohort 2 activities. Number of research blood draws was reduced. Follow-up was clarified.  
• Section 12.7.1 – In the definition of Immune-Related Stable Disease, the confirmation at 4 weeks was removed. This was not clinically meaningful and is not part of the study objectives.  
• Section 13.1.4 – Updated to define SAE reporting procedures  
• Information on Safety Oversight Committee (SOC) (Section 13.5) was removed. This information is not applicable for an industry-sponsored trial.  
• Section 14.1 – Updated to reflect monitoring by the Sponsor, Istari, rather than monitoring by the Duke Cancer Institute.  
• Administrative changes: formatting and cross referencing corrections throughout |
| 06/29/2018 to 10/03/2018 | • Administrative change: corrected pagination in footer of document (error starting on page 7 in previous version), updated title page with protocol number and personnel change, updated table of contents with correct pagination, and updated header with new version date.  
• Section 12: Table 3 – Combined column for Within 6 months with Screening column and clarified with footnote, additional clarifications on biopsies and vital signs were added in footnotes |
| 10/03/2018 to 02/06/2019 | • Section 9.1.1 Added description of Cohort 3 where up to 3 injections are in the same lesion with possible enrollment of 3 additional patients.  
• Figure 5 - Incorporate Cohort 3 doing schema  
• Section 11.1 Inclusion Criterion 8 – added Cohort 3  
• Section 12.2 Treatment Period - Cohort 3 information was added into the descriptions of each visit. |
| 02/06/2019 to 05/15/2019 | • Section 12: Table 3 and Section 12.2 Treatment Period - Reduced stool sample collection to one time at 10 days |
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<td>aPTT</td>
<td>Activated Partial Thromboplastin Time</td>
</tr>
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<td>AE</td>
<td>Adverse Event</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>CAP</td>
<td>College of American Pathologists</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete Blood Count</td>
</tr>
<tr>
<td>CED</td>
<td>Convection-enhanced Delivery</td>
</tr>
<tr>
<td>CLIA</td>
<td>Clinical Laboratory Improvement Amendments</td>
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<tr>
<td>CMP</td>
<td>Comprehensive Metabolic Panel</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>CPC</td>
<td>Cancer Protocol Committee</td>
</tr>
<tr>
<td>CRF</td>
<td>Case Report Form</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CTCAE</td>
<td>Common Terminology Criteria for Adverse Events</td>
</tr>
<tr>
<td>DCI</td>
<td>Duke Cancer Institute</td>
</tr>
<tr>
<td>DLT</td>
<td>Dose Limiting Toxicity</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSMB</td>
<td>Data and Safety Monitoring Board</td>
</tr>
<tr>
<td>DUHS</td>
<td>Duke University Health System</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>Epidermal Growth Factor Receptor (variant III)</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic Initiation Factor</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FLAIR</td>
<td>Fluid-Attenuated Inversion Recovery</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>Gadolinium Diethylene Triamine Pentaacetic Acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>HGG</td>
<td>High Grade Glioma</td>
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<tr>
<td>HRPP</td>
<td>Human Research Protections Program</td>
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<tr>
<td>HRV2</td>
<td>Human Rhinovirus Type 2</td>
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<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
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<tr>
<td>HSRC</td>
<td>Duke Human Subject Research Compliance Office</td>
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<tr>
<td>ICH</td>
<td>Intracerebral Hemorrhage</td>
</tr>
<tr>
<td>ICS</td>
<td>Investigational Chemotherapy Service</td>
</tr>
<tr>
<td>IDH</td>
<td>Isocitrate Dehydrogenase</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IND</td>
<td>Investigational New Drug</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosomal Entry Site</td>
</tr>
<tr>
<td>IV (or iv)</td>
<td>Intravenously</td>
</tr>
<tr>
<td>KPS</td>
<td>Karnofsky Performance Status</td>
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<td>IND</td>
<td>Investigational New Drug</td>
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<tr>
<td>LSQ</td>
<td>Lymphocyte Subset Quantitation</td>
</tr>
<tr>
<td>MGMT</td>
<td>O(^6)-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum Tolerated Dose</td>
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<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NED</td>
<td>No Evidence of Disease</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human Primate</td>
</tr>
<tr>
<td>NSICU</td>
<td>Neuro-Surgical Intensive Care Unit</td>
</tr>
<tr>
<td>OARC</td>
<td>Office of Audit, Risk, and Compliance</td>
</tr>
<tr>
<td>OS</td>
<td>Overall Survival</td>
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<td>PD</td>
<td>Progressive Disease</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PJP</td>
<td>Pneumocystis jiroveci pneumonia</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton Pump Inhibitor</td>
</tr>
<tr>
<td>PRTBTC</td>
<td>Preston Robert Tisch Brain Tumor Center</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin Time</td>
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<td>PV</td>
<td>Poliovirus</td>
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<td>PV1S</td>
<td>Poliovirus Serotype 1 (Sabin)</td>
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<tr>
<td>PVSRIPO</td>
<td>Polio/Rhinovirus Recombinant</td>
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<td>RANO</td>
<td>Response Assessment in Neuro-Oncology</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious Adverse Event</td>
</tr>
<tr>
<td>SGOT</td>
<td>Serum Glutamic Oxaloacetic Transaminase</td>
</tr>
<tr>
<td>SGPT</td>
<td>Serum Glutamic Pyruvic Transaminase</td>
</tr>
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<td>SOC</td>
<td>Safety Oversight Committee</td>
</tr>
<tr>
<td>TCID</td>
<td>Tissue Culture Infectious Dose</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>WHO</td>
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5 PROTOCOL SYNOPSIS AND RESEARCH SUMMARY

5.1 PURPOSE
A Phase I study of oncolytic polio/rhinovirus recombinant (PVSRIPO) in patients with unresectable, recurrent, American Joint Commission of Cancer (AJCC) Stage IIIB, IIIC, or IV melanoma at Duke is planned to address the following objectives.

See Table 1 in Section 8 for specific objectives and endpoints.

5.2 BACKGROUND AND SIGNIFICANCE
The American Cancer Society estimates that 87,110 people will be diagnosed with melanoma and 9,730 people will die of melanoma of the skin in 2017. Although melanoma accounts for less than 5% of skin cancer cases, it causes a majority of skin cancer deaths. Melanoma is the most common fatal malignancy among young adults, and results in an average of 17.1 years of productive life lost per death for adults between 20 to 49 years of age. The number of new cases of melanoma in the United States has been increasing for at least 30 years.

Despite appropriate initial surgical therapy for melanoma, one-third of patients will develop recurrence. Cutaneous and subcutaneous recurrences with or without concurrent systemic disease affects a large portion of patients with recurrence. The site of first relapse in stage III disease was found to be local/in-transit in 28% of cases, regional nodal in 21% of cases, and systemic in 51% of cases in a large review. In patients that recur after resection of stage II disease, 12% of first recurrences were local/in-transit, 7% nodal, and 11% systemic. Local recurrence can be isolated tumor deposits close to the primary lesion known as satellite lesions or in-transit disease, which represents a specific form of recurrence whereby melanoma tumor deposits occur in the dermal or subcutaneous lymphatic vessels. Tumor recurring in this fashion usually appears as multiple nodules throughout the extremity as shown Figure 1. These lesions can also result in disabling effects, causing pain, bleeding, and/or tremendous anxiety and psychological distress. Because this pattern of recurrence represents multifocal involvement of the extremity’s lymphatic system, local excision of these “in-transit” lesions is frequently followed by rapid recurrence or, due to extensive disease,
rese ction is not possible. Furthermore, systemic disease is either present initially or eventually develops in about 50% of patients with satellite or in-transit disease.

![In-transit Melanoma](image)

**Figure 1. In-transit Melanoma.**

### 5.3 Current Treatment Options for In-Transit Extremity Melanoma

Current treatment options for patients with recurrent melanoma limited to the extremity only include surgical resection, intra-lesional therapy with sclerosing agents or immune adjuvants, isolated limb infusion, hyperthermic isolated limb perfusion (HILP), or amputation. Surgical excision is a reasonable procedure when the lesion(s) are few in number and/or can be encompassed without extensive skin grafting. However, surgical excision of in-transit lesions is often futile, with new lesions appearing before surgical wounds have healed. Intra-lesional therapy with bacillus Calmette-Guerin (BCG) or interferon can provide relief for small lesions that are primarily dermal in location. Regional chemotherapy which involves vascular isolation and perfusion of the extremity with chemotherapy can achieve regional drug concentrations several orders of magnitude higher than could be attained with systemic administration, with minimal systemic toxicity. Regional chemotherapy is associated with complete response rates of 30% but has minimal impact on overall survival. Although control of the extremity disease is important, most patients with advanced disease of the extremity will die of systemic disease, and more effective strategies are needed. Regional chemotherapy (isolated limb perfusion or isolated limb infusion) does not treat disease outside the extremity, is limited to patients with extremity disease only (cannot perfuse head neck), and can be toxic. Although regional chemotherapy is still an option, it’s becoming increasingly rare due to more effective systemic therapy.

### 5.4 Current Novel Systemic Therapies for Metastatic Melanoma

Metastatic melanoma has historically been associated with a poor prognosis and survival of less than one year. Historic systemic therapies include IL-2 and temozolomide, with associated overall response rates of 16% and 9%, respectively. Since 2011, multiple new therapies have become FDA approved for the treatment of metastatic melanoma including unresectable in-transit disease. Examples of novel therapy include mutation based targeted therapies such as vemurafenib, a BRAF inhibitor, or trametinib, a MEK inhibitor. For the 50% of melanoma patients with a BRAF mutation, dramatic initial responses can occur in the majority of patients, but resistance develops within 6-9 months.
Immune blockade checkpoint inhibitors represent other recently developed novel systemic therapies. Immune blockade therapies appear to have more long-term efficacy, do not appear as susceptible to emergence of rapid resistance like mutation based therapy, and are not restricted to patients harboring specific mutations. Melanoma is a highly immunogenic tumor related, in part, to the high mutational load\(^{14}\). Melanoma has thus been the prototype for investigating several forms of immunologic therapy\(^{15}\) including ipilimumab and pembrolizumab. Ipilimumab, a monoclonal antibody directed against the CTLA-4 protein, promotes immune recognition of self-antigens and thus unleashes cytotoxic activity of endogenous melanoma antigen-specific T lymphocytes\(^{16}\). Pembrolizumab is a monoclonal antibody that blocks programmed cell death ligand 1 (PD-1) and therefore counteracts negative regulation of cytotoxic T-lymphocyte activity\(^{12}\). As a monotherapy, pembrolizumab had an objective response rate of 45% in treatment naïve patients and twelve month progression free survival (PFS) was 52% in treatment naïve patients\(^{17}\). PD-1 based therapy has thus been established as a cornerstone of systemic therapy for patients with advanced melanoma, yet the development of both primary and late resistance remains a problem\(^{18,19}\). Dual checkpoint inhibitor therapy can result in higher response rates, but considerable toxicity remains a concern\(^{20}\). The combination of ipilimumab and nivolumab (another PD-1 inhibitor) resulted in response rates of 57% and improved overall survival (OS) for patients with metastatic melanoma, however treatment-related adverse events of grade 3 or 4 occurred in 16.3% of the patients in the nivolumab group, 55.0% of those in the nivolumab-plus-ipilimumab group, and 27.3% of those in the ipilimumab group\(^{18}\). Thus toxicity with nivolumab (PD-1 inhibitor) alone appears acceptable while the combination of nivolumab plus ipilimumab results in unacceptable toxicity for more than half the patients that receive the combination\(^{19}\). While multiple strategies to optimize response are underway including dose reduction in immunomodulatory agents, combination therapies, and predicted response by biomarkers or clinical characteristics, there is opportunity for more directed and specific therapies.

Another strategy for melanoma treatment is oncolytic immunotherapy (proinflammatory effects of viral tumor infection). Specifically, talimogene laherparpvec (T-VEC) is a modified herpes virus that includes insertion and expression of a gene encoding human granulocyte macrophage colony-stimulating factors (GM-CSF) which results in local GM-CSF production to recruit and activate antigen presenting cells\(^{21}\). T-VEC was FDA approved for unresectable melanoma based on results of a Phase 3 randomized trial that showed durable response rates of 16.3% in the T-VEC arm compared to 2.1% in the GM-CSF arm\(^{21,23}\). While this was an improvement over direct GM-CSF injection, few would advocate subcutaneous GM-CSF as an effective therapy, therefore making a potentially poor control arm therapeutic. Notably, T-VEC was given intralesional with resulting grade 3 or 4 adverse event occurring in $\geq 2\%$ of T-VEC treated patients\(^{22}\).

Despite important recent advances in the treatment of metastatic melanoma, the ranges of durable response rates are suboptimal and novel combination therapies with higher response rates may come with unacceptable toxicity. Therefore a need to develop therapies with specific, sustained antineoplastic effects remains.
5.5 PVSRIPO

Oncolytic virotherapy (direct tumor cytotoxicity) has potential to elicit broad sustained, anti-neoplastic effects stemming from combined direct viral cytotoxicity and innate antiviral activation. PVSRIPO is the live attenuated, serotype 1 poliovirus vaccine (SABIN) that was modified by exchange of the entire genetic cognate internal ribosomal entry site (IRES) with the corresponding segment from human rhinovirus type 2, thus eliminating its neurovirulence. Since PVSRIPO is a version of the serotype 1 live-attenuated poliovirus vaccine (PV1S), its immunogenic properties and potential for long-term sequelae are expected to be similar. Poliovirus serotype 1 (PV1S) has been safely administered to >10 billion individuals worldwide without untoward long-term immunogenic sequel.

