Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Supplementary Appendix

Oncolytic HSV-1 G207 Immunovirotherapy for Pediatric High-Grade Gliomas

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SUPPLEMENTARY APPENDIX

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Supplementary Methods

HSV G207:

The $\gamma_1$34.5 gene deletion precludes G207 from evading Protein Kinase R-mediated translational arrest in normal cells; however, G207 is capable of replicating in tumor cells due to altered or mutated signaling pathways that lead to non-functional Protein Kinase R and late viral gene expression.[1] The disruption of viral ribonucleotide reductase provides additional protection and further limits replication in normal cells, whereas proliferating tumor cells often have upregulated ribonucleotide reductase for DNA synthesis that the virus can utilize during replication in tumor cells.[2]

Trial Design:

Each cohort or dose level consisted of 3 participants, and if no dose-limiting toxicities occurred, the study was advanced to the next cohort until the maximum planned dose (cohort 4) was completed. If a dose-limiting toxicity occurred in any participant, 3 additional participants would be enrolled to that cohort. If two dose-limiting toxicities occurred at a dose level, the prior dose level immediately below would be considered the maximum tolerated dose and recommended Phase II dose. Thus, the sample size was up to 24 participants with a minimum of 12 participants needed to complete the maximum planned dose level with no dose-limiting toxicities. While on study, patients were monitored in the outpatient clinic at day 7, 14, 28; and at month 3, 5, 7, 9, 12, 18, 24. HSV-1 serology was performed at screening and outpatient visits starting at day 28. G207 shedding was assessed at baseline, day 1 and 3, and all outpatient visits. Complete blood counts with lymphocyte subsets were obtained at baseline, day 2, and all outpatient visits.

Catheter Placement and Virus Infusion:

Up to four intratumoral catheters (PIC-030, Sophysa) were placed stereotactically and exteriorized. The following day, G207 was infused over 6 hours using infusion tubing (PIT-400, Sophysa) connected to the catheters and microprocessor-controlled infusion pumps. Based on previous Good Laboratory Practices stability testing that showed stable virus activity for up to 8 hours, the 6-hour infusion time was selected to facilitate G207 spread throughout the tumor.

Imaging Analysis: Generation of Fraction Tumor Burden (FTB) maps:

We used a workstation equipped with Horos (Version 3.3.6; https://horosproject.org/) and commercially available plug-ins (IB Neuro, IB Rad Tech, IB Delta Suite, Version 2.0; Imaging Biometrics, Elm Grove, Wisconsin), which uses well-validated multistep process to calculate FTB in a semi-automated fashion. The multi-step process, in brief, includes a) generation of delta T1 maps, which are standardized difference

maps computed from the difference of co-registered pre- and postcontrast T1-weighted images; b) quantification of volume of contrast enhancement from the delta T1 map in a semiautomatic fashion and generation of a lesion mask of the enhancing volume; c) registration of the raw data of the perfusion sequence with the post contrast T1 weighted sequence; d) voxel-by-voxel calculation of relative cerebral blood volume (rCBV) and converting it into standardized rCBV; e) transfer of the contrast enhancing lesion mask to the standardized CBV; f) using predefined standardized rCBV thresholds, classification of the contrast enhancing lesion mask as FTB_{LOW} if percentage of contrast-enhancing voxels with standardized rCBV of ≤1.0, FTB_{MID}, if percentage of voxels with standardized rCBV between 1.0 and 1.55; and FTB_{HIGH} percentage of voxels with standardized rCBV of ≥1.55; and g) coding each FTB class in color (FTB_{LOW} = blue; FTB_{MID} = yellow; FTB_{HIGH} = red) and superimpose them on the post contrast T1 weighted sequence. FTB_{HIGH} correlates with global histologic tumor content and overall survival.[3-5]

Immunohistochemistry and Quantification:

Briefly, 4 μm sections were cut from formalin-fixed, paraffin-embedded block preparations. Immunostaining was performed using a fully automated immunostainer (Bond IHC stainer; Leica Biosystems; Buffalo Grove, IL). Clinical immunohistochemistry was performed with ready-to-use antibodies from Leica Biosystems against CD3 (clone: LN10), CD4 (clone: 4B12), CD8 (clone 4B11), CD20 (clone L26); CD138 (clone MI15), and HSV-1/2 (Abcam; monoclonal; clone number 3B6). Appropriate positive and negative control slides were prepared. The negative control slides consisted of tissue sections of each case processed without the addition of primary antibody. An experienced neuropathologist (RL) quantified CD3+, CD4+, CD8+, CD20+, and CD138+ cells in 6 random regions of interest from each matched pre- and post-G207 tissues. Data points were graphed, with a central-line representing the median value, in GraphPad Prism (version 8.4.2, GraphPad Software).

Polymerase Chain Reaction:

G207 and HSV-1 F strain and the patient's saliva sample were used for preparation of DNA. DNA was purified with the QIAamp Ultrasens Virus (Qiagen, Cat#53704) according to the manufacturer's instruction. DNA was purified using DNeasy columns. Taq PCR Master Mix (Qiagen, Cat# 201443) was used with the following thermal cycler conditions: Initial denaturation for 3 min, 94°C; denaturation for 1 min, 94°C; annealing for 1 min, 56°C; extension for 1 min, 72°C; and final extension for 10 min, 72°C. After PCR, products were mixed with 6x sample solution (Biolabs Cat# B70255) and loaded in 1.5% gel (LE Agarose Cat# BMA5000). DNA ladder (100BP) was used for standard. Pictures of PCR gels were taken. All the primers were purchased from Sigma-Aldrich. The following primers were used: G207-F1: CGT CCC AAC CGC ACA GTC; G207-F2: CAG TCC CAG GTA ACC CTT GT; G207-Rev2: ATA CCG

GGG TTG CCC ATT AAG; LacZ-Fwd1: CAA TTT AAC CGC CAG TCA GG; LacZ- Fwd2: ATG GCG ATT ACC GTT GTT GAT GT; LacZ-Rev1: GGC CTC TTC GCT ATT ACG C; LacZ- Rev2: TGG AAA TCG CTG ATT TGT GT; UAB Primer1: GTC CCG CCG AAC GCA TAC AT; UAB Primer2: CAT ACT TCA GGG CCG ATT GC.

Statistical Analyses:

To compare CD3+, CD4+ or CD8+ cells per high-power field in 6 random regions of interest for both matched pre- and post-treatment tissue, a two-tailed unpaired Student's t-test was performed and differences in the median values between post- and pre-treatment samples with 95% confidence intervals were calculated in GraphPad Prism. To assess the potential role of pre-treatment, baseline (Day -2) peripheral blood NK cell numbers on post-G207 survival, Spearman correlation was performed for all subjects (n = 8) and subjects who had died (n = 6). Patients seropositive for HSV-1 at baseline were excluded due to the potential for HSV-1 antibodies limiting G207 infection. Scatterplots of NK cells at baseline as a function of time from G207 treatment were created and the best-fit line was computed.

