A multi-institutional phase 1 clinical trial exploring upfront multimodal standard of care and combined immunotherapies for newly diagnosed glioblastoma

Patrick Y Wen, Andrea Manzanera, Caroline Duault, Edgar Gonzalez-Kozlova, Lenika Lopez, Stuart A Grossman, Xiaobu Ye, Joy Fisher, Ian Lee, Tobias Walbert, James Snyder, Steven Brem, Arati Desai, Stephen J Bagley, Chandana Kakani, Roy Strowd, Stephen Tatter, Adrian Laxton, Glenn Lesser, Nduka Amankulor, Frank Lieberman, Jan Drappatz, Megan Mantica, Dan Triggs, Cara Haymaker, Ignacio I Wistuba, Gheath al-Atrash, Julia Mendoza Perez, Andrew Futreal, Latasha D Little, M D Habibul Islam, Dzifa Duose, Peixin Jiang, Alexandre Reuben, Sean E Lawler, Mina Pichavant, Andrew Gentles, Sean Bendall, Alex Kong, Christine Camacho,

A multi-institutional phase 1 clinical trial exploring upfront multimodal standard of care and combined immunotherapies for newly diagnosed glioblastoma

Patrick Y. Wen[1,2], Andrea Manzanera[3,] Caroline Duault[4], Edgar Gonzalez-Kozlova[5], Lenika Lopez[3], Stuart A. Grossman[2,6], Xiaobu Ye[2,6], Joy Fisher[2,6], Ian Lee[7], Tobias Walbert[7], James Snyder[7], Steven Brem[8], Arati Desai[8], Stephen J. Bagley[8], Chandana Kakani[8], Roy Strowd[9], Stephen Tatter[9], Adrian Laxton[9], Glenn Lesser[9], Nduka Amankulor[8], Frank Lieberman[10], Jan Drappatz[10], Megan Mantica[10], Dan Triggs[1,11], Cara Haymaker[12], Ignacio I Wistuba[12], Gheath al-Atrash[12], Julia Mendoza Perez[12], Andrew Futreal[12], Latasha D. Little[12], MD Habibul Islam[12], Dzifa Duose[12], Peixin Jiang[12], Alexandre Reuben[12], Sean E. Lawler[1,11,13], Mina Pichavant[4], Andrew Gentles[4], Sean Bendall[4], Alex Kong[4], Christine Camacho[4], Diane Del Valle[5], Seunghee Kim-Schulze[5], Sacha Gnjatic[5], Elad Sharon[14], M. Oskar Nowicki[1,11], Pierpaolo Peruzzi[1,11], Doug Lane[3], Estuardo Aguilar-Cordova[3], Laura K. Aguilar[3], Garrett Nichols[3], Jessica Dwyer[3], Paul Peter Tak[3], Holden Maecker[4], Francesca Barone[3*], E. Antonio Chiocca[1,11,*]

[1]*Center for Neuro-oncology, Dana Farber Cancer Institute, Boston, MA.*

[2]*Adult Brain Tumor Consortium, Baltimore, MD.*

[3]*Candel Therapeutics, Needham, MA,*

[4]*Stanford University, Stanford, CA*,

[5]*Mount Sinai, New York, NY*,

[6]*Department of Neurosurgery, Division of Neuro-oncology Johns Hopkins University Medical Center, Baltimore, MD.*

[7]*Department of Neurosurgery, Henry Ford Health Sciences Center, Detroit, MI.*

[8]*Department of Neurosurgery, Glioblastoma Translational Center of Excellence, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.*

[9]*Department of Neurosurgery, Wake Forest Health Sciences Center, Salem, NC.*

[10]*Hillman Cancer Center, Departments of Neurology and Medicine, University of Pittsburgh Medical School, Pittsburgh, PA;*

[11]*Department of Neurosurgery, Brigham and Women's Hospital and Center for Nervous System Tumors, Mass General Brigham, Boston, MA.*

[12]*Departments of Translational Molecular Pathology (CH, IIW, JMP, DD), Department of Stem Cell Transplantation Ga-A), Dept of Genomic Medicine and Sheikh Khalifa Bin Zayed Al Nahyan Institute, (AF) Dept of Genomic Medicine (LDL, MHI), MD Anderson Cancer Center, Houston, TX.*