Figure 2: Cytotoxicity of PVSRIPO in Melanoma Cell Lines. Melanoma cell lines DM6 (left) and DM440 (right) were infected with PVSRIPO. Lysates were collected and tested for direct viral cytotoxicity (eIF4G cleavage), host cell demise (PARP cleavage), and innate antiviral response (p-eIF2α).

PVSRIPO retains translation competence and cytotoxicity in neoplastic cells, which offers ideal conditions for viral IRES-mediated ribosome recruitment due to unhinged protein synthesis control in cancer. To be successful, oncolytic viruses must also have tropism for tumor. The poliovirus receptor (PVR), CD155, is a cell adhesion molecule of the Ig-like superfamily I1 expressed during embryonic development. CD155 is broadly expressed in glioblastoma (GBM) and melanoma. Upon binding CD155, viral RNA enters the cytoplasm and begins direct viral cytotoxicity through engagement of anti-viral interferon response. PVSRIPO causes cytopathogenicity in melanoma cell lines as shown in Figure 2. PVSRIPO also induces non-lethal infection of tumor associated macrophages and recruits immune effector responses directed against tumor neoantigens.

In a phase 1 trial of intratumoral delivery of PVSRIPO in patients with recurrent GBM, there was one dose limiting toxicity resulting from intracranial hemorrhage at removal of the catheter used to infused PVSRIPO. A recent trial update of all 61 patients treated on the phase 1 trial of PVSRIPO showed a median OS of 12.3 months for the PVSRIPO group versus 10.5 months for the historical controls. The 24 month OS rate of patients infused with 5x10⁷ TCID₅₀ of PVSRIPO was 21.1%.
compared to 13.5% in the historical control group (data not yet published). Encouraging results with PVSRIP0 led the FDA to grant PVSRIP0 breakthrough therapy designation as a potential treatment for patients with recurrent GBM in 2016. For more information on the pre-clinical work and clinical work in GBM with PVSRIP0, please refer to the Investigator’s Brochure.

The key for a sustained suppression is lasting antigen specific immunity. Strategies that promote antigen presentation in the tumor microenvironment may revive pre-existing antitumor immunity and/or initiate new responses, thereby increasing the anti-tumor immune repertoire. Recent data suggests that the tumor cytotoxicity and interferon dendritic cell activation of PVSRIP0 elicit a tumor antigen specific cytotoxic T cell response\textsuperscript{29}. Specifically, in addition to direct infection and killing of cancer cells, PVSRIP0’s ability to promote anti-tumor responses may result in its ability to infect and activate antigen presenting cells which mediate stimulation of specific cytotoxic T cells \textit{in vitro}. These cytotoxic T cells can recognize tumor-associated antigens such as MART1, a melanoma tumor-associated antigen\textsuperscript{29}. The ability of oncolytic virotherapy to target tumor directly and provide specific, sustained anti-tumor responses is worthy of further exploration in solid malignancies such as melanoma.

5.6 RATIONALE FOR PVSRIP0 IN MELANOMA

The pathogenesis of GBM and melanoma have many similarities given their shared neuroectodermal origin. CD155 (PVR) is expressed on the majority of melanoma cell lines and PVSRIP0 causes cytotoxicity of melanoma cells as shown in Figure 2\textsuperscript{29}. There is compelling data in preclinical \textit{in vitro} and \textit{in vivo} models of melanoma demonstrating the potential therapeutic benefit of PVSRIP0. PVSRIP0 infection of melanoma cell lines (DM6, DM440) results in cytotoxicity and lysis from an interferon response and cleavage of elf4G (associated with the viral protease 2A) as shown in Figure 2\textsuperscript{29}. \textit{In vitro}, PVSRIP0 infection of dendritic cells (DCs) led to DC activation and MART1 (a melanoma associated antigen) specific cytotoxic T cell responses\textsuperscript{29}. Furthermore, predictors of response to immunotherapy include an immune active tumor profile with type I interferon emerging as important in CD8 mediated tumor response\textsuperscript{31,32}. \textit{In vitro}, PVSRIP0 infection of DCs induced sustained type I IFN-dominant responses and cytokine production\textsuperscript{29}. Human DC cells exposed to PVSRIP0 oncolysate were able to pick up released tumor antigen, present antigen, and prime T cells in an \textit{in vitro} DC-T cell stimulation assay as shown Figure 3\textsuperscript{29}.

\textit{In vivo}, single intratumoral injection of PVSRIP0 elicits significant anti-tumor efficacy in the murine immunocompetent B16 melanoma model\textsuperscript{29}. Importantly, PVSRIP0 injection into mice was linked to systemic anti-melanoma antigen-specific cytotoxic T cell lymphocyte (CTL) responses that were evident in lymphocytes isolated from tumor-draining lymph nodes and spleen. Confirming observations in mouse xenotransplantation models, PVSRIP0 infusion in the immunocompetent B16 mouse model elicited a series of immune cell invasion steps: 1) neutrophils (peak at 1-2 days post PVSRIP0 and rapid disappearance thereafter); 2) DCs (infiltration setting in at day 6 and continuing thereafter); 3) CD4/CD8 T cells (infiltration setting in at day 6-7 and continuing thereafter)\textsuperscript{29}. This series of events indicates that, by unleashing a natural innate pro-inflammatory response, PVSRIP0 oncolysis turns the immunological ‘cold’ tumor microenvironment into a hotbed of inflammation and immunogenicity. PVSRIP0 infection of tumors, by damaging neoplastic cells and activating IFN responses in antigen-presenting cells, elicits anti-tumor immunity as shown in\textsuperscript{29}. 

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These preclinical models provide the rationale for PVSRIP0 use in recurrent cutaneous melanoma. The subcutaneous delivery is also attractive as a less invasive approach and the possibility of reduced systemic toxicities. However, if clinical efficacy is demonstrated in the subcutaneous disease model, there is the possibility of injecting or infusing other metastatic lesions (lung, lymph node, brain). Importantly, this trial will lead to a phase 1/2 trial of PVSRIP0 on combination with immune checkpoint blockade (PD-1/PD-L1). The combination therapy has potential to overcome resistance to monotherapy and generate a more robust immune response.33

Figure 3: Human Dendritic Cells (DC) Co-Incubated with DM6 Melanoma Cell Line Oncolytic Stimulate Tumor Antigen-Specific T Cell Responses In Vitro. Top panel: Schema of assay. Bottom panel: T cells were co-cultured with autologous DCs pulsed with oncolysate from PVSRIP0 treated DM6 (melanoma cell line) cells and the stimulated effector T cells were then harvested and tested in a CTL assay against tumor cells (red bar), tumor antigens (black bars), irrelevant tumor antigens (white bars). Each bar represents average % specific lysis and SD of triplicate samples.

5.7 DESIGN AND PROCEDURE
Patients must have histologically proven unresectable, recurrent, melanoma, stage IIIIB/C, or stage IV (AJCC staging must be documented in patient’s medical record, as determined by CT of the chest, abdomen and pelvis, and/or whole body PET scan, and MRI of the brain within 4 weeks prior to administration of study drug). Patient must have directly injectable disease (at least 1 cutaneous or subcutaneous melanoma lesion at least 10 mm in size (measurable lesion) when 1 dose is planned, at
least 2 measurable lesions when 2 doses are planned, and 3 measurable lesions when 3 doses as planned according to dose escalation). Patients must have at least one measurable lesion that will not be injected. Therefore, the number of injectable lesions may make a subject ineligible for the study depending on when they enroll. Patients with injectable disease must have disease that is palpable or able to be visualized and easily accessible for intratumoral injection. If superficial, the lesion(s) must be documented photographically. Patients must have at least 2, 3, or 4 lesions, depending on when they are enrolled in the study, that qualify as measurable (index) lesions per irRC (Immune related response criteria); except for cutaneous lesions, where sum of diameter for superficial lesions must be at least 10 mm. Eligible patients may have received prior therapies including nivolumab, ipilimumab, pembrolizumab, and BRAF/MEK inhibitors. Major exclusion criteria include active severe co-morbidities, autoimmune diseases, or active infections.

Patients can have up to 3 separate lesions injected with PVSRIPO according to the dose escalation/reduction criteria described in Section 9.1.1. Index lesions that are ulcerated or oozing will not be eligible for injection. The PI or designated personnel will select the index lesions for injection. Unresectable disease is required for eligibility, however if the disease becomes resectable, the disease can be surgically resected at any time, at the discretion of the PI.

5.8 **SELECTION OF SUBJECTS**

All inclusion/exclusion criteria may be found in Section 11.

5.9 **DATA ANALYSIS AND STATISTICAL CONSIDERATIONS**

A maximum of 9 patients will be accrued to this trial at an accrual rate of about 1-2 patients per month.

The Primary Objective is to characterize the safety and tolerability of PVSRIPO in melanoma. Common Terminology Criteria for Adverse Events (CTCAE) version 4 will be used to assess adverse events. The DLT criteria are defined in Section 9.1.2

Three patients minimum will be treated with PVSRIPO. See Section 9.1.1 for dose escalation and dose reduction plan. Between each cohort a minimum of three weeks is required before additional enrollment.

Upon completion of the trial, all toxicities will be tabulated by type and grade.

Response to PVSRIPO will be determined with immune-related response criteria (irRC). Full detail of irRC is listed in Table 4 Section 12.7.1.
6 STUDY SCHEMA

Figure 4: Study Schema
7 BACKGROUND AND SIGNIFICANCE

7.1 STUDY DISEASE
Melanoma is the most deadly form of skin cancer and has been increasing in incidence for 30 years. Despite important recent advances in the treatment of metastatic melanoma, reviewed thoroughly in Section 5.2, the ranges of durable response rates are suboptimal and novel combination therapies with higher response rates may come with unacceptable toxicity. Therefore, a need to develop therapies with specific, sustained, antineoplastic effects remains.

7.2 STUDY AGENT: PVSRIPO

7.2.1 PVSRIPO Overview
PVSRIPO is based on the prototype neurovirulent poliovirus (PV) serotype 1 PV1S vaccine modified by exchange of its internal ribosomal entry site (IRES) with its counterpart from human rhinovirus type 2 (HRV2), generating PVSRIPO. PVSRIPO is characterized by loss of the inherent neurovirulence of PV. For more details on the make-up, mechanism of action, and formulation of PVSRIPO, please refer to the Investigator’s Brochure.

7.2.2 PVSRIPO Tumor Tropism
Every aspect of PVSRIPO oncolytic immunotherapy is dominated by its relationship with its host cell receptor, CD155. CD155 is broadly expressed in neuroectodermal malignancies, e.g. malignant glioma and melanoma.

7.2.3 PVSRIPO Pre-Clinical Experience
In animal tumor models including the B16 model, oncolytic PVs elicit efficient anti-neoplastic effects resulting in tumor regression and, eventually, destruction. There is histologic evidence for direct, virus-mediated tumor cell killing and indirect, host-mediated inflammatory responses directed against melanoma. Preclinical data in melanoma is reviewed in Section 5.6.

PVSRIPO was subjected to extensive dose-range finding, toxicology, biodistribution, shedding and neutralizing antibody tests with intrathalamic inoculation of up to $5 \times 10^9$ TCID$_{50}$ of PVSRIPO in M. Fascicularis. These revealed: (i) absence of morbidity and mortality; (ii) absence of neuropathological signs consistent with virus-induced CNS damage; (iii) absence of virus dissemination from the brain or viremia; (iv) absence of extraneural replication; (v) absence of shedding with saliva, urine or stool; (vi) presence of a neutralizing antibody response. For more information on pre-clinical studies of PVSRIPO, please refer to the Investigator’s Brochure.

7.2.4 PVSRIPO Clinical Experience
PVSRIPO has been tested in a phase 1 study in subjects with GBM and has generated a durable clinical response in approximately 20% of patients. These responses occurred despite prior disease progression following surgical resection, cranial radiation therapy, chemotherapy (temozolomide, TMZ), and (sometimes) bevacizumab. Twenty-one patients were enrolled into the PVSRIPO dose-finding portion of this phase 1 study. The dose was rapidly escalated from $1 \times 10^8$ TCID$_{50}$ (dose level 1) to $1 \times 10^{10}$ TCID$_{50}$ (dose level 5) after which, the dose was reduced to $5 \times$
$10^7$ TCID$_{50}$ due to long-term steroid or bevacizumab therapy to control inflammatory reaction secondary to PVSRIPO-mediated immune response. For more information about the clinical findings of PVSRIPO, refer to the Investigator’s Brochure for PVSRIPO.

Based on the original dose finding and safety study in patients with recurrent GBM (ClinicalTrials.gov Identifier: NCT01491893; Duke IRB Pro00031169), this study will start at a dose of $1 \times 10^8$ TCID$_{50}$ (dose level 1) in melanoma patients. See Section 9.7 for rationale for starting dose.