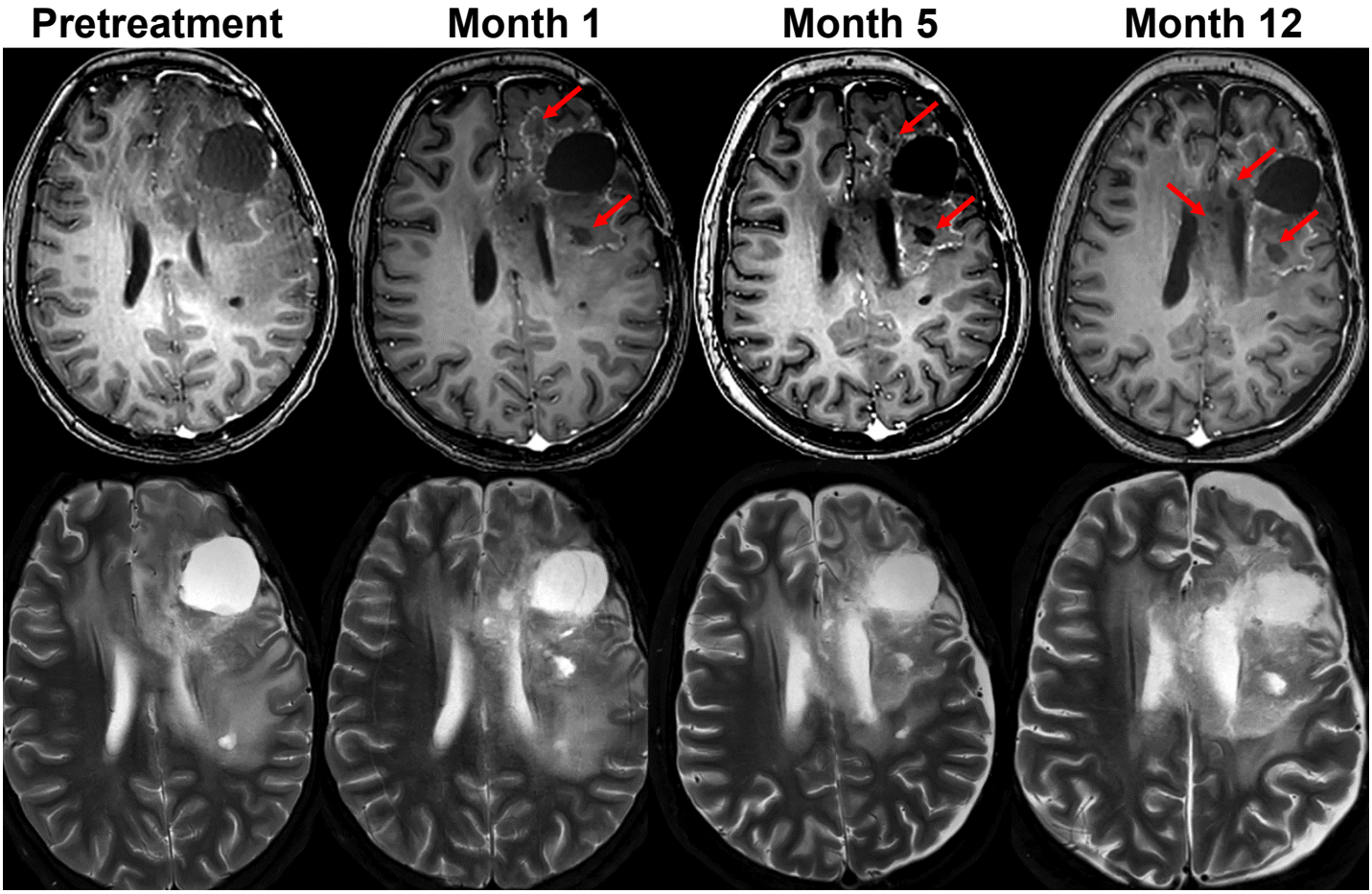

Figure S1. Response in patient 005 treated with G207. Top Row: Post-contrast T1-weighted images show stable area of enhancement with formation of multiple cysts (red arrows) where G207 was inoculated. (Bottom Row) Axial T2 sequence shows decreased signal abnormality over time.

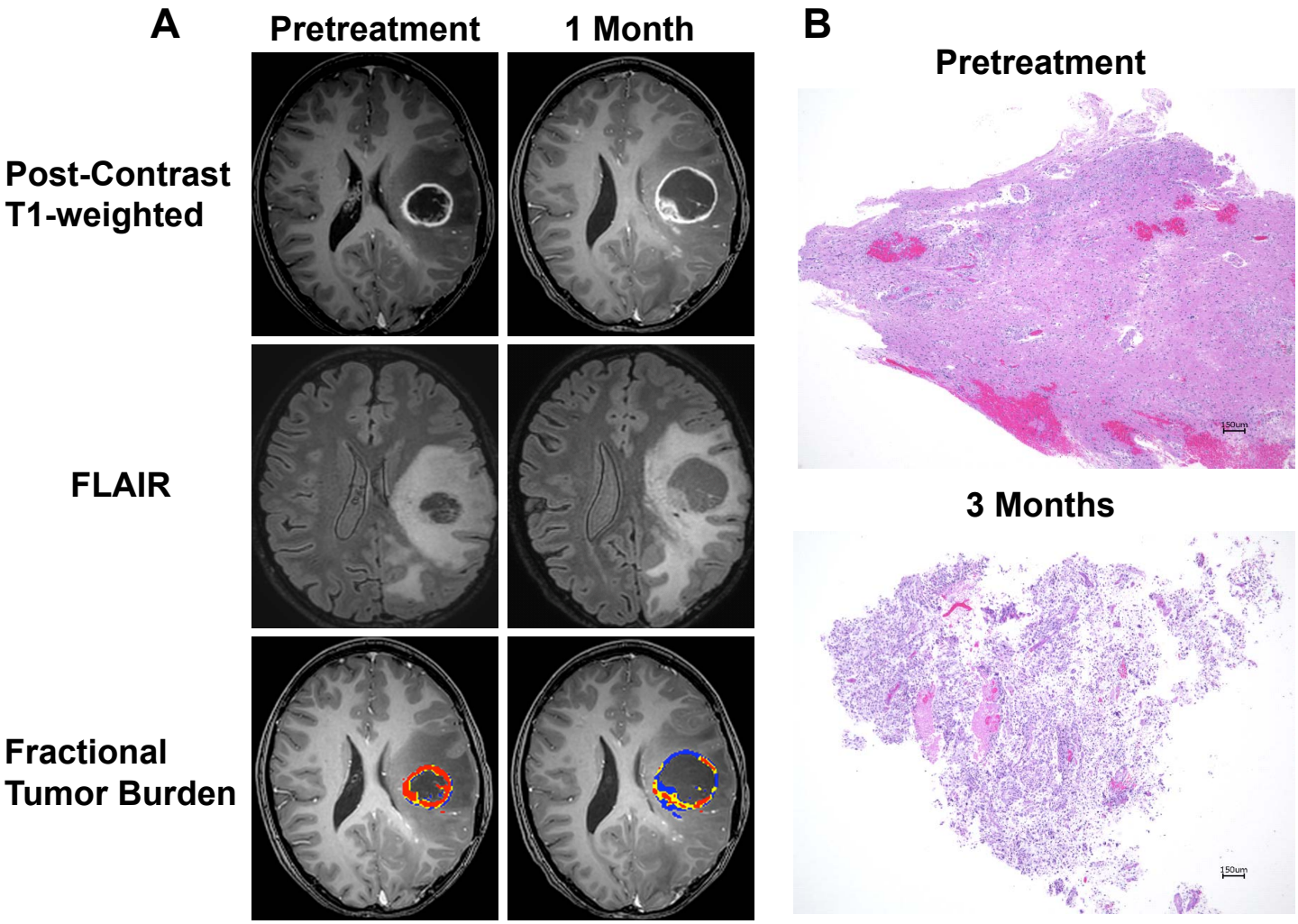

Figure S2. Response in patient 007 treated with G207. Panel A shows the comparison of axial images from pretreatment to 1 month post-G207. Post-contrast T1-weighted images show interval enlargement of the enhancing component of the tumor with increase in the central necrotic component and thinning of the enhancing margin. FLAIR images show worsening of the peri-enhancing FLAIR abnormal areas. Fractional Tumor burden (FTB) maps, a parametric perfusion MRI map used to differentiate between tumor (FTB_{HIGH}; red color) and treatment effect (blue color), show a decrease of FTB_{HIGH} from 63% to 38% from pre- to post-G207 treatment. Panel B shows hematoxylin and eosin stains of pretreatment and 3-month post-G207 biopsy tissue. There were changes in the architecture after G207; the tissue was friable and semi-liquefied. Scale bar = 150 μM.