[13]*Brown University, Providence, RI.*

[14]*Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA.*

*These two authors equally contributed to the manuscript as co-senior authors. Correspondence should be sent to: Dr. E.A. Chiocca, Center for Nervous System Tumors, Mass General Brigham Cancer Institute and Department of Neurosurgery, Mass General Brigham, Boston, MA 02115, EAChiocca@mgb.org

Summary: This multi-institutional clinical trial for patients with newly diagnosed high grade glioma treated with 2 immunotherapies and standard of care discovers molecular and immunologic signatures in subjects with improved survival.

Abstract

Background: For newly diagnosed glioblastoma (GBM), combination of surgical upfront immunotherapy with aglatimagene besadenovec (CAN-2409), followed by chemoradiation and then adjuvant nivolumab has not been tested. The aim of this study was to test the safety of this regimen and determine metrics of immune activation that may correlate with clinical outcomes.

Methods: 41 patients with suspected newly diagnosed GBM by imaging were enrolled in this multi-institutional, open label, phase 1b clinical trial before surgical resection. Frozen section confirmation of high-grade glioma was required for administration of aglatimagene besadenovec. This was then followed with chemoradiation and adjuvant nivolumab. Tumor and blood were assayed for genetic and immune markers before and during treatment.

Results: The regimen was well tolerated and generated measurable immune activation. Factors linked to survival were identified, such as baseline mutated gene pairs (e.g. *MED15/ HRC),* tumor immune cell composition, and changes in systemic cytokine, immune cells, and T cell diversity. The most significant serial systemic immune changes were observed in a long-term survivor subset of patients with gross total resection (GTR)/ methylated methylguanine methyltransferase (MGMT) promoter tumors. Median overall survival (mOS) in these patients was 30.6 months, while it was less for patients with unmethylated or subtotal resections.

Conclusions: These findings suggest the opportunity for patient stratification and the potential for more durable antitumor immune responses in future clinical trials of this multimodal standard of care and combined immunotherapy regimen. ClinicalTrials.gov identifier: NCT03576612.

Key words: Clinical trial, gene therapy, immunotherapy, brain tumor, glioma..

Key points:

- Two immunotherapies with standard of care were safe in 41 newly diagnosed GBM/HGG.
- Genomics and tumor immune cell composition at baseline associated with outcomes.
- Blood changes in cytokines, immune cells, and T cell diversity linked with outcome.

Importance of the study: Previously we have shown that the immunotherapy, aglatimagene besadenovec (CAN-2409), administered in newly diagnosed GBM/HGG at the time of surgical resection was well tolerated and in a prospective phase 2 cohort-matched study had shown preliminary evidence of efficacy. Mouse studies had shown evidence that immune-evasion via the PD-1/ PD-L1 pathway was likely. This provided the immunologic rationale to add a PD-1 checkpoint inhibitor. In this current trial, conducted via the American Brain Tumor Consortium (ABTC) multi-institutional group, the regimen was safe and well tolerated. We further evaluated the presence of possible tissue and blood correlates of outcome.

INTRODUCTION

High-grade gliomas (HGG) are malignant primary brain tumors characterized by poor prognosis[1]. The most aggressive subtype is glioblastoma (GBM), affecting more than 13,000 new cases in the US annually[1]. Hypermethylation of the promoter for methylguanine methyltransferase (MGMT) is a biomarker associated with better response to treatment and improved survival[2]. Despite optimal therapy with standard of care (SoC) surgery, radiotherapy, temozolomide (TMZ) chemotherapy, and tumor-treating fields (TTF), the median overall survival of GBM patients remains approximately 20 months for GBMs without MGMT promoter hypermethylation[3] and about 2 years for patients with MGMT promoter hypermethylation. Poor survival is associated with paucity of intratumoral T cell infiltrates and highly immunosuppressive tumor microenvironment (TME)[4]. To date, trials of tumor vaccines and checkpoint inhibitors have failed to demonstrate benefit in this indication[5-8].