### 7.3 STUDY PURPOSE/RATIONALE

Local oncolytic immunotherapy is a promising biologic approach to treatment that not only induces viral mediated tumor destruction, but also harnesses a complex immune response that can serve in disease control for the injected tumor and possibly distant lesions. The effects of local oncolytic immunotherapy may help to induce inflammation and generate systemic immunity. The data gained from this trial of PVSRIPO may support a future trial where the inflammation generated by PVSRIPO in combination with a checkpoint inhibitor (eg anti-PD-1 therapy) could generate a more robust immune response. The genetically engineered oncolytic PV (PVSRIPO) has tropism for melanoma cells by virtue of expression of CD155 in tumor cells. To facilitate concentration of the therapeutic agent at the tumor site, while minimizing systemic exposure, PVSRIPO will be subcutaneously injected directly into the tumor. The main toxicity attributable to PVSRIPO in brain tumors has been post-treatment peri-tumoral inflammation that has required prolonged steroid therapy and/or low dose bevacizumab to control edema. Because of the subcutaneous injection without the confinement of the skull, there should not be any complications due to peri-tumoral edema in this study.
8 OBJECTIVES AND ENDPOINTS

Table 1: Objectives

<table>
<thead>
<tr>
<th>Objective</th>
<th>Endpoint</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
<td>To characterize the safety and tolerability of PVSRIPO in AJCC Stage III B, III C, or IV melanoma.</td>
<td>The proportion of patients with DLTs by cohort (see Section 9.1.2 for definition of DLT)</td>
</tr>
<tr>
<td><strong>Exploratory</strong></td>
<td>To describe the response rates of PVSRIPO-injected versus non-injected lesion(s).</td>
<td>Measurement of cutaneous lesions every 3 weeks</td>
</tr>
<tr>
<td><strong>Exploratory</strong></td>
<td>To describe the number of CD8 positive T cells present in the tumor biopsies before and after injection of PVSRIPO.</td>
<td>Number of CD8 positive T cells by IHC on pre treatment and post treatment biopsy</td>
</tr>
<tr>
<td><strong>Exploratory</strong></td>
<td>Determine the pathologic response in tumor biopsies after PVSRIPO.</td>
<td>The change in tumor pathology from baseline to after PVSRIPO injection</td>
</tr>
<tr>
<td><strong>Exploratory</strong></td>
<td>Determine changes in the tumor microenvironment from biopsies after PVSRIPO.</td>
<td>The change in inflammatory cells and markers after PVSRIPO and detection of viral replication in injected versus non injected lesions.</td>
</tr>
<tr>
<td><strong>Exploratory</strong></td>
<td>Describe how systemic immune cell populations change after treatment with PVSRIPO.</td>
<td>Change relative to basline in type and function of T cells via flow cytometry</td>
</tr>
</tbody>
</table>

9 INVESTIGATIONAL PLAN

9.1 STUDY DESIGN

Within this phase 1 study, patients will have one to three lesions of melanoma injected with PVSRIPO, depending on when they begin the first injection.
9.1.1 Dose Reduction/Escalation

A modified 3+3 phase 1 trial design as shown in Figure 5 will be used in this study. Patients in cohort 0 will receive PVSRIPO at a dose of $1 \times 10^8$ TCID$_{50}$ on day 0 into one index lesion (as described in Section 9.1.4). A minimum of three patients will have one lesion injected with PVSRIPO at $1 \times 10^8$ TCID$_{50}$. If 0 or 1 of 3 have a DLT (defined in Section 9.1.2) during the DLT period (21 days after injection), three patients will be enrolled to cohort 1, which consists of PVSRIPO at a dose of $1 \times 10^8$ TCID$_{50}$ on day 0 into one index lesion and a second injection at the same dose ($1 \times 10^8$ TCID$_{50}$) into a separate lesion on day 21 ($\pm 2$ days). If 2 or more patients have a DLT in cohort 0, the dose will decrease to $5 \times 10^7$ TCID$_{50}$ and 3 patients will have 1 lesion injected on day 0 and this reduced dose will be cohort 1. Additional cohorts will be based on the number of toxicities as detailed in Figure 5. We will treat a maximum of 9 patients. A second injection of a second lesion may not be given in an individual patient if that patient experiences a DLT following their first injection. If an individual patient in cohort 1 or 2 has a DLT, they will not be replaced; rather the trial will continue as detailed in Figure 5. We will enroll in 3 patient cohorts. There must be a minimum of 3 weeks after the last dose in the third patient of the cohort before additional patients can enroll. If 2 of 3 patients in cohort 1 have a DLT, we will temporarily suspend accrual, review DLTs, and re-assess before proceeding to enroll additional patients. If the trial resumes, will proceed to a lower second dose per outline below in Cohort 2, or possibly stop the trial. If 2 of the first 3 patients in cohort 2 have a DLT, we will pause and re-assess before treating a third patient in cohort 2.

Cohort 3 will also treat 3 patients with 3 injections but into the same lesion; each injection will be 3 weeks apart. The proposed dose for each injection will be $1 \times 10^8$ TCID. We will also include a dose reduction plan (schema below). If the patient has a DLT after any dose, they will not be able to get additional doses. The trial now will enroll a minimum of 6 patients and a maximum of 12 patients.

In the study calendar, patients cohort 3 will follow the same schedule as cohort 2 patients with the exception that the 3 injections will be into the same lesion.
9.1.2 Definition of Dose-Limiting Toxicity

Toxicities will be graded according to the NCI CTCAE version 4 criteria. Any possible DLTs will be identified from each PVSIROPO injection until the end of a 21-day observation period, during which the patient will be evaluated in clinic on day 21 (± 4 days). DLTs will be defined as any of the below events that do not resolve to pre-treatment baseline or ≤ Grade 1 within 21 days or any toxicity indicated below that resolves within the 21-day period, but then recurs as Grade 4 or higher during that same 21-day period.

DLTs will include:
- Any Grade 4 or higher non-hematologic toxicities probably, possibly, or definitely related to PVSIROPO; with the exception of vitiligo
- Any Grade 4 or higher hematologic toxicities probably, possibly, or definitely related to PVSIROPO.

9.1.3 PVSIROPO Injection

The entire volume of the agent will be pre-loaded into a 1 mL syringe by the investigational pharmacist and connected to a 25 G needle. PVSIROPO at each time point will be given in 1 standard dose (either 1×10^8 TCID₅₀, or 5×10^7 TCID₅₀ according to Section 9.1.1). The injection volume will be 0.5 mL.

Table 2: Treatment Schedule Overview

<table>
<thead>
<tr>
<th>Treatment Timepoint</th>
<th>Max. Injection Volume</th>
<th>Dose</th>
<th>Injection Criteria</th>
</tr>
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</table>

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Melanoma index lesions present in patients in different quantities and sizes (from 10 mm and larger but rarely no larger than 5 cm in diameter). To facilitate logistics of drug preparation, we will have 1 volume of the same dose (either $1 \times 10^8$ TCID$_{50}$ or $5 \times 10^7$ TCID$_{50}$ according to Section 9.1.1). A volume of 0.5 mL will be given to all lesions, the priority of lesions is shown in Table 2. A maximum total of $1 \times 10^8$ TCID$_{50}$ will be given per lesion. When lesions are clustered together (sum equals 10 mm), we will inject them as a single lesion with a total of 0.5 mL.

9.1.4 Administration
The following steps will be followed for the administration of PVSRIPO:

Pre-Injection
1. The lesion and surrounding areas will be cleaned with an alcohol swab and let dry.
2. The injection site may be treated with a topical or local anesthetic agent, if necessary, at the discretion of the administering physician. The anesthetic agent will be injected around the periphery of the lesion, not directly into the lesion.

Injection
1. PVSRIPO will be injected intralesionally into a cutaneous or subcutaneous lesion that is visible and/or palpable (Figure 6) PVSRIPO will be injected along multiple tracks, using a single insertion point, as far as the radial reach of the needle allows within the lesion to achieve even and complete dispersion. Multiple insertion points may be used if a lesion is larger than the radial reach of the needle.
Figure 6: Injection of Cutaneous (left) or Subcutaneous (right) Lesions.

2. PVSRIPO will be injected evenly and completely within the lesion by pulling the needle back without exiting the lesion. The needle will be redirected as many times as necessary while injecting the remainder of the dose of PVSRIPO.

3. The needle will be withdrawn from the lesion slowly to avoid leakage of PVSRIPO at the insertion point.

Post-Injection

1. Pressure will be applied to the injection site(s) with sterile gauze for at least 30 seconds.

2. The injection site(s) and the surrounding area will be swabbed with alcohol.

3. Following a glove change, the injected lesion will be covered with an absorbent pad and dry occlusive dressing.

4. The exterior of the occlusive dressing will be wiped with alcohol.

5. Patients will be advised to:
   - Keep the injection site(s) covered for at least the first week after each treatment visit or longer if the injection site is weeping or oozing.
   - Replace the dressing if it falls off.

Up to 10 index lesions can be selected for response determination and up to 3 index lesions may be injected over the course of the study. See Section 12.7.1 for definition of index and non index lesions.

9.1.5 Acute Reaction

Any reaction symptoms determined to be an acute reaction to the study drug will be managed by the administering physician.

If a subject experiences any of the following treatment-related toxicities, further PVSRIPO administration should be delayed until the toxicity has resolved to at least CTCAE grade 1 or has returned to baseline:
• grade 2 or greater immune-mediated adverse events, with the exception of vitiligo
• grade 2 or greater allergic reactions
• any other grade 3 or greater hematologic or non-hematologic toxicity

9.2 BIOPSY SAMPLING AND ANALYSES

Biopsy material will be obtained from tumor tissue prior to first virus administration. This tissue material may be subjected to routine histology to confirm tumor recurrence by the study pathologist, Angelica Selim or her designate. If the patient already had documented recurrence by fine needle aspiration or previous biopsy within 4 weeks prior to PVSRIPO injection, no additional routine histology to confirm tumor will be necessary until after PVSRIPO therapy. Molecular genetic tests, as described in Section 12.8 will also be conducted on extracts of tumor cells from the protocol-specified biopsy prior to PVSRIPO injection, if there is sufficient tissue sample remaining after the standard clinical pathologic testing. All tests will be performed on fresh, frozen, or fixed tissue.

Patients may undergo biopsies of injected lesions up to 28 days prior to injection but no less than 7 days before injection, day 10, and day 84 following each injection. Given that each patient has different sizes and numbers of lesions not all biopsies may be possible; only larger lesions can be biopsied 3 times. The priority for biopsies will be 1) pre-treatment; 2) day 84 biopsy; 3) day 10 biopsy. Additionally, biopsies may be performed to assess pathologic response. Surgical excisions will also include examination of pathologic response. Non injected lesions may also be biopsied pre-treatment, day 10, and day 84, if tumor burden is sufficient. More than 1 biopsy can be performed on injected or non-injected lesions at the timepoints at the discretion of the PI. For example 2 or more non-injected lesions can be biopsied, or more than 1 biopsy (for large lesions) can be done.

9.3 SAFETY CONSIDERATIONS

• **Biopsy Complications:** 3-5 mm punch biopsy is generally a non-hazardous procedure; however, possible hazards may include: 1) reaction to anesthetic (numbing medicine), 2) excessive bleeding, 3) bruising, 4) infection and 5) excessive scarring.

• **Poliomyelitis:** PVSRIPO has been tested in non-human primates according to the World Health Organization (WHO) standardized monkey neurovirulence tests. These tests revealed the absence of neuropathogenic properties, evident as failure to induce symptoms of poliomyelitis in non-human primates after intracerebral inoculation of virus. However, PVSRIPO is a replication-competent viral agent that, in principle, retains the potential to cause motor neuron damage. Possible complications include transient or permanent mono- or paraparesis, paralysis, and life-threatening respiratory insufficiency.

• **Virus Recombination and Mutation:** PVSRIPO retains the ability to alter its genome during replication upon administration to patients. Various mechanisms are known to lead to genetic adaptation, including spontaneous mutagenesis and recombination that may give rise to viral progeny with changed properties. Empirical studies in tissue culture and laboratory animals demonstrated that prolonged passaging in GBM cells does not select for genetic changes in viral progeny. However, the occurrence of such events cannot be categorically excluded in patients receiving intracerebral PVSRIPO. Genetic changes may cause an altered phenotype of PVSRIPO, including adaptation to improved virus replication in the normal CNS.
• **Long-Term Sequelae of Virus Injection:** PVSRIPO does not encode foreign genetic material; PVs are unable to insert viral genetic material in the host genome and PVs are incapable of inducing latent or chronic CNS infection. Therefore, PVSRIPO is unable to induce long-term expression of foreign polypeptides or long-term persistence. Thus, there are no long-term neurologic consequences to intracerebral PVSRIPO administration in study subjects. For these reasons, no specific measures to analyze the potential for persistence of virus replication in the CNS of long-term survivors are indicated.

• **Gastrointestinal Infection and Virus Excretion:** After oral uptake, poliovirus replicates in the gastrointestinal tract and is excreted by infected individuals within stool. Gastrointestinal viral replication usually is asymptomatic, but may cause mild symptoms of gastrointestinal discomfort. Tests of PVSRIPO in non-human primates suggest that excretion of virus does not occur after intracerebral administration, implying the absence of gastrointestinal replication. However, PVSRIPO excretion within stool cannot be categorically excluded in patients enrolled in this study. Therefore, treatment entails collection of stool specimens to assess virus excretion during the study.

• **Risk of Phlebotomy:** Drawing blood or inserting an intravenous catheter into an arm vein may result in bruising or swelling in the area of the insertion, bleeding at the site of the needle puncture, light headedness, fainting and very rarely, local infection, which may be severe. These risks are reduced by the fact that the blood will be drawn by a qualified physician, nurse or phlebotomist (a professional trained to draw blood).

• **Risks to Household Contacts of Study Subjects:** Primate toxicology studies showed that intracerebral infusion of PVSRIPO does not lead to extraneural dissemination or replication and, hence, is not associated with virus shedding. Therefore, and because PV transmission occurs via the fecal-oral route, the likelihood of unintended exposure of patient household contacts is exceedingly low. If accidental exposure occurred, it would equal the risk of exposure to any type 1 Sabin vaccine virus or vaccine virus derivative. Thus, in essence, exposure with PVSRIPO is equal to oral immunization with a safe version of type 1 Sabin. Since type 1 Sabin vaccine virus or vaccine virus derivatives have to be considered part of the human environment, exposure to PVSRIPO would not represent an added risk beyond the possibility for exposure that already exists. Study subjects will be advised of the risks of exposure to unvaccinated household contacts. We will also keep the injected lesion covered for 1 week or longer if the tumor becomes weeping or oozing.