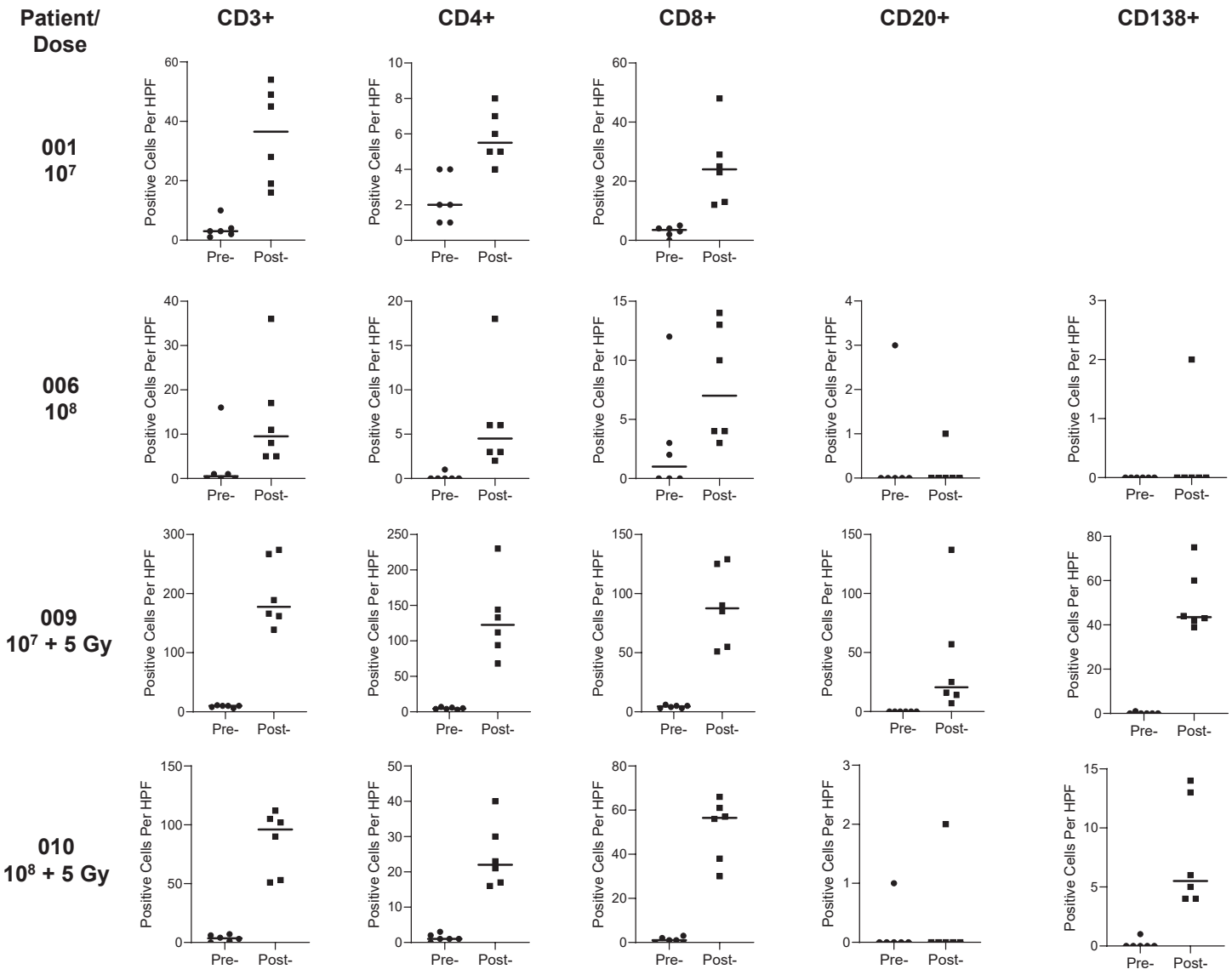

Figure S3. Quantification of CD3+, CD4+, CD8+ CD20+, and CD138+ per high-power field (HPF) in matched pre- and post-G207 tissues. Positive cells for CD3, CD4, CD8, CD20 and CD138 were counted by a trained neuropathologist in 6 random HPF (40x) regions of interest in each tissue sample. The data is shown as a scatter plot with the central line representing the median. CD20 and CD138 staining were not performed on patient 001 due to biopsy tissue being exhausted.

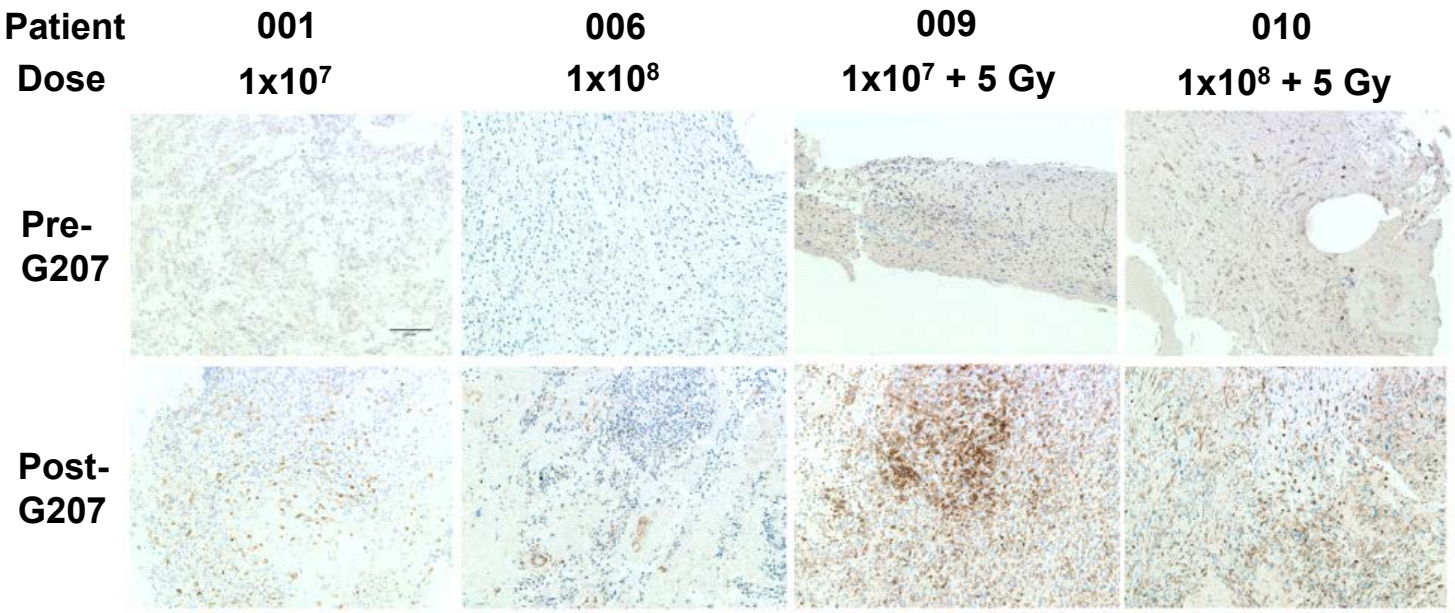

Figure S4. Immunohistologic staining for CD4+ helper T lymphocytes pre- and post-G207 treatment in 4 patients. (Top row) The initial core biopsies prior to G207 administration demonstrate few CD4+ cells. **(Bottom row)** Between 3-6 months after G207, tumor tissues from the same 4 subjects revealed a brisk infiltration of CD4+ cells. 10x magnification. Scale bar = 150 μM.

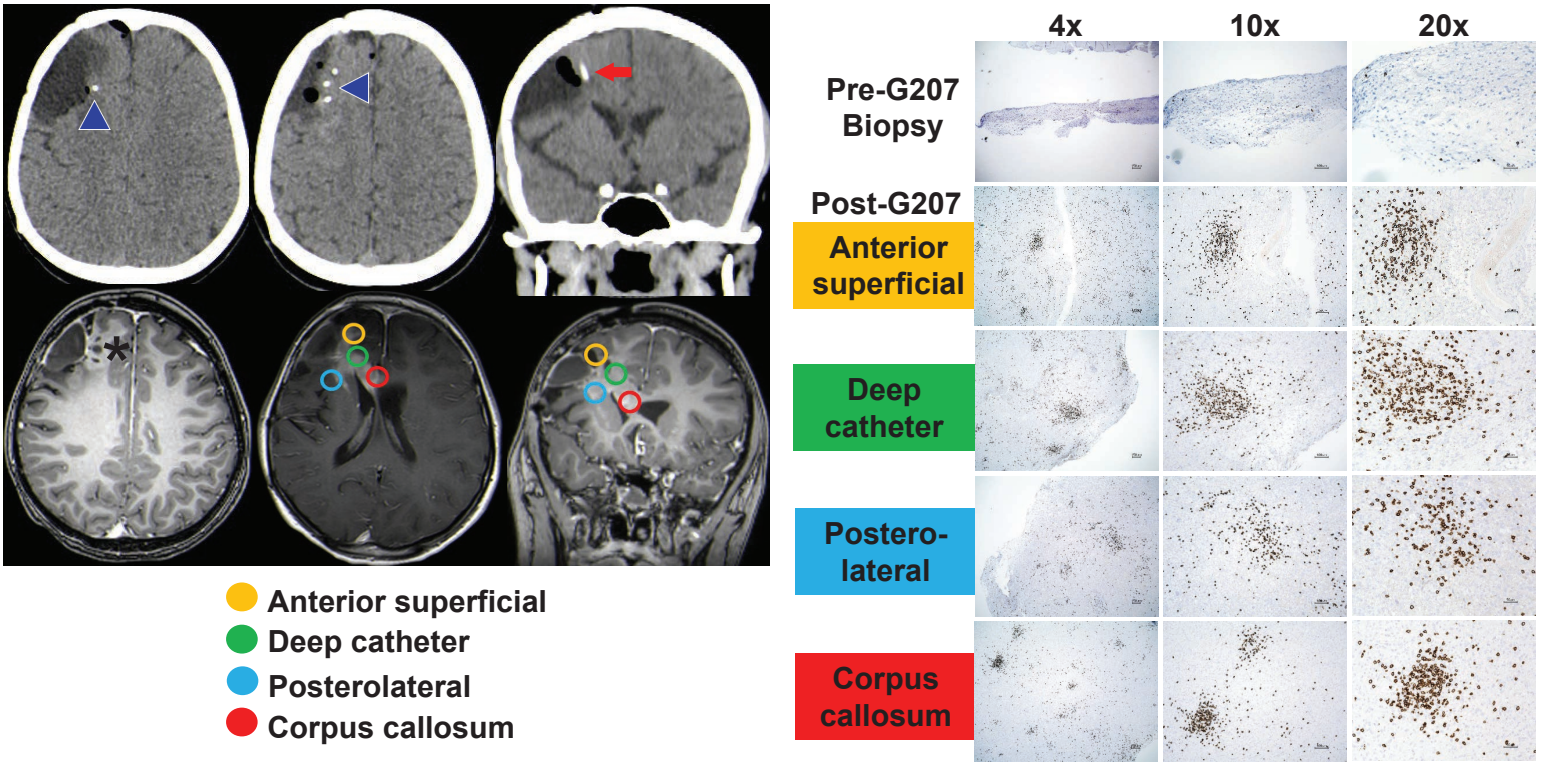

Figure S5. Immune response throughout tumor in areas adjacent and distant from G207 inoculation in patient 009. The top row of the left panel shows CT scan, which demonstrates placement of four catheters: 3 superficial (blue arrow heads) and one deep (red arrow). The bottom row of the left panel shows the MRI 5 months after G207 prior to resection. Left image shows cystic changes where G207 was infused (black asterisk). The middle image shows wedge shaped contrast enhancement. Because of uncertainty whether this represented pseudoprogression or progression, tumor resection was performed. Color coded areas in the images indicate regions that were marked during resection for pathological assessment. The right panel shows Immunohistochemistry photomicrographs (4X, 10X and 20X magnification) for CD8+ cytotoxic T cells from tissue obtained from the color coded tissue regions. Large clusters of CD8+ cells were seen in all areas including several centimeters from the initial G207 inoculation site (corpus callosum).