Aglatimagene besadenovec (CAN-2409, gene-mediated cytotoxic immunotherapy, GMCI)[9,10] is a genetically modified, replication-defective adenoviral vector expressing the herpes simplex virus (HSV) thymidine kinase (tk) gene. CAN-2409 is injected directly into the tumor and administered in combination with a prodrug (valacyclovir or acyclovir), leading to local production of nucleotide analogs that, once intercalated in the tumor cell DNA, induce immunogenic cell death with release of tumor (neo)antigens in the TME. The presence of highly immunogenic proteins present in the adenovirus backbone contributes to antigen presentation

and activation of resident and newly recruited cells in the TME, thought to foster the development of responses against the patient's tumor antigens[11-13].

A previous phase 1b/2a clinical trial of CAN-2409 plus valacyclovir (NCT00589875) in 48 newly-diagnosed HGG patients undergoing standard radiochemotherapy showed improvement in median overall survival (mOS) (17.1 months in CAN-2409 treated patients compared to 13.4 months in a pre-planned matched case contemporaneous cohort who received SoC only)[10]. Improved survival was most evident in patients undergoing gross total resection (GTR), at 25 months with CAN-2409 versus 16.9 months for SoC. A subsequent preclinical study suggested the ability of CAN-2409 to further increase survival and induce tumor regression when combined with anti-PD1 antibodies in two distinct syngeneic mouse models of GBM[14]. Analysis of post-treatment tumor samples demonstrated an increase in interferon-γ positive T cells and a lower proportion of exhausted CD8+ PD1+ TIM-3+ T cells in the group receiving combination therapy, suggesting that the addition of CAN-2409 to immune checkpoint inhibitors (ICI) could overcome resistance to immunotherapy.

Those studies supported the rationale for a phase 1 clinical trial (NCT03576612) of CAN-2409 plus valacyclovir in combination with nivolumab and SOC surgery and radiochemotherapy in patients with newly diagnosed GBM/HGG. Our results suggest that this therapeutic approach is feasible, well tolerated, and results in systemic immune activation.

Materials and Methods (see also Supplemental Methods)

Ethics. Institutional review board approval was obtained from all participating institutions. All patients provided written consent prior to study, screening evaluations and enrollment. Patient management and assessment were performed by the investigators and reported to the sponsor. Reporting to the FDA and other regulatory entities was performed as required. This study was monitored by the sponsor and ABTC, who was responsible for compiling and submitting data to the Cancer Therapy Evaluation Program (CTEP) for all patients and for providing the data to the principal investigator for review.

Patient population. Eligible patients were ≥18 years of age presumed to have newly diagnosed HGG (nHGG)/ newly diagnosed GBM (nGBM) based on clinical and radiographic evaluation with a planned intent to undergo a gross total surgical resection by the neurosurgeon. During surgical resection, the neuropathologist had to confirm the presence of tumor on frozen section analysis for enrollment prior to intraoperative CAN-2409 peritumoral injection. Tumor sites were required to be supratentorial and suitable for injection. All patients with neuropathologic frozen identification of tumor were injected. Confirmation of extent of resection was obtained by MRI scan after the procedure.

Study design. This was an open-label, multicenter, phase 1 clinical trial in patients with nHGG/nGBM (NCT03576612) conducted by the American Brian Tumor Consortium (ABTC). It evaluated safety and initial efficacy of CAN-2409 plus valacyclovir and SoC plus nivolumab. In addition, exploratory analyses of scientific correlates on tumor and longitudinal peripheral blood samples were performed by the Cancer Immune Monitoring and Analysis Centers (CIMACs) and Cancer Immunologic Data Center (CIDC) Network at the NCI's Division of Cancer Treatment and Diagnosis to characterize local and systemic immune response to treatment (https://dctd.cancer.gov/ResearchNetworks/cimacs_cidc_network.htm).