• **Healthcare providers:** Healthcare providers who are immunocompromised or pregnant should not prepare or administer PVSRIPO and should not come into direct contact with the PVSRIPO injection sites, dressings, or body fluids of treated patients. Wear personal protective equipment (protective gown or laboratory coat, safety glasses or face shield, and gloves) while preparing or administering PVSRIPO. Avoid accidental exposure to PVSRIPO, especially contact with skin, eyes, and mucous membranes. Cover any exposed wounds before handling. In the event of an accidental occupational exposure (e.g., through a splash to the eyes or mucous membranes), flush with clean water for at least 15 minutes. In the event of exposure to broken skin or needlestick, clean the affected area thoroughly with soap and water and/or a disinfectant.

• **Unknown Risks:** The overall risk classification of this research is unknown.
9.4 **CONCOMITANT MEDICATIONS**

9.4.1 **Steroids**
Corticosteroids should be used at the lowest dose to control symptoms of edema or reactions to PVSRIPO. Use of corticosteroids should be recorded in the electronic database. If the subject requires corticosteroid dosing of >10 mg prednisone daily (or equivalent) for related toxicities, PVSRIPO dosing must be withheld until the corticosteroid dose has decreased to <10 mg prednisone daily (or equivalent).

9.4.2 **Growth Factors**
Routine use of growth factors (i.e. G-CSF, GM-CSF, and erythropoietin) is not permitted. However, therapeutic use of G-CSF in patients with serious neutropenic conditions, such as sepsis, may be used at the investigator’s discretion. Use of such growth factors should be recorded in the electronic database.

9.4.3 **Anti-emetics**
The use of anti-emetics will be at the investigator’s discretion. Use of anti-emetics should be recorded in the electronic database.

9.4.4 **Proton Pump Inhibitors**
The use of proton pump inhibitors (PPI) (e.g. rabeprazole, omeprazole, pantoprazole, lansoprazole or esomeprazole) is allowed on this study.

9.4.5 **Febrile Neutropenia**
Febrile neutropenia should be managed according to the local institutional guidelines. Measures include laboratory testing, blood and urine cultures, and institution of broad spectrum antibiotics.

9.4.6 **Pneumocystis Jiroveci Pneumonia (PJP) Prophylaxis**
The use of medication (i.e., Bactrim) for PJP prophylaxis in patients on chronic steroids is recommended, but is at the investigator’s discretion.

9.4.7 **Epidermal Procedures**
If an epidermal procedure is required for a reason other than tumor progression, these procedures should be documented, but will not constitute criteria for declaring the patient “off study”.

9.5 **STUDY DRUG BLINDING**
Not applicable

9.6 **RANDOMIZATION**
Not applicable

9.7 **RATIONALE FOR SELECTION OF DOSE, REGIMEN, AND TREATMENT DURATION**
The dose of PVSRIPO for this trial was selected based on IND-directed toxicity studies and on experience from a phase 1 dose-escalation study in adults with recurrent WHO Grade IV malignant
glioma. Dose-range finding and toxicology studies in non-human primates (NHP) documented the absence of viral encephalomyelitis, poliomyelitis and meningitis with intracerebral injection of PVSRIPO up to a dose of $5 \times 10^9$ TCID$_{50}$ In the adult WHO Grade IV malignant glioma study, 4 patients were dosed at the maximum intended dose of $10^{10}$ TCID$_{50}$, injected intracerebrally, without signs of viral encephalomyelitis, poliomyelitis or meningitis. In the phase 1 GBM clinical trial, the starting dose was $1 \times 10^8$ TCID$_{50}$, which is 1/10th of the highest non-toxic dose in NHPs from the definitive, IND-directed toxicology study and 1/50th of the highest non-toxic dose in NHPs from the dose-range finding toxicology study. For more information on these pre-clinical studies, please refer to the Investigator’s Brochure. Based on the original dose finding and safety study in patients with recurrent GBM (ClinicalTrials.gov Identifier: NCT01491893; Duke IRB Pro00031169), this study will start at a dose of $1\times 10^8$ TCID$_{50}$ (dose level 1) in melanoma patients. 1x10$^8$ TCID$_{50}$ (dose level 1 from the GBM trial) was chosen for the following reasons.

1. In the dose escalation study conducted in patients with GBM, patient 1 received dose level 1, $1\times 10^8$ TCID$_{50}$. This patient responded favorably to PVSRIPO therapy and was disease-free 60+ months after receiving a single intratumoral injection of PVSRIPO.

2. Brain inflammation in the area of the tumor or immediately adjacent was the main toxicity associated with intratumoral infusion of PVSRIPO and the primary reason for dose reduction in the phase 1 brain tumor study. There were no signs of encephalitis, systemic toxicity or systemic inflammatory processes. Given that melanoma lesions are not in physiologically confined areas, e.g. the brain in the cranium, the effects of inflammation due to PVSRIPO are not of significant clinical impact.

3. A higher dose is warranted in this setting given that there may be some systemic leakage upon injection, therefore requiring an overall higher dose to ensure that a substantial amount of the PVSRIPO remains in the tumor. We will therefore start with dose level 1 ($1\times 10^8$ TCID$_{50}$) from the GBM study, to ensure that we elicit a response in tumor (both tumor killing and immune consequences) post-PVSRIPO in this Phase 1 study.

9.8 **Rationale for Correlative Studies**

While tumor-selective PVSRIPO propagation is an important mediator of cytotoxicity, significant intra- and peritumoral inflammation likely ensues. This indicates that an immunogenic response is likely being generated, certainly against the virus itself and most likely against the tumor as well. While the exact host immune response to PVSRIPO oncolysis is currently unknown, but is being investigated, host innate antiviral defenses are likely to trigger a broad immune effector cascade that needs to be examined in patients receiving PVSRIPO therapy. Blood will therefore be collected for immune function studies before and at periodic intervals following treatment with PVSRIPO. The accessibility of tumor tissue in the cutaneous melanoma also allows for correlative studies directly on tumor tissue and the tumor microenvironment. This tumor tissue may be extracted as biopsies or as complete tumor resection, if available.

9.9 **Definition of Evaluable Subjects, On Study, and End of Study**

Any patient that receives at least one PVSRIPO injection will be evaluable for toxicity and efficacy analyses. Any patient that does not receive PVSRIPO injection will be withdrawn from the study and replaced.
9.10 EArLY SToDY TERRMINATION

This study can be terminated at any time for any reason by the PI or the sponsor. If this occurs, all subjects on study should be notified as soon as possible. Additional procedures and/or follow up should occur in accordance with Section 12.5, which describes procedures and process for prematurely withdrawn patients.

10 STUDY DRUG

10.1 PVSRIPO

10.1.1 Names, Classification, and Mechanism of Action

PVSRIPO is a modified version of the serotype 1 live-attenuated (Sabin) PV vaccine (PV1S) and its immunogenic properties and potential for long-term sequelae are expected to be similar. PV1S has been safely administered to > 10 billion individuals worldwide without untoward long-term sequelae. The only known effect of PV1S administration to human subjects is neutralizing immunity to PV.

PVSRIPO is PV1S containing a heterologous IRES of HRV2. The IRES is a cis-acting, non-coding genetic element within the 5' untranslated region (UTR) of all enteroviruses and is essential for translation of the viral genome. PVSRIPO is a non-enveloped, positive-sense ssRNA virus with a genome of ~7300 nucleotides (nt) in length. PVSRIPO particles consist of a proteinaceous capsid composed of 60 copies of each of 4 capsid proteins (VP1-VP4) arranged in icosahedral geometry. Since the coding regions for the viral polyprotein (giving rise to all viral polypeptides) of PVSRIPO and PV1S are the same, the physical structure of the viral capsid and all non-structural viral polypeptides of PVSRIPO and PV1S are identical.

10.1.2 Packaging and Labeling

PVSRIPO is formulated in 50 mM sodium phosphate in 0.9% sodium chloride, pH 7.4 with 0.2% human serum albumin. It is provided in sterile, single use containers. PVSRIPO was manufactured at the Biopharmaceutical Development Program/National Cancer Institute (NCI)-Frederick.

10.1.3 Supply, Receipt, and Storage

The study agent and vehicle will be supplied to the Investigational Pharmacy by the sponsor or their representative. The study agent will be shipped via approved methods in the appropriate packaging on dry ice. Storage of the study agent at -70°C has been empirically determined to not compromise biological and biophysical properties for at least 5 years. The agent will be stored long-term in an ultra-low temperature freezer (less than or equal to -70°C). Once thawed, it is a clear colorless liquid with no evidence of particulates or foreign matter.

10.1.4 Dispensing, Preparation, Administration

For aliquot preparation, the agent will be thawed slowly on ice (4°C) and kept at that temperature. Thawed vials of PVSRIPO are stable at 4°C for 48 hours. PVSRIPO contained in the clinically intended delivery apparatus (i.e., ready for injection) is stable at room temperature for 18 hours,
but should be used as soon as possible. The manufacturer will provide the study agent’s potency (as tissue culture infectious doses, TCID) and will also supply the appropriate vehicle for aliquot preparation. Aliquot preparation will occur in the ICS. All handling of the study agent will occur in a biosafety cabinet or a similarly contained environment.

Any materials in contact with the study agent, e.g. pipettes, vials, etc., will be disposed of as biological waste. The final desired aliquot of the study agent will be prepared at the intended volume (0.5 ml) and drawn into the intended delivery device (1 ml syringe) using a needle. The disposable needle will be a safer hypodermic needle with a safety sheath to cover the needle using a one-handed technique after the agent is drawn into the syringe. The sheathed needle is then removed from the syringe containing the study agent aliquoted at the desired dose, and the syringe will be capped and connected to a 25 G needle. The capped needle and syringe will be transported to the study site in a ‘ziplock’ bag placed in a portable cooler while maintaining a temperature of 4°C.

10.1.5 Compliance and Accountability

Drug accountability records will be maintained for all clinical trial supplies. All empty and partially used clinical trial supplies will be destroyed in accordance with institutional guidelines. ICS will maintain detailed documentation of the receipt and/or destruction of the study agent. All materials coming in contact with the study agent, the syringe delivered from ICS, dressings or coverings used to protect the injected lesion site, will be disposed of as biological waste in the treatment room.

10.1.6 Disposal and Destruction

All materials coming in contact with the study agent, the syringe delivered from the Investigational Pharmacy, tubing, dressings or coverings used to protect the injected lesion site, will be disposed of as biological waste in the treatment room. Disposal of any unused or partially used study agent will be the responsibility of the Investigational Pharmacy. Used sharps will be disposed in a biohazard sharps container and incinerated in final disposal. All personnel caring for the patient will be wearing appropriate safety devices, e.g. disposable gloves, gown, and face protection, which will be disposed of as biological waste in the treatment room. Appropriate hand hygiene is required before and after any handling of the study agent.

10.1.7 Spill Procedure

PVSRIPO can be easily and completely inactivated with household bleach. In the event of a spill, the liquid will be absorbed with gauze then treated as biohazardous waste as above. The spill area will then be liberally wiped down with a 20% household bleach solution for chemical disinfection. The disinfectant will be left in contact with the contaminated area for at least 30 minutes, then wiped away with wet towels, which will be disposed as biohazard waste as well.

10.1.8 Exposure Follow-Up

Any virus exposures will be reported to the Employee Health Exposure Hotline (919-684-8115), and Sponsor followed by submission of the Report of Occupational Injury or Illness form. There is no specific preventative treatment. Based on the circumstances of exposure, employee health may choose to monitor for infection by submitting stool for enterovirus culture at 7, 14, and 21 days.
after an exposure event.

**11 SUBJECT ELIGIBILITY**

### 11.1 INCLUSION CRITERIA

1. Positive serum anti-poliovirus titer prior to biopsy.
2. The patient must have received a boost immunization with trivalent inactivated IPOL™ (Sanofi-Pasteur) at least 1 week prior to administration of the study agent.
3. Patient must have histologically proven unresectable, recurrent, melanoma, stage IIIB, IIIC, or stage IV (AJCC staging must be documented in patient’s medical record, as determined by CT of the chest, abdomen and pelvis, and/or whole body PET scan, and MRI of the brain within 4 weeks prior to administration of study drug)
4. Patients with BRAF mutations, must have failed at least 2 lines of therapy, specifically one BRAF targeted therapy and at least one anti-PD-1 based therapy. For BRAF wild type, patients must have failed at least one anti-PD-1 based therapy.
5. Patient must be ≥18 years of age.
6. Patient must have an ECOG/Zubrod status of 0-1.
7. Patient’s disease must be bi-dimensionally measurable by caliper or radiological method as defined in the irRC criteria.
8. At least 1 injectable cutaneous, subcutaneous or nodal melanoma lesions ≥ 10 mm in longest diameter or, multiple injectable melanoma lesions which in aggregate have a longest diameter of ≥ 10 mm (Cohorts 0, 3 and possibly 1). For cohorts where 2 or 3 injections are planned (Cohorts 1 and 2), the patient must have at least 2 injectable melanoma lesions (when 2 doses are planned) or 3 injectable melanoma lesions when 3 doses are planned in different lesions.
9. At least one measurable lesion that will not be injected.
10. Serum lactate dehydrogenase (LDH) levels less than 1.5 x upper limit of normal (ULN)
11. Patient must have adequate bone marrow, liver and renal function as assessed by the following:
   a. Hemoglobin > 9.0 g/dl
   b. White blood count (WBC) of > 2000 m3
   c. Absolute neutrophil count (ANC) > 1,000/mm3
   d. Platelet count > 75,000/mm3
   e. Total bilirubin < 2.0 x ULN
   f. ALT and AST < 2.5 x the ULN
   g. Creatinine < 2.0 x ULN
12. Patients must have a life expectancy of > 6 months.
13. Patient must provide a signed and dated written informed consent prior to registration and any study-related procedures.
14. Ability to read and understand English and the ability to complete paper and electronic survey assessments.
11.2 EXCLUSION CRITERIA