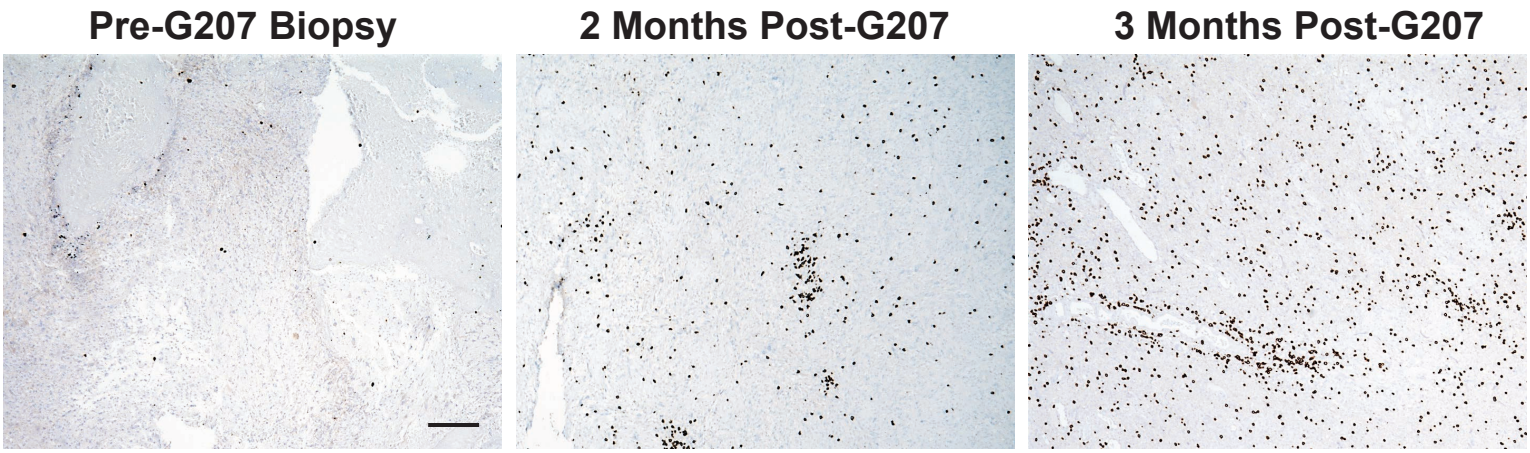

Figure S6. Neuropathologic response in patient 010 treated with G207 at 1x10^8 pfu followed by a single 5 Gy dose of radiation. Immunohistochemistry for CD8+ T cells was performed on pre-treatment biopsy tissue and on resection tissue at 2 and 3 months post-treatment. Pre-treatment tissue shows few CD8+ T cells consistent with a “cold tumor”. Post-treatment tissue revealed that G207 induces a robust CD8+ T cell response by 2 months that is increased in magnitude at 3 months post-G207 treatment indicating the tumor shifting from immunologically ‘cold’ to ‘hot’. This occurred even in the setting of severe lymphopenia (absolute lymphocyte count of 499) at screening prior to G207. Scale bar = 300 μM.

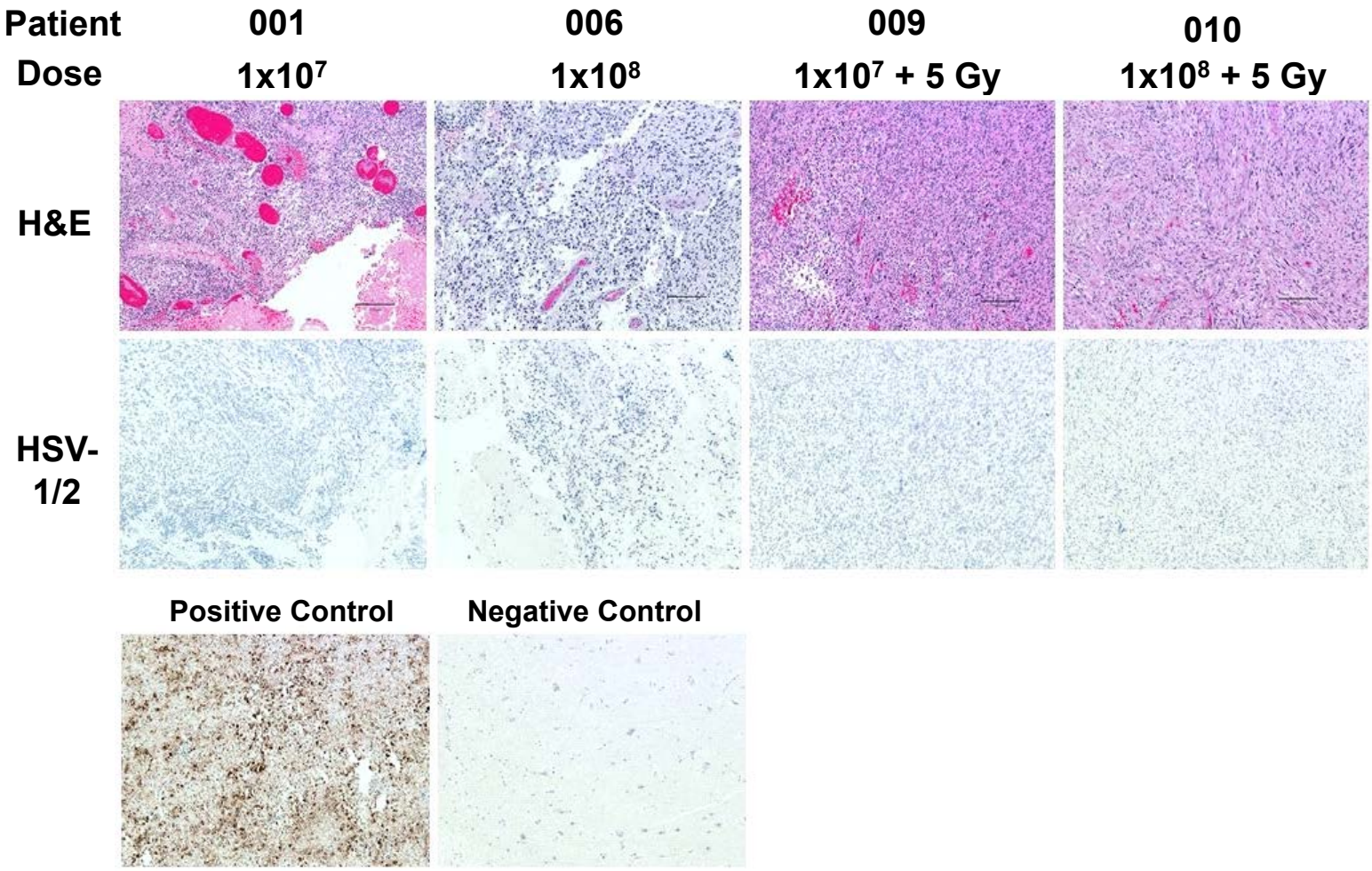

Figure S7. Hematoxylin and eosin (H&E) and HSV-1/2 stains of tumor tissue 3 months (patient 001 and 010) and 5 months (patient 006 and 009) after G207. All tissues were negative for HSV-1 staining indicating there was not active G207 infection. 10x magnification. Scale bar = 150 μM.