CAN-2409 was "free-hand" injected by the neurosurgeons at the time of surgery to the tumor bed at a dose of $2.5x10^{11}$ viral particles (vp) in a total volume of 1ml solution which could be divided across up to 10 sites (approximately 100µL per site in the wall of the resection cavity) based on previous phase 1 and 2 monotherapy trial data[9,10]. Valacyclovir (prodrug) was given orally 1-3 days after the CAN-2409 injection and continued for fourteen days at a dose of 2g three times daily or adjusted based on the calculated creatinine clearance. Radiation was started within 8 (±4) days of surgery. Concomitant treatment with TMZ started within 15 (±3) days of surgery and about one week after the start of radiation. All patients began treatment with TMZ unless already known to be MGMT unmethylated. TMZ subsequently was discontinued in patients found to have an unmethylated MGMT promoter. Nivolumab was administered by intravenous infusion on an outpatient basis within 15 (±3) days of surgery at a dose of 240mg every 2 weeks (12-16 days from prior dose) for up to 52 weeks.

Safety. Patients were continuously monitored throughout the study for determination of safety. All patients who received one of the study drugs were counted for safety and toxicity evaluation of the combination regimen.

Assessments. Pre-treatment evaluations included medical history, clinical examination, KPS, corticosteroid dosage, brain MRI, 12-lead ECG echocardiogram and laboratory analyses. Patients underwent surgery with the intent for gross total resection defined as removal of >95% of the contrast-enhancing area[10]. To assess tumor and progressive disease, patients were evaluated 1 to 3 days after surgery and re-evaluated at the end of every second cycle (approximately every 8 weeks) and after completion of radiation with a gadolinium-enhanced cranial MRI scan following RANO (radiographic assessment in neuro-oncology) criteria with special consideration for clinical trials with immunotherapies (iRANO criteria)[15,16]. There was no central review of pathology or imaging for this phase 1 trial.

Exploratory scientific analyses (see supplemental methods). The following methodologies were used: cytometry time of flight (Cytof) for cells (Cytof), Olink for cytokines, Nanostring for transcripts on blood, whole exome sequencing (WES) for genomics, T-cell receptor sequencing for T cell clonotypes on blood/tumor and MIBI for immune cell identification on tumor.

Statistical Analyses (see supplemental methods)

RESULTS

Patient Demographics and Baseline Characteristics

This multi-institutional, phase 1 clinical trial for patients with (magnetic resonance imaging (MRI) consistent with nHGG/nGBM involved intraoperative peritumoral injection of CAN-2409 after histologic confirmation of glioma on frozen tissue. Injections were followed by treatment with two weeks of valacyclovir, standard of care (SOC) chemoradiation therapy (initiated within a week of surgery[9,10]) and nivolumab 240 mg every 2 weeks (beginning 3 weeks after surgery) **(Figure S1a)**. Patients were enrolled before surgery (intent to treat, ITT, population) and treated only if the frozen tissue confirmed presence of tumor (Safety Population). While this phase 1 trial was not statistically designed to test efficacy or correlates of responses, we retrospectively attempted to determine if there were genetic or immunologic profiles that significantly correlated with patient outcome. These correlative studies included baseline tumor whole exome sequencing (WES), baseline tumor immunophenotyping, longitudinal peripheral blood immune cell and cytokines phenotyping and longitudinal peripheral blood T cell receptor (TCR) analysis (**Figure S1b)**.

Between February 2019 and March 2021, 41 patients were enrolled and treated at five participating sites (**Figure 1a**). Demographic and baseline clinical characteristics are reported in

Table S1. Median age was 62 years (IQR 28-81), with 27 males (66%) and 14 females (34%). Because of the timing of trial initiation, enrolled patients were classified using WHO 2016[17]. One of the patients originally classified as GBM, would now be classified as IDH-mutant grade IV diffuse astrocytoma, based on WHO 2021[18]. Twenty-five patients (61%) had an unmethylated MGMT promoter and 16 patients (39%) had a methylated MGMT promoter. The neurosurgeon preoperatively assessed that a GTR was possible and thus all 41 patients received CAN-2409 injection after tumor resection. However, postoperative MRI confirmed that GTR was achieved only in 30 patients (73%) with subtotal resection occurring in 11 patients (27%). Thirty-five patients completed treatment per protocol, 6 received less than 80% of the valacyclovir and nivolumab regimen and thus were considered mid-treatment dropouts.