1. Females who are pregnant or breast-feeding.
2. Adults of reproductive potential not employing an effective method of birth control.
3. Patients with severe, active co-morbidity, defined as follows:
   a) Patients with an active infection requiring treatment or having an unexplained febrile illness (Tmax > 99.5°F/37.5°C)
   b) Patients with impaired cardiac function or clinically significant cardiac disease, such as congestive heart failure requiring treatment (New York Heart Association Class ≥ 2), uncontrolled hypertension or clinically significant arrhythmia; QTcF > 470 msec on ECG if performed or congenital long QT syndrome; acute myocardial infarction or unstable angina pectoris < 3 months prior to study
   c) Patients with known lung (FEV1 < 50%) disease or uncontrolled diabetes mellitus (HgbA1c > 7)
   d) Patients with albumin allergy.
   e) Autoimmune disease: History of or current active autoimmune diseases, [e.g. including but not limited to inflammatory bowel diseases [IBD], rheumatoid arthritis, autoimmune thyroiditis, autoimmune hepatitis, systemic sclerosis (scleroderma and variants), systemic lupus erythematosus, autoimmune vasculitis, autoimmune neuropathies (such as Guillain-Barre syndrome)]. Vitiligo and adequately controlled endocrine deficiencies such as hypothyroidism are not exclusionary.
   f) Known immunosuppressive disease, human immunodeficiency virus (HIV) infection, or chronic Hepatitis B or C
4. Patients with a previous history of neurological complications due to PV infection
5. Patients who have not recovered from the toxic effects of prior chemo- and/or radiation therapy. Guidelines for this recovery period are dependent upon the specific therapeutic agent being used. Toxicities must have resolved to CTCAE grade 1 or less with the following exceptions (alopecia, fatigue, vitiligo)
6. Patients with undetectable anti-tetanus toxoid IgG
7. Patients with known history of agammaglobulinemia
8. Patients on greater than 10 mg per day of prednisone within the 2 weeks prior to admission for PVSRIPO injection
9. Patients with worsening steroid myopathy (history of gradual progression of bilateral proximal muscle weakness, and atrophy of proximal muscle groups)
10. Patients with prior, unrelated malignancy requiring current active treatment with the exception of cervical carcinoma in situ and adequately treated basal cell or squamous cell carcinoma of the skin
11. Clinically active cerebral or bone metastases
12. Greater than 3 visceral metastases (this does not include nodal metastases associated with visceral organs)
13. Prior allogeneic stem cell transplantation
14. Concomitant therapy with any of the following: IL-2, interferon, or other non-study immunotherapy regimens; cytotoxic chemotherapy; immunosuppressive agents; other investigation therapies; or chronic use of systemic corticosteroids (used in the management of...
cancer or non-cancer-related illnesses). However, during the course of the study, use of corticosteroids is allowed if used for treating irAEs, adrenal insufficiencies, or if administered at doses of prednisone 10 mg daily or equivalent.

15. Active clinically serious infection > CTCAE Grade 2.

16. Antineoplastic therapy, radiotherapy, or any other investigational drug within 15 days prior to first study drug administration.
# 12 SCREENING AND ON-STUDY TESTS AND PROCEDURES

## Table 3: Schedule of Study Tests/Procedures

<table>
<thead>
<tr>
<th>Day</th>
<th>Screening: Within 28 days prior to PVSRIPO</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>General Evaluations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Informed Consent</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Physical Exam±17</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Lesion Measurements</td>
<td>x (6)</td>
<td>x (1)</td>
</tr>
<tr>
<td>Performance Status</td>
<td>x (1)</td>
<td>x</td>
</tr>
<tr>
<td>Adverse Events</td>
<td>Continuous (11)</td>
<td></td>
</tr>
<tr>
<td>Laboratory Evaluations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EKG</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>PV Booster</td>
<td>X16</td>
<td></td>
</tr>
<tr>
<td>Tetanus toxoid titer</td>
<td>X16</td>
<td></td>
</tr>
<tr>
<td>PV titer (blood)</td>
<td>X16</td>
<td>X (9)</td>
</tr>
<tr>
<td>LSQ Titer</td>
<td>X16</td>
<td></td>
</tr>
<tr>
<td>PV titer (stool)</td>
<td>X16</td>
<td></td>
</tr>
<tr>
<td>CBC w/diff</td>
<td>x</td>
<td>X (1)</td>
</tr>
<tr>
<td>PT, aPTT</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

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J Immunother Cancer

Pro00090774: PVSRIPO in melanoma
Version: 05/15/2019

<table>
<thead>
<tr>
<th>Day</th>
<th>Screening: Within 28 days prior to PVSRIPO</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>CMP</td>
<td>x</td>
<td>x&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum pregnancy</td>
<td>x</td>
<td>x&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVSRIPO injection</td>
<td>X</td>
<td>X&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Disease Evaluations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>irRC skin lesion</td>
<td>X&lt;sup&gt;(1)&lt;/sup&gt;</td>
<td>X&lt;sup&gt;(1)&lt;/sup&gt;</td>
</tr>
<tr>
<td>PET/CT&lt;sup&gt;(12)&lt;/sup&gt;</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Brain MRI</td>
<td>x</td>
<td>X&lt;sup&gt;(10)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Correlative aspects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>whole blood (35 ml) immune analysis&lt;sup&gt;(18)&lt;/sup&gt;</td>
<td>X&lt;sup&gt;(14a,16)&lt;/sup&gt;</td>
<td>X&lt;sup&gt;(14b)&lt;/sup&gt;</td>
</tr>
<tr>
<td>tumor biopsy lesion 1</td>
<td>x&lt;sup&gt;(2)(3)(4)&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>tumor biopsy lesion 2&lt;sup&gt;7&lt;/sup&gt;</td>
<td>X&lt;sup&gt;7&lt;/sup&gt;</td>
<td>X&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>tumor biopsy lesion 3&lt;sup&gt;7&lt;/sup&gt;</td>
<td>X&lt;sup&gt;7&lt;/sup&gt;</td>
<td>X&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>tumor biopsy non injected&lt;sup&gt;(15)&lt;/sup&gt;</td>
<td>X&lt;sup&gt;(4)&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>tumor resection</td>
<td>x&lt;sup&gt;(5)&lt;/sup&gt;</td>
<td>X&lt;sup&gt;(5)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1=done before pvsripo is given, but can be within 48 hours of PVSRIPO

2= if not yet histologically confirmed, will need histologic confirmation, biopsy for correlative studies should be done up to 21 days before and must be done no less than 7 days before injection

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3=correlative and histology can be done on same biopsies
4=histology can be done on lesion planning to inject or not inject
5=tumor resection can be done at discretion of PI, the resection can be used for histologic and correlative aspects
6=only need to measure 1 to 4 lesions to qualify depending on dose escalation cohort
7=when applicable per dose escalation guidelines
8=these visits and all study procedures for that visit will only occur if the patient received a second and/or third PVRSIPO injection per dose escalation cohort
9=after booster, but before PVRSIPO preferably just before PVRSIPO at same time as immune blood draw
10=at discretion of treating physician
11=collection of adverse events will continue for 90 days post last dose of study drug
12=if patient has limited metastatic disease, irRC reads will also come from PET/CT scans, if patients do not have any metastatic lesions, irRC will only be performed on measurements of skin lesions
13=patients without progressive disease will be followed up to 2 years with procedures in this column; for patients with progressive disease, chart review only will occur every 3 months at the time of progression and they may be contacted by the study team
14a=needs to be done just BEFORE booster
14b=JUST BEFORE PVRSIPO injection
14c=at follow up visit 1, around 6 months after PVRSIPO injection #1
15=if patient has sufficient disease
16=does not need to be repeated during 28 day screening period, if performed within 6 months prior to PVRSIPO injection
17=Physical exam includes vital signs
18=whole blood collection for immune analysis can be done at discretion of PI
12.1 SCREENING EXAMINATION

The screening examination will take place within 28 days before PVSRIPO injection. An informed consent must be signed by the patient or their legal guardian(s) before any screening procedure takes place. Within 4 weeks before receiving the PVSRIPO injection, the patient must have received a PV booster. The patient must also have whole blood drawn for LSQ and tetanus titer, and PV titer to document immunity.

Pre-treatment evaluations within 28 days before PVSRIPO injection to determine eligibility will include the following, unless otherwise indicated:

- History and physical exam, including a full assessment, performance status (ECOG), and measurement of lesions that determine eligibility (1-3 depending on dose escalation)
- Informed Consent
- Laboratory Evaluations:
  - CBC with differential
  - CMP
  - PT, aPTT
  - LDH
  - Beta-HCG, if appropriate
  - EKG
  - Whole blood (10 mL) for PV titer (pre vaccine boost), LSQ, and anti-tetanus toxoid titer
- Whole blood (35 mL) for immunologic analysis before polio vaccine boost, Baseline PET/CT or CT c/a/p plus brain MRI
- Biopsy of the lesion that will be injected (can be up to 21 days before must be no less than 7 days before injection) and planned non injected lesion if patient has sufficient disease.

If a subject is found to be ineligible to participate in the study and does not receive PVSRIPO, minimal records regarding the subject and the reason for screen failure will be retained in the study database.

12.2 TREATMENT PERIOD

Day 0
- CBC w/diff, CMP, serum pregnancy before PVSRIPO (no more than 48 hours prior)
- PV titer before PVSRIPO
- Whole blood (35 ml) for immune analysis before PVSRIPO
- irRC skin lesion before PVSRIPO
- Collection of Adverse Events
- Lesion measurements before PVSRIPO
- Performance status before PVSRIPO
- PVSRIPO injection (lesion #1)
- Monitor for DLT (dose limiting toxicities) until day 21

Day 2
- Performance status (ECOG)
- Collection of Adverse Events
- Blood for immunologic assay (35 mL)
Day 10 (± 2 days)
- physical exam
- Performance status (ECOG)
- PV titer blood
- PV titer stool
- CBC w/ diff/CMP
- Collection of Adverse Events
- Whole blood (35 mL) for immunologic analysis
- Tumor biopsy (lesion #1 and a non-injected lesion)
- Tumor biopsy (of intended lesion #2), if applicable

Day 21 (± 2 days)
- physical exam
- Performance status (ECOG)
- Serum pregnancy test, CBC/CMP, (no more than 48 hours prior)
- irRC skin lesion before PVSRIPO
- Collection of Adverse Events
- Lesion measurements
- PVSRIPO injection (#2)
- Begin DLT assessment for injection #2 for 21 days. End DLT assessment for injection #1

Day 23 (~2 ±2 days after #2 injection, only for patients in Cohort 1, 2, and 3 who receive a second PVSRIPO injection)
- Performance status
- Physical exam
- Collection of Adverse Events

Day 31 (~10 ±3 days after second PVSRIPO injection, only for patients in Cohort 1, 2, and 3 who receive a second PVSRIPO injection)
- Tumor biopsy (#2 and non injected)
- Tumor biopsy (site of injection #3), if applicable
- 35 ml Whole blood for immune analysis
- Physical exam
- Performance status
- PV titer blood
- CBC w/ diff, CMP
- Collection of Adverse Events

Day 42 (± 3 days)
- physical exam
- Performance status (ECOG)
- Lesion measurements
- irRC skin lesion before PVSRIPO
- Collection of Adverse Events
- CBC w/diff, CMP, serum pregnancy before PVSRIPO (no more than 48 hours prior)
- PVSRIPO injection (#3)
- Begin DLT assessment for injection #3 for 21 days. End DLT assessment for injection #2

**Day 44 (~2 ±2 days after #3 injection, only for patients in Cohort 2 and Cohort 3 who receive 3 PVSRIPO injections)**
- Physical exam
- Performance Status (ECOG)
- Collection of Adverse Events

**Day 52 (~10 ±3 days after third PVSRIPO injection, only for patients in Cohort 2 and Cohort 3 who receive a third PVSRIPO injection)**
- Tumor biopsy (#3 and non-injected)
- Physical exam
- Performance status
- CBC w/diff, CMP
- Collection of Adverse Events

**Day 63 (± 5 days)**
- physical exam
- Performance status (ECOG)
- Collection of Adverse Events
- End DLT assessment for injection #3

**Day 84 (± 7 days)**
- physical exam
- Lesion measurements
- Performance Status (ECOG)
- irRC
- CBC w/diff, CMP
- LDH
- Collection of Adverse Events
- PET/CT and brain MRI
- Whole blood (35 mL) for immunologic analysis, at the discretion of the PI
- Tumor biopsy of relevant lesions (injected index lesion No.1 and non-injected tumor)
- Surgical resection at discretion of PI

**Day 105 (± 7 days)**
- physical exam,
- Lesion measurements
- irRC
- CBC w/diff, CMP
- CMP
• Collection of Adverse Events
• Tumor biopsy of relevant regions (injection site No. 2 and non-injected tumor)
• Surgical resection, at the discretion of the PI

Day 126(±7 days, for cohort 2 and cohort 3 only)
• Physical Exam
• Performance status
• Lesion measurements
• CBC w/diff, CMP
• irRC
• Collection of Adverse Events
• Tumor biopsy of relevant lesions (injection site No. 3 and non-injected tumor)

PRN Every 2-3 months after last study visit (last study visit is Day 84 (1 dose), 105 (2 doses), or 126 (3 doses)) up to 2 years for subjects who do not progress
• physical exam
• Lesion Measurements
• LDH
• Whole blood (35 mL) for immunologic analysis (At the discretion of the PI, this blood sample, at least 2 years post-study drug infusion, may be obtained.) at 6 months from PVSRIPO injection #1
• Surgical resection, at discretion of PI
• Collection of Adverse Events will continue for 90 days post last dose of study drug or for 30 days after a patient is off study. For injection #3 AE collection is study day is +7 days of Day 132

If progression occurs, then subjects may be contacted by the study team approximately every 3 months up to 2 years after the last study visit.