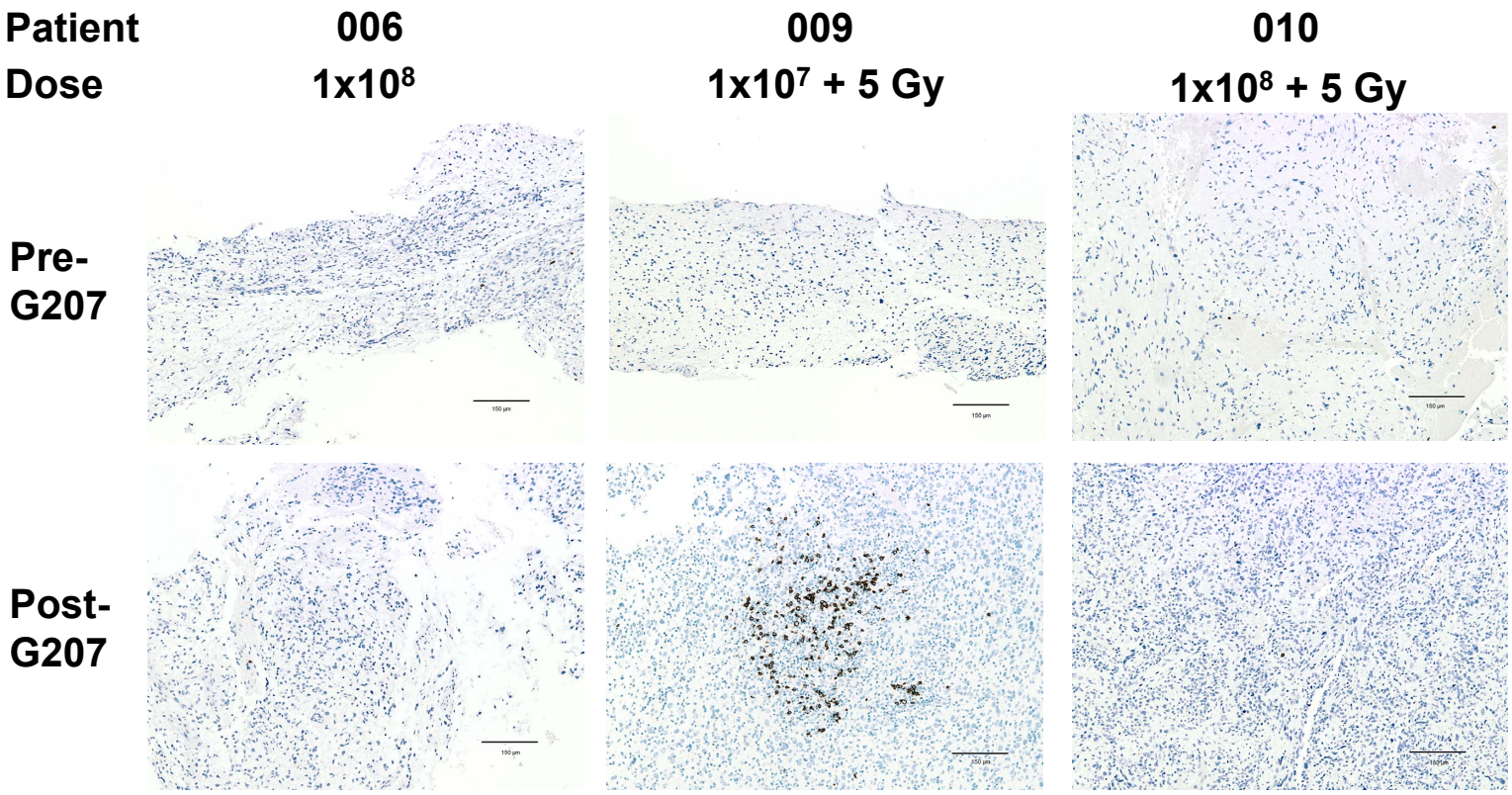

Figure S8. Immunohistologic staining for CD20+ B lymphocytes pre- and post-G207 treatment in 3 subjects. (Top row) The initial core biopsies prior to G207 administration demonstrate few CD20+ cells. **(Bottom row)** Infiltration of CD20+ cells was seen in subject 009 5 months after G207 but not in subject 006 or 010 **(**Between 3-6 months after G207. 10x magnification. Scale bar = 150 μM.

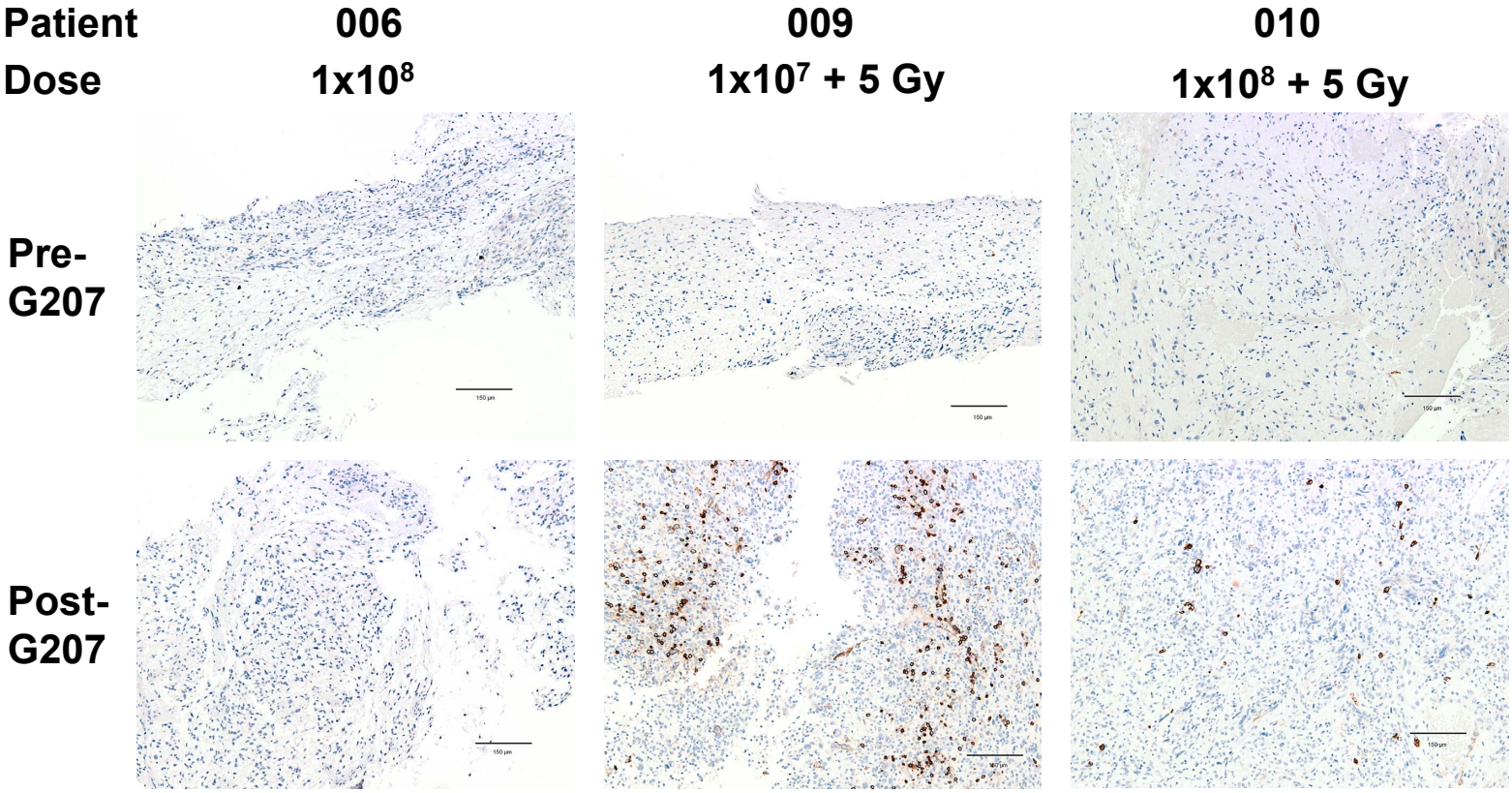

Figure S9. Immunohistologic staining for CD138+ plasma cells pre- and post-G207 treatment in 3 subjects. (Top row) The initial core biopsies prior to G207 administration demonstrate few CD138+ cells. **(Bottom row)** Infiltration of CD138+ cells was seen in subject 009 and 010 at 5 and 3 months after G207, respectively, but not in subject 006 6 months after G207. 10x magnification. Scale bar = 150 μM.