In this study, the use of corticosteroids was left to physician's choice. At baseline, 22 out of 35 patients (62.9%) were treated with steroids. This percentage increased to 91.4% after surgery, representing 32 out of 35 (91.4%) evaluable patients. At day 15 post-surgery, 29 out of 35 patients (82.9%) were still receiving corticosteroid, with 62.9% receiving a high dose (>8 mg/day dose) of dexamethasone. At days 43 and 71 of study, 57.1% and 22.9% of 35 evaluable patients were still receiving glucocorticoid therapy, respectively (**Table S2**).

Safety

The safety population included 41 treated patients. No dose limiting toxicity (DLTs) related to CAN-2409 were reported. However, five events unrelated to CAN-2409, met the criteria during the evaluation period: a grade 3 stroke (related to the surgery), a grade 4 neutropenia, and three cases of grade 4 thrombocytopenia that were considered related to TMZ, with a possible contribution of nivolumab in one case (**Table S3**). With 15.1 months of median follow-up, the most common adverse events (AEs), attributable to the combination of CAN-2409, valacyclovir, nivolumab, and radio chemotherapy, occurred in ≥10% of patients and these were anemia, nausea, vomiting, fatigue, fever, increased aspartate transaminase (AST)/ alanine transaminase (ALT), increased blood bilirubin, hyponatremia, anorexia, and headache (**Table S4**). Most of the events were grade 1 and 2. Other grade 3 or higher treatment-related AEs that occurred in more than one patient included lymphopenia, acute kidney injury, and hypertension. No treatment-related deaths or unexpected serious adverse events were observed. Nine patients discontinued treatment due to AEs unrelated to CAN-2409 plus valacyclovir. Two discontinuations were considered possibly related to nivolumab, including one with increased ALT and AST, and one grade 3 case of aseptic meningitis. Among the remaining discontinuations, one case of pancytopenia was attributed to nivolumab, while the rest were attributed to TMZ and/or underlying disease. Aseptic meningitis was reported in a 65-year-old male (IDH wild-type, MGMT methylated with GTR) approximately three months after CAN-2409 treatment and the sixth cycle of nivolumab. A biopsy was performed at the time of symptom onset and revealed presence of necrotic tissue. After treatment with dexamethasone and

improvement of the clinical presentation, the patient survived more than 30 months, even after nivolumab discontinuation.

Clinical outcome

Figure 1b shows the clinical course and treatment timelines for the evaluable (n=35) patients**.** Median OS (mOS) for the evaluable (n=35) and the intent-to-treat (ITT) population (n=41) was similar at 15.1 months (**Figure 1c, Figure S2).** mOS for patients with unmethylated MGMT promoter was 13.2 and 15.9 months for patients with GTR and STR, respectively (**Figures 1d**). mOS in patients with methylation of the MGMT promoter was 30.6 and 12.6 months for patients with GTR and STR, respectively.

Baseline tumor gene mutations association with mortality

We asked if there were gene mutations that correlated with outcome. WES was performed on baseline tumors. Missense mutations were the dominant variant classifications **(Figure S3a)**. Single nucleotide polymorphisms and deletions represented the most common variants. . The median number of variants identified per sample was 247 (**Figure S3b)**. Commonly altered genes included MUC16 (64% of the patients), ARID1B (82%), PABPC3 (50%), and PAXIP1 (55%) (**Figure S3c)**. Tumor mutational burden in our dataset was compared to The Cancer Genome Atlas (34 available GBM datasets) and found to be fourth highest of the available datasets with a median of 4.8 per Mb **(Figure S3d)**. Nanostring expression analysis revealed visual clustering

of patients based on high expression of a subset of genes but no correlation between gene expression, baseline clinical features or outcome (**Figure S3e**).

There was a subset of mutated gene pairs that negatively associated with survival. These included *MED15/HRC, SKIDA1/ARID3A, SKIDA1/CACNA1A,* and *PAXIP1/ARID3A* (**Figure 2a**). While these combinations were more frequently present in unmethylated MGMT patients, this frequency was not statistically different, except for a trend for the *SKIDA1/CACNA1A* pair (p = 0.052) (**Figure 2b**). To discriminate if the increased mortality association of the combined mutated genes was a consequence of their lower frequency in the methylated tumors, we focused on the unmethylated cohort. Despite the small number of patients, a statistically significant association for the *MED15/HRC* pair was observed (p=0.036) (**Figure 2c**), suggesting a novel association between the mutational pair *MED15/HRC* with mortality, in addition to the known association between mortality and MGMT promoter hypomethylation.