While patients are on study, their melanoma may not be treated with any modality other than PVSRIPO. Patients will be considered off study upon treatment of the tumor with another modality. When subjects are considered off study, this indicates that subjects will no longer be obligated to undergo study-related tests and procedures, but the data described below will still be collected from these subjects as feasible.

Due to the mechanism of action of PVSRIPO, subjects may experience transient growth in existing tumors or the appearance of new tumors prior to maximal clinical benefit of PVSRIPO. Subjects who experience growth in existing tumors or the appearance of new tumors will be allowed to remain on therapy until the 12th week after therapy unless, in the opinion of the investigator, immediate surgical resection or any other treatment for melanoma is warranted.

12.3 FOLLOW-UP PERIOD
Subjects will be followed for collection of the additional data if possible, but it is not mandatory and will not be considered a deviation if the data cannot be obtained. Follow up can be obtained by a phone call or...
in person visit. Subjects’ medical records will be reviewed for the remainder of their life, in order to follow survival, as well as subjects’ imaging. Subjects will also be followed for progression and final results of subsequent treatments. Patients without progressive disease will be followed up to 2 years with procedures in the study calendar; for patients with progressive disease, chart review only will occur every 3 months at the time of progression and they may be contacted by the study team.

12.4 END OF STUDY
The study will be considered complete once enrollment has been met, follow-up procedures outlined in Section 12.3 have been conducted on all subjects, and data analysis is concluded. The study may also be terminated early for any reason by the PI-sponsor.

Subjects that are lost to follow-up will be documented in the patient record and in the 21 CFR Part 11 database. In the compliant database, the subject will be marked as “Patient Status Unknown,” along with a corresponding explanation, if any.

12.5 EARLY WITHDRAWAL OF SUBJECT(S)

12.5.1 Criteria for Early Withdrawal
Subjects may voluntarily withdraw from the study at any time. The PI may also withdraw a subject from the study at any time based on his/her discretion. Reasons for PI-initiated withdrawal may include, but are not limited to the following:
- Progressive disease as documented by PET/CT or physical examination at any time
- Treatment with non-protocol therapy
- Pregnancy
- Upon request of the subject
- If, in the investigator’s medical judgment, further participation would be injurious to the subject’s health or wellbeing
- Development of intolerable symptoms
- Protocol deviation
- Non-compliance of the subject
- Administrative issues

12.5.2 Follow-Up Requirements for Early Withdrawal
Subjects who received at least 1 injection of PVSRIPO should be seen in clinic or contacted at a minimum of every 3 months for 1 year, if possible.

12.5.3 Replacement of Early Withdrawal(s)
Subjects that withdraw from the study prior to receiving PVSRIPO injection, either voluntarily or due to ineligibility, will be considered non-evaluable; those subjects will be replaced.
12.6 STUDY ASSESSMENTS

12.6.1 Medical History
Standard medical history will be obtained and documented per institutional guidelines.

12.6.2 Physical Exam
Standard physical exam and performance status assessment will be conducted and documented per institutional guidelines.

12.6.3 Radiographic Review
A PET/CT will be obtained prior to PVSRIPO injection to evaluate and quantify disease. Patients with greater than 3 visceral metastases will be excluded. A PET/CT will also be obtained 3 months after the first injection of PVSRIPO to document tumor response.

12.6.4 Laboratory Evaluations
The timing of laboratory assessments that will be obtained during the course of the study is given above in Table 3. A list of each evaluation is below.

- CBC w/differential
- CMP
- PT, aPTT (one prior to beginning trial)
- Beta-HCG, if applicable, within 48 hours prior to receiving PVSRIPO

12.7 TUMOR ASSESSMENTS
Response will be determined according to immune related response criteria. Tumor measurement data in combination with photographic records of lesions obtained prior to intervention will be used to establish baseline measurements.

Using a standard centimeter calibrated caliper, the longest axis and the perpendicular axis of the tumor will be measured and recorded in metric notation.

Primary source documentation of disease burden will consist of digital photographs taken at baseline prior to treatment, Day 21, Day 42, Day 63, and Day 84 after each PVSRIPO injection. Photographs should adequately document the number and size of recorded lesions (a millimeter-scale ruler should be placed in field of image to provide scale).

12.7.1 Definition of Overall Response Using irRC

Baseline Measurement: Sum of products of the 2 largest perpendicular diameters (SPD) of all index lesions. The sum of the products of the two largest perpendicular diameters (SPD) of all index lesions will be calculated and reported.

Baseline Documentation of “Non-index Lesions”: All other lesions (or sites of disease), including any measurable lesions that were not chosen as index lesions will be identified as non-index lesions. Non-index lesions should be recorded and assessed qualitatively over the course of therapy.

Definitions:
**Index lesions**: Up to 5 cutaneous lesions and up to 5 metastases (no more than 2 visceral organs) can be chosen. The distribution of these index lesions should be representative of the subject’s overall disease status. Index lesions should be selected on the basis of their size (lesions with longest bi-dimensionally perpendicular diameters) and suitability for accurate repeated measurements by imaging techniques (CT, MRI or ultrasound) and/or other method such as clinical exam. The sum of the products of the two largest of perpendicular diameters (SPD) of all index lesions will be calculated and reported.

**Measurable Disease**: Measurability is defined by the ability to measure a lesion bi-dimensionally with surface area determined by multiplying the longest diameter by the diameter perpendicular to the longest diameter as defined below. An individual lesion measure is therefore provided by the product of a tumor’s longest diameter and the diameter perpendicular to that. All measurements will be determined using a ruler or calipers and reported in metric notation (mm) and will be recorded bi-dimensionally.

**Measurable Lesions**: Measurable lesions are defined at baseline as lesions that can be accurately and serially measured in at least 2 dimensions and for which the longest diameter is:

- ≥ 10 mm as measured by CT scan, MRI, or ultrasound for nodal/soft tissue disease (including lymph nodes)
- ≥ 10 mm caliper measurement by clinical exam for superficial cutaneous or subcutaneous melanoma lesion as measured by caliper
- Multiple superficial melanoma lesions which in aggregate have a total diameter of ≥ 10 mm

**Nonmeasurable Lesions**: All other lesions, including small lesions (longest diameter < 10 mm by CT/MRI/ultrasound for nodal/soft tissue disease [including lymph nodes] or < 10 mm caliper measurement by clinical exam for superficial cutaneous melanoma lesion) and other truly nonmeasurable lesions are considered nonmeasurable and characterized as non-index lesions. This will include any measurable lesions beyond the maximum number of 5 lesions that were not chosen as index lesions.

**Follow-up “Index Lesions”**: At each subsequent tumor assessment, the SPD of the index lesions are added together to provide the total tumor burden.

**Follow-up “Non-index Lesions”**: Non-index disease measurements are not required and these lesions should be followed as “present”, “absent”, or in rare cases “unequivocal progression”.

**Follow-up “New Lesions”**: At each subsequent tumor assessment, if new measurable lesions have appeared they should be added to SPD of the index lesions to provide the total tumor burden. Any nonmeasurable new lesions should be followed as non-index lesions and described as “present”, “absent”, or in rare cases “unequivocal progression”.

**Definition of Overall Response Using irRC**

Overall response using irRC will be based on these criteria:
Immune-Related Complete Response (irCR): Complete disappearance of all tumor lesions (index and non-index together with no new measurable/unmeasurable lesions) for at least 4 weeks from the date of documentation of complete response.

Immune-Related Partial Response (irPR): The sum of the products of the two largest perpendicular diameters of all index lesions is measured and captured as the SPD baseline. At each subsequent tumor assessment, the sum of the products of the two largest perpendicular diameters of all index lesions and of new measurable lesions are added together to provide the Immune Response Sum of Product Diameters (irSPD). A decrease, relative to baseline of 50% or greater is considered an immune Partial Response (irPR). The decrease must be confirmed 4 weeks later.

Immune-Related Stable Disease (irSD): irSD is defined as the failure to meet criteria for immune complete response or immune partial response, in the absence of progressive disease.

Immune-Related Progressive Disease (irPD): It is recommended in difficult cases to confirm PD by serial imaging. Any of the following will constitute progressive disease:

- At least a 25% increase in the sum of the products of all index lesions and new measurable lesions (irSPD) over the nadir SPD calculated for the index lesions. PD must be confirmed 4 weeks later.

**Table 4: Immune-Related Response Criteria Definitions**

<table>
<thead>
<tr>
<th>Index Lesion Definition</th>
<th>Non-Index Lesion Definition</th>
<th>New Measurable Lesions</th>
<th>New Unmeasurable Lesions</th>
<th>Percent change in tumor burden (including measurable new lesions when present)</th>
<th>Overall irRC Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Response</td>
<td>Complete Response</td>
<td>No</td>
<td>No</td>
<td>-100%</td>
<td>irCR</td>
</tr>
<tr>
<td>Partial Response</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>≥ -50%</td>
<td>irPR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;-50% to &lt;+25%</td>
<td>irSD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;=+25%</td>
<td>irPD</td>
</tr>
<tr>
<td>Stable Disease</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>&lt;-50% to &lt;+25%</td>
<td>irSD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;=+25%</td>
<td>irPD</td>
</tr>
<tr>
<td>Progressive Disease</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>&gt;=+25%</td>
<td>irPD</td>
</tr>
</tbody>
</table>

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12.8 Correlative Assessments

- 35 mL of whole blood for tests of anti-tumor immune responses prior to start of therapy (pre polio boost and just before PVSRIPO injection #1), day 2, and day 10 after PVSRIPO injection #1, then at day 31, day 105, and 6 months after PVSRIPO injection #1. Immune monitoring samples may be analyzed by Dr. Smita Nair, Dr. Jing Li, or their designate in the Department of Immunology at Duke University. Peripheral blood mononuclear cells (PBMCs) will also be collected pre-treatment and at selected times post treatment. Analysis will take place in the laboratory of Smita Nair, PhD. To examine blood for inflammatory and immune signature using arrays and immune cell composition (antigen presenting cells, B cells and T cells), T cell activation by flow cytometry and B cell activation by ELISA and peptide arrays on day -7 (prior to polio vaccine booster), day -2 (prior to PVSRIPO), day 2, 10, and day 84 (after PVSRIPO), and in follow-up (if available).

- Tumor biopsy will be performed before treatment begins. If not already done, this biopsy will be for pathologic confirmation of melanoma. Tumor biopsy will take place prior to PVSRIPO injection (no less than 7 days prior), days 10 after each injection, day 84 after injection and additional biopsies, at the discretion of the PI. If surgical resection is performed, tumor from the specimen will be used after standard histologic assessment and may be used for any of the aforementioned correlative assessments with tissue. The pre-treatment and day 84 biopsy must include pathologic examination for presence of tumor. Additional tumor biopsies will examine histologic response. If additional tissue remains, it may be used for RNA immune signatures and possibly calculation of mutational burden.

- Tumor biopsy studies will include:

  1. **Detect presence of PVSRIPO activity in both injected and non-injected lesions.** Viral replication will be assessed in the laboratory of Matthias Gromeier using the following techniques: IHC and/or immunoblot detection of non-capsid viral protein (only produced during viral replication; not present in the virus inoculum), plaque assay detection of infectious PVSRIPO, and qRT-PCR for PVSRIPO negative strand RNA (only produced during viral replication; not present in the inoculum). Viral replication in non-injected lesions will be particularly important. If non-injected lesions demonstrate a clinical response, knowing if viral replication is occurring in non-injected lesions will help delineate if response is related to direct viral cytotoxicity or broader immune response.

  2. **Exam markers of lymphoid subsets, immune correlates and CD155 (poliovirus receptor) expression.** Tumor biopsy studies will include a panel of use of a multiplex IHC machine. The panel includes: CD4, CD8, PD-1, FoxP3, PD-L1, PVR (CD155), and TIGIT. These will be performed in a CLIA certified lab. CD155 expression will be important to verify PVSRIPO tumor tropism. T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is a candidate for novel immune checkpoints; CD155 is a ligand for TIGIT. The other markers will help elucidate the type of inflammation and changes in immune cell subtypes.

  3. **Histologic examination of tumors.** In our previous experience with in-transit melanoma, treated lesions to regional chemotherapy can flatten with remaining pigment. Although
pigment may remain, no viable tumor remains. Thus we will examine lesions at 84 days post injection for confirmation or absence of viable tumor cells.

4. RNA for interferon immune signatures. Given the importance of type I interferon production, we will examine interferon signatures in tumor specimens. Only if enough tissue remains will we do this analysis.

5. DNA isolation for calculation of mutational burden. Response to immune checkpoint inhibitors seems to correlate with higher mutational load, though studies have been inconsistent. In limited biopsies from 15 patients with recurrent GBM, a lower mutational load was associated with a clinical benefit after PVSRIPO treatment (unpublished). Only if enough tissue remains will we do this analysis. This is not a defined exploratory analysis of this trial but if tissue remains we may do this analysis at a later time point.

13 SAFETY MONITORING AND REPORTING

13.1 ADVERSE EVENTS

The PI is responsible for the identification and documentation of adverse events and serious adverse events, as defined below. At each study visit, the PI or designee must assess, through non-suggestive inquiries of the subject or evaluation of study assessments, whether an adverse event (AE) or serious adverse event (SAE) has occurred.

An adverse event (AE) is any untoward medical occurrence in a subject receiving study drug and which does not necessarily have a causal relationship with this treatment. For this protocol, the definition of AE also includes worsening of any pre-existing medical condition. An AE can therefore be any unfavorable and unintended or worsening sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of PVSRIPO, whether or not related to use of either drug. Abnormal laboratory findings without clinical significance (based on the PI’s judgment) should not be recorded as AEs. Laboratory value changes that require therapy or adjustment in prior therapy, or changes to the protocol treatment regimen are considered adverse events.