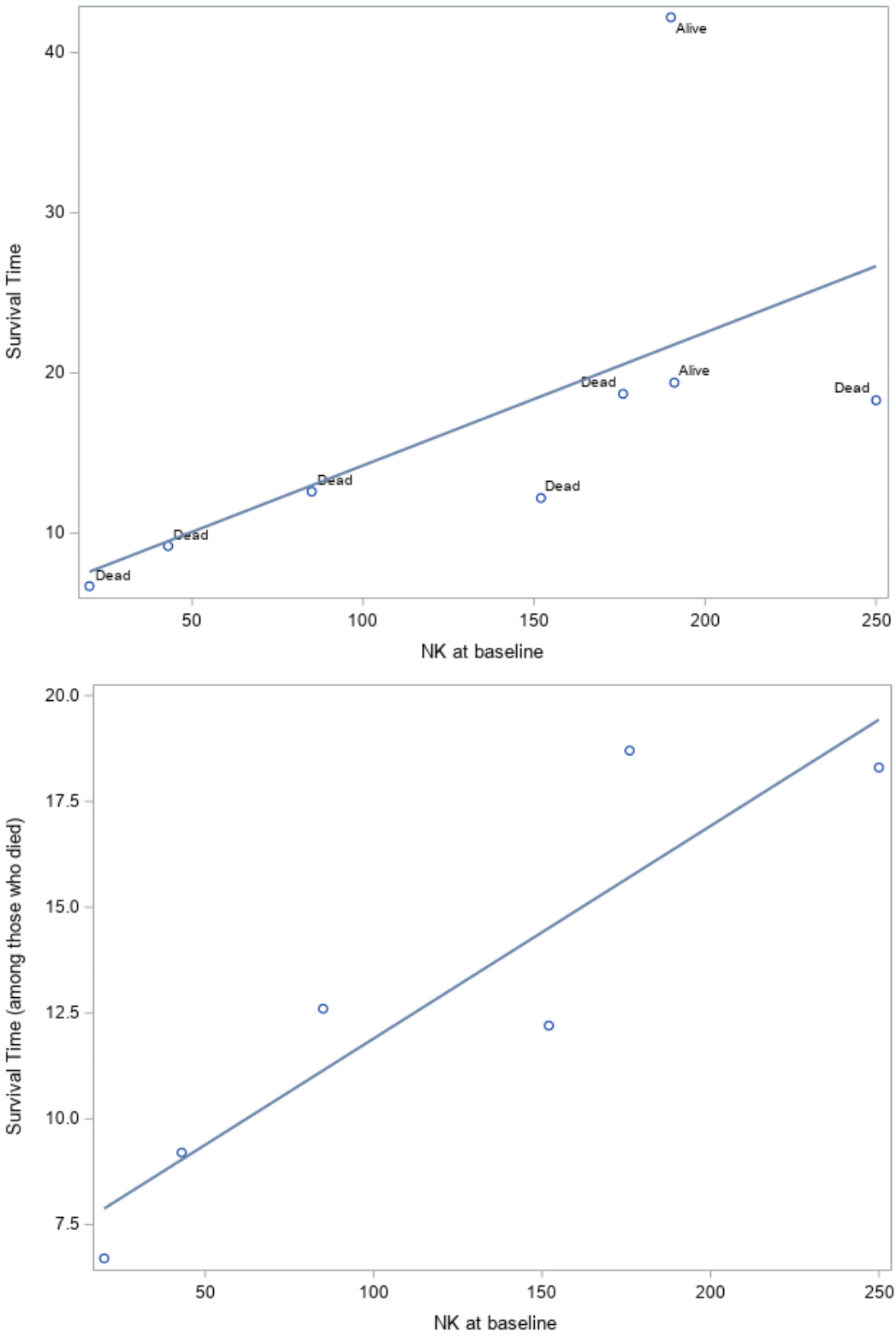

Figure S10. Scatterplots of peripheral blood absolute NK cells at baseline as a function of survival time in months from G207 treatment. (Top panel) All subject (n=8). (Bottom panel) Subjects who had died (n=6). Patients seropositive for HSV-1 at baseline were excluded due to the potential for HSV-1 antibodies limiting G207 infection. The best fit line was computed. Spearman R = 0.81 for all subjects and R = 0.88 for subjects who had died.

Table S1. Inclusion and exclusion criteria for UAB 1472 (NCT02457845)

Inclusion Criteria	Exclusion Criteria
• Age ≥ 36 months and < 19 years • Pathologically proven malignant supratentorial brain tumor (including glioblastoma multiforme, giant cell glioblastoma, anaplastic astrocytoma, primitive neuroectodermal tumor, ependymoma, atypical teratoid/rhabdoid tumor, germ cell tumor, or other high-grade malignant tumor) which is progressive or recurrent despite standard care including surgery, radiotherapy, and/or chemotherapy. A pathologically proven secondary malignant tumor without curative treatment options is eligible • Lesion must be ≥ 1.0 cm in diameter and surgically accessible as determined by MRI • Patients must have fully recovered from acute treatment related toxicities of all prior chemotherapy, immunotherapy or radiotherapy prior to entering this study. • Myelosuppressive chemotherapy: patients must have received their last dose at least 3 weeks prior (or at least 6 weeks if nitrosourea) • Investigational/Biologic agents: patients must have recovered from any acute toxicities potentially related to the agent and received last dose ≥ 7 days prior to entering this study (this period must be extended beyond the time during which adverse events are known to occur for agents with known adverse events ≥ 7 days). For viral therapy, patients must have received viral therapy ≥ 3 months prior to study entry and have recovered from all acute toxicities potentially related to the agent • Monoclonal antibodies: patient must have received last dose ≥ 21 days prior. • Radiation: Patients must have received their last fraction of craniospinal radiation (> 24 Gy) or total body irradiation ≥ 3 months prior to study entry. Patients must have received focal radiation to symptomatic metastatic sites or local palliative radiation ≥ 28 days prior to study entry. • Autologous bone marrow transplant: Patients must be ≥ 3 months since transplant prior to study entry. • Normal hematological, renal and liver function (Absolute neutrophil count > 1000/mm3, Platelets > 100,000/mm3, PT or PTT < 1.3 x control,	• Any treatment outside the allowable guidelines in the inclusion criteria • Acute infection, granulocytopenia or medical condition precluding surgery • Pregnant or lactating females • Prior history of encephalitis, multiple sclerosis, or other CNS infection • Tumor involvement which would require ventricular, cerebellar or brainstem inoculation or would require access through a ventricle in order to deliver treatment • Required steroid increase within 1 week prior to injection • Known HIV seropositivity • Concurrent therapy with any drug active against HSV (acyclovir, valacyclovir, penciclovir, famciclovir, ganciclovir, foscarnet, cidofovir) or any immunosuppressive drug therapy (except dexamethasone or prednisone).

Creatinine within normal institutional limits OR creatinine clearance >60 mL/min/1.73 m2 for patients with creatinine levels above institutional normal, Total Bilirubin < 1.5 mg/dl, Transaminases < 3 times above the upper limits of the institutional norm) • Patients < 16 years, Modified Lansky score ≥ 60; patients ≥ 16 years, Karnofsky score ≥ 60 • Patient life expectancy must be at least 8 weeks • Written informed consent in accordance with institutional and FDA guidelines must be obtained from patient or legal guardian	

Table S2. Patient demographics, tumor characteristics, and treatment summary

Subject ID	Age/ Gender	Diagnosis/ Tumor Location	Tumor Size (mm)	Prior Failed Therapies	Catheters Infused	Dose (pfu)
001	12 Female	Glioblastoma Right parietal; occipital	35 x 32	(1) Surgery/radiation/temozolomide (2) Vemurafenib (3) Lomustine/bevacizumab	3	10^7
002	17 Male	Anaplastic astrocytoma Left frontal; insular	51 x 19	(1) Surgery/radiation/temozolomide (2) Surgery	3	10^7
003	14 Female	Glioblastoma Left fronto-temporal; insular	43 x 30 10 x 19 (satellite)	(1) Surgery/radiation/temozolomide/valproic acid (2) Surgery/ re-irradiation (3) Lomustine/procarbazine	4	10^7
004	13 Male	Glioblastoma Left mesial occipital; posterior temporal	39 x 37	(1) Surgery/radiation (2) Surgery/vincristine/etoposide/ cisplatin/cyclophosphamide (3) Irinotecan/temozolomide/bevacizumab (4) Etoposide/sirolimus/celecoxib	4	10^8
005	12 Male	Glioblastoma Left frontal	67 x 57	(1) Surgery/radiation/temozolomide (2) Nivolumab/bevacizumab	4	10^8
006	10 Male	Glioblastoma Right temporal; thalamus	24 x 38	(1) Surgery/radiation/cisplatin/ cyclophosphamide/lomustine/vincristine	4	10^8
007	16 Female	Glioblastoma Left parietal; frontal	33 x 25 12 x 8 (satellite)	(1) Surgery/radiation/temozolomide	3	10^7 5 Gy
008	7 Female	Glioblastoma Left frontal; corpus callosum	49 x 22	(1) Surgery/radiation/temozolomide/lomustine (2) Surgery/lenvatinib/everolimus (3) Surgery	4	10^7 5 Gy
009	10 Female	Glioblastoma Right frontal	24 x 12	(1) Surgery/radiation/temozolomide/lomustine	3	10^7 5 Gy
010	18 Female	Glioblastoma Left temporal	30 x 16 18 x 9 (satellite)	(1) Surgery/radiation/temozolomide/lomustine	4	10^8 5 Gy
011	17 Male	Glioblastoma Right frontal	34 x 15 (Anterior) 26 x 8 (Posterior)	(1) Surgery/radiation/veliparib/temozolomide (2) Re-irradiation/temozolomide	4	10^8 5 Gy
012	15 Male	High-grade glioma, not-otherwise specified Right frontal; Sylvian fissure leptomeningeal disease	33 x 22	(1) Surgery (2) Surgery/radiation/temozolomide/lomustine	3	10^8 5 Gy