Baseline tumor immune cell composition associates with survival

We next asked if tumor immune cell populations were linked to outcome. Resected tumors, before CAN-2409 injection, were stained by Multiplex Ion Beam Imaging (MIBI). Cluster abundance analysis performed using the elastic net model[19] unveiled positive associations between patient survival and abundance of B cells/Dendritic cells (DC), HLA-DR high macrophage clusters, and Memory CD4+ T cells. Conversely, negative association was observed between survival and abundance of HLA-DR low monocytes (c=0.72; **Figure 3a, b**). Importantly

individual cluster frequencies were not associated with survival (**Figure 3c**). Correlations between expression analysis and survival were then performed for each meta-cluster identified in at least 20 baseline specimens. The c statistic for specific clusters was found to be below 0.7 (**data are not shown**). Overall, these data suggest that patient outcome positively associates with the presence, at baseline, of proinflammatory immune cells infiltrates.

Longitudinal peripheral cytokine and immune cell profiles characterize survivors

Next, we investigated the presence of possible associations between changes in peripheral cytokines and clinical outcome. Cytokine profiling was performed via O-link at baseline, before surgery and CAN-2409 treatment and at the 3 (day 15), 5 (day 29), 11 (day 71) week timepoint after craniotomy and peritumoral CAN-2409 injection and then at progression **(see Figure S1).** At the 3-week (day 15) timepoint, patients had been treated with surgical resection of tumor, CAN-2409 injection, had received almost a full course of valacyclovir (VCV) and started radiation (day 4-7 days)**.** By the 5-week (day 29) timepoint, patients had also received 14 days of radiation, 7 doses of TMZ (for patients with MGMT methylation), and one dose of nivolumab. By week 11 (day 71), patients had received the full course of RT, were on TMZ maintenance (if applicable) and received 3 doses of nivolumab.

Levels of 45 circulating cytokines and proteins were analyzed for their association with overall survival (**Figure 4a, b)**. At each time point, some of these significantly associated with changes

in survival (**Figure 4a, b**). Volcano plots of cytokine frequency comparisons from week 3 to baseline and week 5 to week 3 further validated the finding of changes in cytokine levels in peripheral blood during the trial **(Figure 4c)**. However, by the week 11 timepoint, there were no significant differences with baseline pre-treatment cytokine profiles.

Next, we sought to determine changes in peripheral blood immune cells using Cytometry by time of flight (CyTOF) and possible associations between post treatment changes and clinical outcome **(Figures S4a, b).** At the week 3 (day 15) timepoint, we found a significant increase (as compared to pre-treatment baseline) in several naive and effector T cell populations, including CD69+CD4+ T cells (FDR adjusted p=0.0379) and naïve CD4+ T cells expressing CD28, CD69, PD-L1 and CD161 ($p<0.05$) **(Figure S5a and S6a).** At the week 5 timepoint, after the first cycle of nivolumab, we detected a significant increase in activated HLA-DR+CD38+CD4+ T cells (p=0.0011), activated HLA-DR+CD38+CD8+ T cells (p=0.0189), CD69+ CD4+ T cells (p=0.0228), CD69+ Central Memory (CM) CD4+ T cells (p = 0.0324), CD69+ Effector Memory (EM) T cells (p=0.0324), and inducible costimulator (ICOS)+ Effector Memory (EM) CD4+ and CD8+ T cells (p=0.0134 and p=0.0326, respectively. PD1+ T cells and PD1+ NKT cells were also downregulated at week 5 in conjunction with initiation of nivolumab treatment ($p \leq 0.0001$) **(Figures S5a and S6b).** At week 11, HLA-DR+CD38+CD4+ (p=0.0011), CD28+ EM CD4+ (p=0.0375), and ICOS+ EM CD4+ (p=0.0036) were significantly increased from baseline **(Figure S5a)**. By the time of progression these percentages had returned to baseline. This analysis was also validated by Uniform Manifold Approximation and Projection (UMAP) clustering on T cells,