Events that do not meet the definition of an AE include:

- Medical or surgical procedures (e.g., endoscopy, appendectomy). The condition that leads to the procedure is, however, an AE.
- Situations where an untoward medical occurrence did not occur (e.g., social and/or convenience admission to a hospital).
- Anticipated day-to-day fluctuations of pre-existing diseases or conditions present or detected at the start of the study that do not worsen.

From the time the subject signs the informed consent form through the End of Study visit (as defined in Section 12.4), all AEs must be recorded in the subject’s medical record and adverse events case report form.
AEs will be assessed according to the CTCAE version 4. If CTCAE grading does not exist for an AE, the severity of the AE will be graded as mild (1), moderate (2), severe (3), life-threatening (4), or fatal (5).

Attribution of AEs will be indicated as follows:

- **Definite**: The AE is clearly related to the study drug
- **Probably**: The AE is likely related to the study drug
- **Possible**: The AE may be related to the study drug
- **Unlikely**: The AE is doubtfully related to the study drug
- **Unrelated**: The AE is clearly NOT related to the study drug

### 13.1.1 AEs related to study protocol.

See Section 9.3

### 13.1.2 Reporting of AEs

Any AE occurrence (spontaneously volunteered and enquired for, as well as observed AEs) during the study must be documented in the patient’s medical records in accordance with the Principal Investigator’s (PI’s) normal clinical practice and on the AE page of the eCRF. SAEs that occur during the study must be documented in the patient’s medical record, on the AE eCRF, and on the Sponsor’s provided SAE form.

The PI should attempt to establish a diagnosis of the event based on the signs, symptoms, and/or other clinical information. In such cases, the diagnosis should be documented as the AE (and SAE if serious) and not the individual signs/symptoms.

If a clinically significant abnormal laboratory finding or other abnormal assessment meets the definition of an AE, then the AE eCRF page must be completed as appropriate. In addition, if the abnormal assessment meets the criteria for being serious, the SAE form must also be completed. A diagnosis, if known, or clinical signs or symptoms if the diagnosis is unknown, rather than the clinically significant laboratory finding or abnormal assessment, should be used to complete the AE/SAE page. If no diagnosis is known and clinical signs or symptoms are not present, then the abnormal finding should be recorded. If an SAE report is completed, pertinent laboratory data should be recorded on the SAE form, preferably with baseline values and copies of laboratory reports.

The database of all adverse events (not just those considered related to the study drug) will be maintained in a 21 CFR Part 11 Compliant database. The event will be categorized by organ system, relationship to treatment, its grade of severity, and resolution. The PI and study statistician will periodically review the collective adverse events with the intention of identifying any trends or patterns in toxicity. If any such trends are identified, depending on their severity and frequency, a protocol amendment will be considered.

### 13.1.3 Serious Adverse Events

An AE is considered “serious” if it has one of the following outcomes:
13.1.4 Reporting of SAEs

All serious and unexpected adverse events should be reported immediately to the PI or his/her designee. Only adverse events that the PI determines to be serious, unanticipated, and related or possibly/probably (i.e. more likely than not) related to the research must be reported to the IRB. Those adverse events will be submitted to the IRB according the following guidelines:

- Report within 24 hours of learning about any subject’s death that was unanticipated and more likely related to the research than unrelated;
- Report within 5 business days of learning about any serious, unanticipated, and related or possibly/probably related AE;
- Report within 10 business days of learning about any other unanticipated problem or event that was more likely related to the research than unrelated. The Sponsor must report to the FDA, in an IND safety report, any suspected adverse reaction that is both serious and unexpected. Before submitting this report, the Sponsor needs to ensure that the event meets all three of the definitions contained in the requirement:
  - Suspected adverse reaction (i.e. there is a reasonable possibility that the drug caused the adverse event)
  - Serious
  - Unexpected

SAEs must be reported by the PI within 24 hours of discovery if the event occurs during the clinical study or within 28 days of receiving the study drug, whether or not the SAE is considered to be related to the study drug. Applicable forms provided by the Sponsor or designee must be transmitted via secure email to the Sponsor within 24 hours of discovery at pv_istari@klerv.com.

The PI should not wait to receive additional information to document fully the event before notification of an SAE, although additional information may be requested. Where applicable, information from relevant laboratory results, hospital case records, and autopsy reports should be obtained.

Instances of death, congenital abnormality, or an event that is of such clinical concern as to influence the overall assessment of safety, if brought to the attention of the PI at any time after cessation of study drug administration and linked by the PI to this study, should be reported to the Sponsor.

All SAEs must be reported to the IRB by the PI in accordance with the IRB regulations.
If the adverse event does not meet all three of the definitions, it should not be submitted as an expedited IND safety report. The Sponsor is required to report to the FDA all IND Safety reports in writing within 15 days (7 days for unexpected fatal or life-threatening suspected adverse reaction). All other adverse events will be reported to the FDA in the Annual Report.

13.2 **EMERGENCY UNBLINDING OF INVESTIGATIONAL TREATMENT**

Not applicable

13.3 **OTHER REPORTABLE INFORMATION**

Not applicable

13.4 **SPECIAL WARNINGS AND PRECAUTIONS**

Not applicable

13.5 **SAFETY OVERSIGHT COMMITTEE (SOC)**

Not applicable

13.6 **EXTERNAL DATA AND SAFETY MONITORING BOARD (DSMB)**

Due to a potential conflict of interest, an external DSMB-Plus was created for the phase 1 PVSRIPO clinical trial performed at Duke University Medical Center. The current study will be monitored by a DSMB. The DSMB will be responsible for safeguarding the interests of trial subjects and assessing the safety of the interventions during the trial. The DSMB will provide recommendations about stopping or continuing enrollment in the trial. To contribute to enhancing the integrity of the trial, the DSMB may also formulate recommendations relating to the selection, recruitment, and retention of subjects and their management. Additional details regarding the responsibility of the DSMB and its chair may be found in the charter document.

14 **QUALITY CONTROL AND QUALITY ASSURANCE**

14.1 **MONITORING**

This clinical research study will be monitored both internally by the PI and Sponsor (Istari) monitor or their designee, according to their respective SOPs. All monitor(s) will be qualified, trained on the study protocol and will be familiar with all study procedures. The procedures and plan outlined herein, are applicable to monitoring conducted only by Istari or their designee(s). In terms of internal review the PI will continuously monitor and tabulate adverse events. Appropriate reporting to the IRB of record will be made. If an unexpected frequency of Grade III or IV events occur, depending on their nature, action appropriate to the nature and frequency of these adverse events will be taken. This may require a protocol amendment, dose de-escalation, or potentially closure of the study. The PI of this study will also continuously monitor the conduct, data, and safety of this study to ensure that:

- Interim analyses occur as scheduled;
• Stopping rules for toxicity and/or response are met;
• Risk/benefit ratio is not altered to the detriment of the subjects;
• Appropriate internal monitoring of AEs and outcomes is done;
• Over-accrual does not occur;
• Under-accrual is addressed with appropriate amendments or actions;
• Data are being appropriately collected in a reasonably timely manner.

This Monitoring Plan also serves as SOP for the Sponsor (Istari)/Sponsor monitors or their designee(s) and is applicable to the Investigators and site study team. All Sponsor/Sponsor monitors obligations may be delegated to a qualified third party via a transfer of obligations (i.e., contract CRA or clinical research organization (CRO)) and the plan/procedure may be modified if obligations are transferred.

DEFINITIONS & ABBREVIATIONS:

a) MONITORING
The act of overseeing the progress of a clinical study, and of ensuring that it is conducted, recorded, and reported in accordance with the protocol, SOPs, Good Clinical Practice (GCP), and the applicable regulatory requirement(s).

b) Good Clinical Practice (GCP):
A standard for the design, conduct, performance, monitoring, recording, analyses, and reporting of clinical studies to ensure that the data and reported results are credible and accurate, and that the rights, integrity, and confidentiality of study subjects are protected.

c) Monitoring Report:
Is a written report listing the monitor’s findings from a monitoring visit (MV). This report will be sent to the PI and study team after each MV.

d) INVESTIGATOR SITE FILE (ISF)
The repository for the essential documents for the conduct of a clinical trial. These documents individually and collectively permit evaluation of the conduct of the study and the quality of the data produced. These documents demonstrate the compliance of the Sponsor-Investigator and of the monitor with standards of GCP and with all applicable regulatory requirements.

e) SOP (Standard Operating Procedure)
This is a document that describes the agreed procedures of a routine process.

SITE MONITORING SCHEDULE
Initiation, interim/routine and close out monitoring visits are planned by the Sponsor or their designee and will be conducted throughout the study. The site initiation visit will be conducted as soon as:

• All the necessary approvals have been obtained
- Staff recruited
- Investigational product has been delivered to site (is about to be delivered to site)
- CRF and source documents are ready
- Laboratory is ready to start receiving/processing/storing study samples, as applicable.

The first routine monitoring visit is anticipated to occur when the first participant is enrolled and within 60 days of enrollment. It is anticipated that routine/interim monitoring visits will occur after enrollment of every 3-4 subjects (within 60-90 days of enrollment) for an anticipated total of 2 to 3 interim monitoring visits during the enrollment period and again approximately every 6 to 12 months during the follow-up phase once all subjects are enrolled.

However, monitoring frequency will be continually assessed by the study management team, the Sponsor or their designee and the monitor. For example, the monitoring frequency may need to increase if recruitment is faster than predicted, at times of data entry deadlines (such as interim analysis or prior to a DSMB-plus scheduled safety report review), if there is an unexpected frequency of serious and/or unexpected toxicities based on the investigator’s brochure, or “for cause”, if critical issues are noted at previous MVs or for other issues. The monitor can also conduct a MV upon the request of the Sponsor (Istari), Site/Center Leadership, an Investigator, an IRB or DSMB-plus. The monitoring team, in cooperation with the requestor and Sponsor, will evaluate the MV request and determine whether the MV is appropriate and/or timely.

The close out visit will be scheduled after all subjects have completed the last follow-up visit and/or discontinued, all data are entered into the eCRF and all queries are resolved.

Additional monitoring may be prompted by findings from monitoring visits, unexpected frequency of serious and/or unexpected toxicities, or other concerns and may be initiated upon request of Duke University Health System (DUHS) and DCI leadership, the DCI Cancer Protocol Committee, the Safety Oversight Committee (SOC), the Sponsor or their designee, the Principal Investigator, or the IRB. All study documents must be made available upon request to other authorized regulatory authorities, including but not limited to the National Institute of Health, National Cancer Institute, and the FDA. Every reasonable effort will be made to maintain confidentiality during study monitoring.

### 14.2 Audits

The Sponsor or their designee and the Duke Office of Audit, Risk and Compliance (OARC) may conduct confidential audits to evaluate compliance with the protocol and the principles of good clinical practice (GCP). The Principal Investigator (PI) agrees to allow the auditor(s) direct access to all relevant documents and to allocate his/her time and the time of the study team to the OARC auditor(s) in order to discuss findings and any relevant issues.

Audits are designed to protect the right and well-being of human research subjects. Audits may be routine or directed (for cause). Routine audits are selected based upon risk metrics generally geared towards high subject enrollment, studies with limited oversight or monitoring, Investigator initiated Investigational Drugs or Devices, federally-funded studies, high degree of risk (based upon adverse
events, type of study, or vulnerable populations), phase 1 studies, or studies that involve Medicare populations. Directed audits occur at the directive of the IRB or an authorized Institutional Official.

OARC and Sponsor audits may examine research studies/clinical trials methodology, processes and systems to assess whether the research is conducted according to the protocol approved by the IRB of record. The primary purpose of the audit/review is to verify that the standards for safety of human subjects in clinical trials and the quality of data produced by the clinical trial research are met. The audit/review will serve as a quality assurance measure, internal to the institution. Additional goals of such audits are to detect both random and systemic errors occurring during the conduct of clinical research and to emphasize “best practices” in the research/clinical trials environment.

14.3 DATA MANAGEMENT AND PROCESSING

14.3.1 Case Report Forms (CRFs)

The electronic CRF (eCRF) will be the primary data collection document for the study and is developed in conjunction with statistical oversight. The CRFs will be updated in a timely manner following acquisition of new source data. Only the PI, the study coordinator, the data management team, and the clinical trials manager are permitted to make entries, changes, or corrections in the eCRF.

An audit trail will be maintained automatically by the electronic CRF management system. All users of this system will complete user training, as required or appropriate per DCI requirements and other regulations.

14.3.2 Data Management Procedures and Data Verification

Access to electronic databases will be managed by the Melanoma Data Manager.

Completeness of entered data will be checked automatically by the eCRF system and users will be alerted to the presence of data inconsistencies. Additionally, the data management team and the statistical team will cross-reference the data to verify accuracy. Missing or implausible data will be brought to the attention of the PI requiring an appropriate response (i.e., confirmation of data, correction of data, completion or confirmation that data is not available, etc.).

The database will be reviewed and discussed prior to database closure, and will be closed only after resolution of all remaining queries. An audit trail will be kept of all subsequent changes to the data.

14.3.3 Study Closure

Following completion of the studies, the study PI will be responsible for ensuring the following activities:

- Data clarification and/or resolution
- Accounting, reconciliation, and destruction/return of used and unused study drugs
- Review of site study records for completeness
- Shipment of all remaining laboratory samples to the designated laboratories.
15 STATISTICAL METHODS AND DATA ANALYSIS

All statistical analysis will be performed under the direction of the statistician designated in key personnel. Any data analysis carried out independently by the investigator must be approved by the statistician and Sponsor or their designee before publication or presentation.