Table S3. Tumor genetic features

Subject ID	Comprehensive Genomic Profiling*	Timing of Report	Genetic Features
001	No	Diagnosis	*ATRX* loss; *BRAF V600E* mutation; *IDH* wild-type; MGMT unmethylated
002	No	Diagnosis	*IDH* wild-type; *BRAF V600E* negative; *ATRX* wild-type
003	No	First recurrence	*IDH* wild-type; *H3K27M* negative
004	FoundationOne®	Diagnosis	*MLL* R2204Q; *RB1* Q597fs*4; *TP53* R175H
005	FoundationOne®	Diagnosis	*KDR*, *KIT*, *PDGFRA* amplification; *CDKN2A/B* loss
	MSK-IMPACT Solid Tumor Testing	Diagnosis	Same as above and *TEK* loss, *STAG2* splicing mutation, *RHOA* missense mutation; P*DGFRA* rearrangement results in exon 10 deletion MGMT unmethylated
006	FoundationOne®	Diagnosis	*PTEN* M1fs*1; *CDKN2A/B* loss; *PTPN11* G60A; *KMT2C (MLL3)* deletion exons 54-55; microsatellite status stable; tumor mutation burden low (1 per megabase); MGMT unmethylated
007	FoundationOne®	Diagnosis	No reportable genomic alterations; MGMT unmethylated
008	MD Anderson Cancer Center Solid Tumor Genomic Assay 2018	Recurrence (pre-G207)	*CDK4* mutation; *MDM2* mutation; MGMT unmethylated
009	FoundationOne®	Diagnosis	*RAF1* GPHN-RAF1 rearrangement; *ATRX* C235fs*22; *CDKN2A/B* loss; *TP53* R282W
		Recurrence (post-G207)	*RAF1* GPHN-RAF1 fusion; *ATRX* C235fs*22; *MTAP* loss exons 2-8; *TP53* R282W; microsatellite status stable; tumor mutational burden low (4 per megabase)
010	FoundationOne®	Diagnosis	*NF1* R2583fs*13; *ATRX* splice site 6326+1G>A; *CDKN2A/B* loss; *MTAP* loss exons 2-8; *TP53* R110L; microsatellite status stable; tumor mutation burden low (0 per megabase); MGMT unmethylated
011	FoundationOne®	Diagnosis	*ATRX* K183*; *BCL6* R459H; *TP53* H179Y, H214fs*4; *CDKN2A* p16INK4A R80*; *CDKN2A* p14ARF P94L; *H3F3A* G35R subclonal microsatellite status stable; tumor mutational burden low (3 per megabase)
	MSK-IMPACT Solid Tumor Testing	Diagnosis	Same as above and *NOTCH1* F357del, *PDGFRA* G286E; tumor mutational burden 6.1 per megabase
012	FoundationOne®	First recurrence	*BCOR* rearrangement intron 1; *EP300* truncation exon 31

*FoundationOne® assay interrogated 315-324 genes as well as introns of 28-36 genes involved in rearrangements

MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets) identifies specific mutations in 468 genes

MD Anderson Cancer Center Solid Tumor Genomic Assay 2018 is a next generation sequencing-based analysis for detection of somatic mutations in the coding sequence of 134 genes and selected copy number variations (amplifications) in 47 genes (overlap: 146 genes total)

Table S4. Performance score, supportive care, subsequent treatments, time at which iRANO progression criteria was met, overall survival by subject

Subject ID	Dose Level	Performance Score at Screening	Steroids Post-G207 Dose (~Month Started)	Bevacizumab Post-G207 Dose (~Month Started)	Therapy Post-G207 (~Month Started)	iRANO Progression Criteria Met (months)	Overall Survival (months)
001	1	80	Prednisone 30mg twice daily (0.5 months)	None	Partial resection (3 months) Palliative radiation (4 months)	1.2	8.7
002	1	80	None	None	None	—	42.2; alive
003	1	80	Dexamethasone 1mg daily-4mg 4x/day (3 months)	5mg/kg (3 months)	None	4.6	6.7
004	2	90	None	None	Partial resection (2 months) Palliative radiation (3 months)	1.2	12.2
005	2	70	Dexamethasone 0.5mg daily-2mg 3x/day (2 months)	5-10mg/kg (2 months)	SurVaxM (15 months)	14.3	18.3
006	2	100	None	5mg/kg (2 months)	Palliative radiation, TMZ (7 months) IT methotrexate x 3 doses (12 months) Ramucirumab (16 months)	5.9	18.7
007	3	90	None	5-10mg/kg (1 month)	Palliative radiation (3.5 months) Lomustine (5 months) Indoximod, TMZ (7 months)	2.8	9.2
008	3	100	None	5mg/kg (1 month)	Indoximod, TMZ (4 months) Palliative radiation (4 months)	3.0	5.1
009	3	100	None	5-10mg/kg *(9 months)	Partial resection (5 months) Pembrolizumab x 10 doses (5 months) Palliative radiation (6 months) Trametinib (13 months)	4.8	19.4; alive
010	4	100	None	None	Partial resection (2 & 3 months) Palliative radiation (4 months) Pembrolizumab x 1 dose (5 months) Everolimus (9 months)	2.8	12.6
011	4	80	None	None	None	2.3	3.8
012	4	100	None	None	None	—	1.7; alive

*Patient 009 received bevacizumab as supportive care 9 months post-G207 when no longer on study and after initiating other therapies

—, patient did not progress; TMZ, temozolomide

Table S5: Frequency of adverse events attributable to G207

Event	Body System	Grade 1	Grade 2	Grade 3	Grade 4	Total
Diarrhea	Gastrointestinal	1				1
Nausea	Gastrointestinal	2				2
Vomiting	Gastrointestinal	2				2
Chills	General Disorder	1				1
Fatigue	General Disorder	2				2
Fever	Body as a Whole	4				4
Anorexia	Metabolism/Nutrition	1				1
Dizziness	Nervous System	1				1
Headache	Nervous System	2				2
Seizure	Nervous System	2				2
Hemorrhage, post-operative	Nervous System	2				2
	Total	**20**	**0**	**0**	**0**	**20**

Table S6. Cumulative adverse events by patient receiving G207 alone

Subject ID	Event	System Organ Class	Baseline grade	CTCAE grade	Days Post-G207	Relationship to G207	Total
001	Anemia	Blood and lymphatic system disorders		1	0	Unrelated	1
	Bradycardia	Cardiac disorders		1	1	Unlikely	1
	Diarrhea	Gastrointestinal disorders		1	5	Unlikely	2
	Vomiting		1	2	12	Unlikely	
	Fever	General disorders		1	0	Possibly	1
	Other, perirectal abscess	Infections and Infestations		3	29	Unrelated	1
	Postoperative hemorrhage	Injury, poisoning and procedural complications		1	3	Possibly	2
	Other, pseudomeningocele			3	84	Unrelated	
	Alanine aminotransferase increased	Investigations	1	3	12	Unlikely	5
	Aspartate aminotransferase increased		1	2	12	Unlikely	
	Neutrophil count decreased			1	0	Unrelated	
	Platelet count decreased			1	0	Unrelated	
	White blood cell decreased		1	2	0	Unrelated	
	Facial nerve disorder	Nervous system disorders		1	12	Unlikely	8
	Facial nerve disorder			1	84	Unrelated	
	Headache		1	2	12	Unlikely	
	Headache		1	2	53	Unlikely	
	Lethargy			2	53	Unlikely	
	Other, peritumoral vasogenic edema			2	12	Unlikely	
	Other, peritumoral vasogenic edema			3	53	Unlikely	
	Other, possible seizure			1	84	Unrelated	
002	Skin infection	Infections and Infestations		3	163	Unrelated	1
	Obesity	Metabolism and nutrition disorders	2	3	14	Unrelated	1
	Skin disorder	Skin and subcutaneous tissue disorders		1	561	Unrelated	1
003	Anemia	Blood and lymphatic system disorders	2	3	0	Unrelated	1
	Bradycardia	Cardiac disorders		2	111	Unrelated	1
	Blurred vision	Eye disorders		1	49	Unrelated	2
	Eye pain			1	-1	Unrelated	
	Constipation	Gastrointestinal disorders		1	140	Unrelated	5
	Diarrhea			1	12	Unlikely	
	Diarrhea			1	23	Unrelated	
	Vomiting			2	11	Unlikely	
	Vomiting			2	23	Unlikely	
	Fatigue	General disorders		1	11	Unlikely	1
	Postoperative hemorrhage	Injury, poisoning and procedural complications		1	3	Possibly	1
	Alanine aminotransferase increased	Investigations	1	2	28	Unrelated	7

	Aspartate aminotransferase increased		1	2	2	Unrelated	
	Lymphocyte count decreased		2	3	28	Unlikely	
	Neutrophil count decreased		1	2	7	Unlikely	
	Neutrophil count decreased		1	3	14	Unlikely	
	Platelet count decreased		1	1	28	Unlikely	
	White blood cell decreased		2	3	14	Unlikely	
	Anorexia	Metabolism and nutrition disorders		1	11	Unlikely	1
	Cognitive disturbance	Nervous system disorders		2	91	Unlikely	3
	Depressed level of consciousness			4	111	Unlikely	
	Headache		1	2	48	Unlikely	
	Cough	Respiratory, thoracic and mediastinal disorders		1	140	Unrelated	1
004	**Nausea**	Gastrointestinal disorders		2	33	Unrelated	3
	Vomiting			1	1	Unrelated	
	Vomiting			1	33	Unrelated	
	Fever	General disorders		1	1	Possibly	1
	Postoperative hemorrhage	Injury, poisoning and procedural complications		1	0	Unrelated	1
	Headache	Nervous system disorders	1	2	33	Unrelated	2
	Lethargy			1	35	Unrelated	
005	**Hypertension**	Cardiac disorders	1	2	141	Unrelated	2
	Hypertension		1	3	253	Unrelated	
	Cushingoid	Endocrine disorders		1	99	Unrelated	1
	Abdominal pain	Gastrointestinal disorders		1	30	Unrelated	2
	Nausea			1	1	Possibly	
	Fever	General disorders		1	8	Possibly	1
	Postoperative hemorrhage	Injury, poisoning and procedural complications		1	0	Unrelated	1
	Other, vitamin D deficiency	Investigations		2	226	Unrelated	2
	Weight gain			1	99	Unrelated	
	Anorexia	Metabolism and nutrition disorders		1	7	Possibly	7
	Hyperglycemia			2	143	Unrelated	
	Hyperglycemia			2	253	Unrelated	
	Hypoalbuminemia			1	140	Unrelated	
	Hypomagnesemia			1	140	Unrelated	
	Hypophosphatemia			1	140	Unrelated	
	Obesity			3	98	Unrelated	
	Muscle weakness right-sided	Musculoskeletal and connective tissue disorders	2	3	99	Unrelated	1
	Cerebrospinal fluid Leak	Nervous system disorders		2	8	Unrelated	5
	Dizziness			1	1	Possibly	
	Dysphasia		1	2	354	Unrelated	
	Headache		1	2	253	Unrelated	