15.1 ANALYSIS SETS

All patients who receive at least one injection of PVSRIPO will be included in safety analyses.

15.2 PATIENT DEMOGRAPHICS AND OTHER BASELINE CHARACTERISTICS

Socio-demographic and clinical characteristics of patients enrolled and treated on this study will be summarized. For categorical variables, frequencies and percentages will be provided. Means with standard deviations and medians/percentiles will summarize non-categorical variables.

15.3 TREATMENTS

The number of patients that receive PVSRIPO injection will be totaled.

15.4 PRIMARY OBJECTIVE AND METHOD OF ANALYSIS

A maximum of 9 patients will be treated on this study at an accrual rate of about 1-2 patients per month. The Primary Objective is to determine the safety profile of PVSRIPO in Stage IIIB, IIIC, and IV recurrent melanoma patients as determined by DLTs.

Three patients minimum will be injected with PVSRIPO. Please refer to Section 9.1.1 for dose escalation and reduction criteria.

Upon completion of the trial, all toxicities will be tabulated by type and grade according to dose cohort.

15.5 EXPLORATORY OBJECTIVES

One exploratory objective is to describe the response rates of injected versus non-injected lesions from PVSRIPO. This will be done by comparing cutaneous lesion measurements, descriptive measurements only will be obtained. We will estimate 95% confidence intervals for response rates.

Other Exploratory objectives of this study are to describe the number of CD8 positive T cells present on biopsy before and after injection of PVSRIPO, to determine the change in pathologic response of biopsies from baseline to 84 days, to determine changes in the tumor microenvironment and immune cell populations before and after injection of PVSRIPO. Immunotherapy can trigger an inflammatory immune response that is observed by presence of tumor infiltrating lymphocytes and therefore tumor biopsies at baseline, day 10, and day 84 will also be examined for tumor infiltrates.

Another exploratory objective is the assessment of immunologic changes stemming from the administration of PVSRIPO. Peripheral blood will be collected at defined intervals for correlative immune monitoring studies in serum and in peripheral blood monocytes. These include (i) analyses of innate and inflammatory immune events (HMGB1, inflammatory cytokines, NK and NKT-cells and
regulatory immune subsets (Tregs & MDSCs); (ii) analyses of adaptive immune responses (lineage, maturation, induction and activation/functional status of tumor antigen-specific T-cells).

15.6 INTERIM ANALYSIS
If any patient experiences an inflammatory reaction that does not improve within 116 days of PVSRIPO injection or commencing steroid treatment, the inflammatory reaction will be considered “irreversible.” If a patient experiences an “irreversible” inflammatory reaction, further enrollment of patients on the PVSRIPO trial will be temporarily interrupted and data on all previously treated patients will be carefully reviewed to determine if the study needs modification. Options may include dose reductions in future patients (next PVSRIPO dose would be $5 \times 10^7$ TCID$_{50}$, then $1 \times 10^7$ TCID$_{50}$), modification of the approach to treating a patient with an inflammatory reaction, study termination, or accrual continuation without modification.

The FDA and/or the DSMB for this study may be part of this decision-making in the event that there are unacceptable toxicities or inflammatory issues.

15.7 SAMPLE SIZE CALCULATION
We have chosen to have 9 patients participate in this pilot study.

16 ADMINISTRATIVE AND ETHICAL CONSIDERATIONS

16.1 REGULATORY AND ETHICAL COMPLIANCE
This protocol was designed and will be conducted and reported in accordance with the International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines for Good Clinical Practice, the Declaration of Helsinki, and applicable federal, state, and local regulations.

16.2 DUHS INSTITUTIONAL REVIEW BOARD AND DCI CANCER PROTOCOL COMMITTEE
The protocol, informed consent form, advertising material, and additional protocol-related documents must be submitted to the Institutional Review Board (IRB) of record and DCI Cancer Protocol Committee (CPC) for review. The study may be initiated only after the Principal Investigator has received written and dated approval from the CPC and IRB.

The Principal Investigator must submit and obtain approval from the IRB for all subsequent protocol amendments and changes to the informed consent form. The CPC should be informed about any protocol amendments that potentially affect research design or data analysis (i.e. amendments affecting subject population, inclusion/exclusion criteria, agent administration, statistical analysis, etc.).

The Principal Investigator must obtain protocol re-approval from the IRB within 1 year of the most recent IRB approval. The Principal Investigator must also obtain protocol re-approval from the CPC within 1 year of the most recent IRB approval, for as long as the protocol remains open to subject enrollment.
16.3 INFORMED CONSENT
The informed consent form must be written in a manner that is understandable to the subject population. Prior to its use, the informed consent form must be approved by the IRB.

The Principal Investigator or authorized key personnel will discuss with the potential subject the purpose of the research, methods, potential risks and benefits, subject concerns, and other study-related matters. This discussion will occur in a location that ensures subject privacy and in a manner that minimizes the possibility of coercion. Appropriate accommodations will be made available for potential subjects who cannot read or understand English or are visually impaired. Potential subjects will have the opportunity to contact the Principal Investigator or authorized key personnel with questions, and will be given as much time as needed to make an informed decision about participation in the study.

Before conducting any study-specific procedures, the Principal Investigator must obtain written informed consent from the subject or a legally acceptable representative. The original informed consent form will be stored with the subject’s study records, and a copy of the informed consent form will be provided to the subject. The Principal Investigator is responsible for asking the subject whether the subject wishes to notify his/her primary care physician about participation in the study. If the subject agrees to such notification, the Principal Investigator will inform the subject’s primary care physician about the subject’s participation in the clinical study.

16.4 STUDY DOCUMENTATION
Study documentation includes, but is not limited to, source documents, case report forms (CRFs), monitoring logs, appointment schedules, study team correspondence with sponsors or regulatory bodies/committees, and regulatory documents that can be found in the DCI-mandated “Regulatory Binder,” which includes, but is not limited to, approved protocol versions, approved informed consent forms, FDA Form 1572s, and CAP and CLIA laboratory certifications.

Source documents are original records that contain source data, which is all information in original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source documents include but are not limited to hospital records, clinical and office charts, laboratory notes, memoranda, subjects’ diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate copies, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories and at medico-technical departments involved in the clinical trial. DUHS utilizes Epic Maestro Care as an electronic health record. When possible, the original record should be retained as the source document. However, a photocopy is acceptable, provided that it is a clear, legible, and an exact duplication of the original document.

16.5 PRIVACY, CONFIDENTIALITY, AND DATA STORAGE
The Principal Investigator will ensure that subject privacy and confidentiality of the subject’s data will be maintained. Research Data Security Plans (RDSPs) will be approved by the appropriate institutional Site Based Research group.
To protect privacy, every reasonable effort will be made to prevent undue access to subjects during the course of the study. Prospective participants will be consented in an exam room where it is just the research staff, the patient and his family, if desired. For all future visits, interactions with research staff (study doctor and study coordinators) regarding research activities will take place in a private exam room. All research related interactions with the participant will be conducted by qualified research staff who are directly involved in the conduct of the research study.

To protect confidentiality, subject files in paper format will be stored in secure cabinets under lock and key accessible only by the research staff. Subjects will be identified only by a unique study number and subject initials. Electronic records of subject data will be maintained using a dedicated 21 CFR Part 11 compliant database (Medidata Rave), which is housed in an encrypted and password-protected file on a secure network drive. Access to electronic databases will be managed by the PRTBTC Data Manager. The security and viability of the IT infrastructure will be managed by the DCI and/or Duke Medicine.

Upon completion of the study, research records will be archived and handled per DUHS Human Research Protections Program (HRPP) and Sponsor policies. Subject names or identifiers will not be used in reports, presentations at scientific meetings, or publications in scientific journals.

16.6 DATA AND SAFETY MONITORING
Data and Safety Monitoring will be performed in accordance with the DCI Data and Safety Monitoring Plan.

16.7 PROTOCOL AMENDMENTS
All protocol amendments must be initiated by the Principal Investigator and approved by the IRB prior to implementation. IRB approval is not required for protocol changes that occur to protect the safety of a subject from an immediate hazard. However, the Principal Investigator must inform the IRB and all other applicable regulatory agencies of such action immediately.

16.8 RECORDS RETENTION
The Principal Investigator will maintain study-related records for the longer of a period of:

- at least two years after the date on which a New Drug Application is approved by the FDA
- at least two years after formal withdrawal of the IND associated with this protocol
- at least six years after study completion (Duke policy)

17 REFERENCES

20. Friedman CF, Navid-Azbariyan, P.; Shoushtari, A. N.; et al.: Toxicity associated with ipilimumab and nivolumab (Ipi+Nivo) combination therapy in melanoma patients (pts) treated at a single-institution under an expanded-access program (EAP). J Clin Oncol 34, 2016


## 18 APPENDICES

### 18.1 Staging Reference  AJCC 7TH EDITION – 2009

<table>
<thead>
<tr>
<th>T Classification</th>
<th>Thickness</th>
<th>Ulceration Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
| T1               | ≤ 1.0mm   | a: w/o ulceration and mitosis < 1 / mm²  
                        b: with ulceration and mitosis ≥ 1 / mm² |
| T2               | 1.01-2.0mm| a: w/o ulceration  
                        b: with ulceration |
| T3               | 2.01-4.0mm| a: w/o ulceration  
                        b: with ulceration |
| T4               | > 4.0mm   | a: w/o ulceration  
                        b: with ulceration |

<table>
<thead>
<tr>
<th>N Classification</th>
<th># of Metastatic Nodes</th>
<th>Nodal Metastatic Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No evidence of lymph node metastasis</td>
<td></td>
</tr>
</tbody>
</table>
| N1               | 1 node                | a: micrometastasis  
                        b: macrometastasis |
| N2               | 2-3 nodes             | a: micrometastasis  
                        b: macrometastasis  
                        c: In transit metastases/satellites without metastatic nodes |
| N3               | 4 or more metastatic nodes, or matted nodes, or in-transit metastases/satellites and metastatic nodes |

<table>
<thead>
<tr>
<th>M Classification</th>
<th>Site</th>
<th>Serum LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No evidence of metastasis to distant tissues or organs</td>
<td></td>
</tr>
<tr>
<td>M1a</td>
<td>Distant skin, subcutaneous or nodal metastases</td>
<td>Normal</td>
</tr>
<tr>
<td>M1b</td>
<td>Lung metastases</td>
<td>Normal</td>
</tr>
</tbody>
</table>
| M1c              | All other visceral metastases  
                        Or any distant metastases | Normal  
                        Elevated |
18.2 ECOG/ZUBROD PERFORMANCE STATUS SCALE

0  Asymptomatic and fully active.
1  Symptomatic; fully ambulatory; restricted in physical strenuous activity.
2  Symptomatic; ambulatory; capable of self-care; more than 50% of waking hours are spent out of bed.
3  Symptomatic; limited self-care; spends more than 50% of time in bed, but not bedridden.

18.3 SELECTED REGIONAL CLINICAL LIMB TOXICITY (CTCAE 4.03)

Table 5: Selected Regional Clinical Limb Toxicity (CTCAE 4.03)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Adverse Event: Erythroderma</th>
<th>Adverse Event: Skin Ulceration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Erythema covering &gt;90% BSA without associated symptoms; limiting instrumental ADL</td>
<td>Combined area of ulcers &lt; 1 cm; nonblanchable erythema of intact skin with associated warmth or edema</td>
</tr>
<tr>
<td>3</td>
<td>Erythema covering &gt;90% BSA with associated symptoms (e.g., pruritus or tenderness); limiting self care ADL</td>
<td>Combined area of ulcers 1 - 2 cm; partial thickness skin loss involving skin or subcutaneous fat</td>
</tr>
<tr>
<td>4</td>
<td>Erythema covering &gt;90% BSA with associated fluid or electrolyte abnormalities; ICU care or burn unit indicated</td>
<td>Combined area of ulcers &gt;2 cm; full-thickness skin loss involving damage to or necrosis of subcutaneous tissue that may extend down to fascia</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
<td>Any size ulcer with extensive destruction, tissue necrosis, or damage to muscle, bone, or supporting structures with or without full thickness skin loss</td>
</tr>
</tbody>
</table>

Death
<table>
<thead>
<tr>
<th>Grade</th>
<th>Adverse Event: Peripheral Motor Neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asymptomatic; clinical or diagnostic observations only; intervention not indicated</td>
</tr>
<tr>
<td>2</td>
<td>Moderate symptoms; limiting instrumental ADL</td>
</tr>
<tr>
<td>3</td>
<td>Severe symptoms; limiting self care ADL; assistive device indicated</td>
</tr>
<tr>
<td>4</td>
<td>Life-threatening consequences; urgent intervention indicated such as fasciotomy</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>Adverse Event: Peripheral Sensory Neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asymptomatic; loss of deep tendon reflexes or paresthesia</td>
</tr>
<tr>
<td>2</td>
<td>Moderate symptoms; limiting instrumental ADL</td>
</tr>
<tr>
<td>3</td>
<td>Severe symptoms; limiting self care ADL</td>
</tr>
<tr>
<td>4</td>
<td>Life-threatening consequences; urgent intervention indicated such as fasciotomy</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>Adverse Event: Myositis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mild pain</td>
</tr>
<tr>
<td>2</td>
<td>Moderate pain associated with weakness; pain limiting instrumental ADL</td>
</tr>
<tr>
<td>3</td>
<td>Pain associated with severe weakness; limiting self care ADL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>Adverse Event: Pain in Extremity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mild pain</td>
</tr>
<tr>
<td>2</td>
<td>Moderate pain; limiting instrumental ADL</td>
</tr>
<tr>
<td>3</td>
<td>Severe pain; limiting self care ADL</td>
</tr>
</tbody>
</table>