	Seizure			1	139	Unrelated	
	Depression	Psychiatric disorders		1	99	Unrelated	1
	Sore throat	Respiratory, thoracic and mediastinal disorders		1	34	Unrelated	3
	Upper respiratory infection			1	141	Unrelated	
	Upper respiratory infection			2	258	Unrelated	
006	**Other, chalazion**	Eye disorders		1	162	Unrelated	1
	Constipation	Gastrointestinal disorders		1	1	Unlikely	6
	Diarrhea			1	72	Unlikely	
	Nausea			1	86	Unrelated	
	Nausea			1	177	Unrelated	
	Vomiting			1	1	Possibly	
	Vomiting			1	87	Unrelated	
	Fatigue	General disorders		1	0	Possibly	1
	Headache	Nervous system disorders		1	12	Possibly	3
	Headache			1	21	Possibly	
	Paresthesia			1	148	Unlikely	
						Total	**91**

Table S7. Cumulative adverse events by patient receiving G207 + 5 Gy radiation

Subject ID	Event	System Organ Class	Baseline grade	CTCAE grade	Days Post-G207	Relationship to G207	Total
007	Other, peripheral vision loss	Eye disorders		1	82	Unlikely	2
	Blurred Vision			1	82	Unlikely	
	Nausea	Gastrointestinal disorders		1	5	Possibly	3
	Vomiting			1	5	Possibly	
	Diarrhea			1	49	Unlikely	
	Dysphasia	Nervous System disorders		1	72	Unlikely	3
	Headache		1	2	91	Unlikely	
	Paresthesia		1	1	63	Unlikely	
008	Fever	General disorders		1	2	Possibly	1
009	Otitis media	Infections and Infestations	1	1	100	Unrelated	1
	Postoperative hemorrhage	Injury, poisoning and procedural complications		1	3	Unlikely	1
	Seizure	Nervous system disorders	1	1	4	Possibly	1
	Upper respiratory infection	Respiratory, thoracic and mediastinal disorders		1	111	Unrelated	1
	Skin Disorder	Skin and subcutaneous tissue disorders		1	14	Unlikely	1
010	External ear inflammation	Ear and labyrinth disorders		2	1	Unlikely	1
	Postoperative hemorrhage	Injury, poisoning and procedural complications		1	3	Unlikely	1
	Platelet count decreased	Investigations		1	14	Unlikely	1
	Seizure	Nervous system disorders		1	17	Possibly	1
	Other, contact dermatitis	Skin and subcutaneous tissue disorders		2	1	Unlikely	1
011	Fatigue	General disorders		1	28	Possibly	1
012	Bradycardia	Cardiac disorders		1	0	Unlikely	5
	Bradycardia			1	29	Unlikely	
	Tachycardia			1	-1	Unrelated	
	Hypertension			1	-1	Unrelated	
	Hypotension			1	3	Unlikely	
	Diarrhea	Gastrointestinal disorders		1	4	Possibly	1
	Chills	General disorders		1	5	Possibly	1
	Alanine aminotransferase increased	Investigations		1	-2	Unrelated	5
	Hemoglobin increased			1	-2	Unrelated	
	Lymphocyte count decreased			1	-1	Unrelated	
	Weight loss			1	15	Unlikely	
	White blood cell decreased			1	-2	Unrelated	
	Other, hyperphosphatemia	Metabolism and nutrition		1	7	Unlikely	1
	Headache	Nervous system disorders		1	0	Unrelated	1
						Total	**34**

Table S8. Cumulative summary of serious adverse events

Patient ID	Day Post-G207	Duration (days)	SAE Description	Body System	Causality	Comments	Outcome
001	29	2	Hospitalization for perirectal abscess	Skin and subcutaneous tissue disorders	Unrelated	Treated with antibiotics. HSV PCR on drainage negative	Resolved
001	53	3	Hospitalization for headache and lethargy and noted to have enlargement of lesion on MRI with edema and midline shift	Nervous system disorder	Unlikely related	Underwent removal of lesion and pathology showed areas of necrosis and residual tumor	Resolved
001	84	2	Hospitalization with facial paresthesia and possible seizure. Found to have pseudomeningocele	Nervous system disorder	Unrelated	Had surgery to correct pseudomeningocele from day 53 surgery	Resolved
002	163	9	Hospitalization for herpes zoster of right leg and foot	Skin and subcutaneous tissue disorders	Unrelated	HSV-1 and HSV-2 PCR negative; varicella PCR +	Resolved
003	111	4	Hospitalized for altered mental status, vomiting, bradycardia, probable seizure	Nervous system disorder	Unlikely related	Symptoms improved with steroids, anti-epileptics	Resolved
005	8	4	Hospitalized for a single fever to 101°F and cerebrospinal fluid leak (CSF)	Nervous system disorder	Unrelated	Stitch oversewn; CSF culture negative. CSF HSV PCR 5480 copies. No additional leak	Resolved
005	139	5	Hospitalized for new onset seizures	Nervous system disorder	Unrelated	Clinically improved with anti-epileptics	Resolved

Table S9: Difference in median positive cells in six high-powered field regions of interest for each patient's post-treatment compared to pre-treatment tissue samples

Subject #	CD3+ (95% CI)	CD4+ (95% CI)	CD8+ (95% CI)	CD20+ (95% CI)	CD138+ (95% CI)
001	33.5 (14, 48)	3.5 (1, 6)	20.5 (9, 43)	Not performed	Not performed
006	9 (4, 20)	4.5 (2, 17)	6 (1, 13)	0 (-2, 0)	0 (-2, 0)
009	167.5 (133, 263)	83 (48, 123)	118 (65, 223)	20.5 (7, 137)	43.5 (39, 74)
010	92.5 (48, 105)	21 (15, 37)	55.5 (30, 63)	0 (0, 1)	5.5 (4, 13)

See figure S3 for graphical representations

Table S10. Serologic responses to single dose of intratumoral G207

Patient	G207 Dose	HSV-1 IgG Baseline	HSV-1 IgG Post-G207	Month Serology Last Checked/ Month Patient Seroconverted	Overall Survival
001	10^7	+	+	1	8.7
002	10^7	-	-	24	42.2; alive
003	10^7	-	-	5	6.7
004	10^8	-	-	1	12.2
005	10^8	-	+	3	18.3
006	10^8	-	+	5	18.7
007	10^7	-	-	3	9.2
008	10^7	+	+	3	5.1
009	10^7	-	-	5	19.4; alive
010	10^8	-	+	1	12.6
011	10^8	+	+	1	3.8
012	10^8	-	-	1	1.7; alive

+, positive; -, negative; red color signifies patients that seroconverted; blue color signifies patients that had baseline antibodies to HSV.

Table S11. Peripheral blood counts at baseline and day 7, 14 and 28 after treatment

Patient	Baseline					Absolute Activated T Cells				CD4/CD8 Ratio				Overall Survival
	WBC	ANC	ALC	Absolute T cells	Absolute NK Cells	Baseline	Day 7	Day 14	Day 28	Baseline	Day 7	Day 14	Day 28	
1	3.4	2240	750	416	29	82	84	69	191	1.14	0.9	0.73	1.01	8.7
2	4.9	3080	1190	707	190	134	105	83	55	0.37	0.37	0.4	0.42	42.2; alive
3	2.4	1560	600	495	20	98	110	105	67	1.1	1.1	1.72	1.47	6.7
4	4.1	2140	1520	1171	152	125	68	72	48	1.21	1.18	1.1	1.11	12.2
5	9.2	5570	2780	1508	250	139	120	67	136	1.1	1.15	0.82	1.14	18.3
6	5.1	2730	1600	1059	176	149	137	100	180	1.16	1.38	1.36	1.26	18.7
7	4.6	2810	1430	1143	43	90	119	181	96	0.72	0.79	0.65	0.55	9.2
8	5.7	2130	3020	2072	574	284	68	231	180	0.7	0.96	0.97	1.23	5.1
9	5.4	2590	1740	1200	191	17	25	47	87	1.33	1.5	1.52	1.23	19.4; alive
10	2.7	1840	499	324	85	15	50	66	31	0.79	0.52	0.87	0.93	12.6
11	4.5	2630	1440	1156	86	29	29	ND	52	1.36	1.26	ND	1.2	3.8
12	4.3	1930	1750	1825	ND	ND	ND	ND	ND	0.99	1.24	ND	1.03	1.7; alive

ALC, absolute lymphocyte count; ANC, absolute neutrophil count; ND, no data; NK, natural killer; WBC, white blood count

Supplementary References

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