showing an increased expression of the activation markers CD38, HLA-DR, CD69, and ICOS **(Figure S5b).** Both week 3 and week 5 timepoint samples showed a decrease in frequency of immunosuppressive TIM3+ NK cells (p=0.0337 at week 3; p=0.0324 at week 5) (**Figure S6c**), consistent with evidence of systemic activation following treatment with CAN-2409 and nivolumab. At the week 11 timepoint, the TIM3+ NK cell immune cell changes were not significantly different compared to pre-treatment baseline.

Taken in conjunction, the longitudinal changes in both cytokines and immune cells in peripheral blood were consistent with systemic immune activation during the early phase of the clinical trial; while immune cell changes were still present at week 11, peripheral blood cytokine levels had returned to baseline values at this timepoint.

Longitudinal changes in peripheral T cell clonotypes

We then investigated the relationship between systemic longitudinal immune changes and changes in the peripheral T cell clonotypes. Beta chain T-cell receptor (TCR) sequencing was performed in the peripheral blood for the timepoints described. Consistent with the activation markers seen in protein analyses, we observed a significant increase in TCR density, a marker of individual cell activation (p=0.0066), and of T cell richness, a marker of overall TCR diversity (p=0.021), after CAN-2409 injection (week 3 as compared to baseline) (**Figure 5a**). TCR density continued to be significantly different up to week 11 (p =0.0046) but had returned to baseline

at the time of progression. TCR richness was not significantly different at either week 5 or 11. We observed no significant changes in clonality after week 3, suggesting that the systemic immune activation observed in the periphery was not driven by specific tumor clones. This was confirmed by analyses of tumor-derived clones in PBMCs, which did not demonstrate a particular clone expansion after treatment initiation or the addition of nivolumab to the therapeutic regimen (**data not shown**). The observed significant changes in peripheral T cell clonotype density, richness, and clonality at week 3 were also associated with patient survival ($p = 0.032$, 0.044, and 0.003 respectively) **(Figure 5b).** However, these associations were not statistically significant at later timepoints, except for density, which was significant at Week 11 ($p = 0.017$) (**Figures S7a, b).** This result suggests that changes in immune activation, observed through cytokines profiling and peripheral immune cell analysis were reflected in systemic increases in T cell clone density and richness.

Changes in discrete cytokines, immune cell sub-populations, and T cell metrics characterize survivors in the MGMT methylated/ GTR sub-population.

Finally, we focused our analyses on the small patient cohort characterized by methylation of the MGMT promoter who had undergone GTR. Of note, there were 10 patients with methylation of the MGMT promoter who had a GTR: six of these lived more than 30 months and 5/6 were still alive at the time of data cut **(see Figure 1d)**. Four out of 10 survived less than 25 months (**Table S5).** We thus queried our data to evaluate differences in the biomarker profile of long-term (>30 months) vs. short-term (< 25 months) survivors in this population. Longitudinal changes in cytokine levels in peripheral blood, detected at week 3 and 5 post-treatment, were indeed associated with outcome **(Figure 6a)**. PDCD1 (week 3 log-rank $p = 0.0018$), MMP12 (week 3 log-rank $p = 0.032$) and EGF (week 5 log-rank $p = 0.0049$) were

statistically significantly associated with survival **(Figures 6b-d)** and CD40L and ANGPT1 showed a trend towards association with survival **(Figure S8c).** The time course analysis of average fold changes for key cytokines is illustrated in **Figures S8a, b.**

We next evaluated differences in peripheral blood immune cell sub-populations in the long- vs. short-term survivors in the MGMT methylated/GTR patients. Changes in ICOS+CD4+ TEMRA cells, detected at the Week 3 timepoint were significantly associated with survival (t-test p = 0.017), while there was a non-significant trend for a positive association between changes in CD137+ CD4+ TEMRA cells and survival (p = 0.057) (**Figure 6e, Figures S9a, b)**. At the Week 5 timepoint, there was also a trend for changes in OX40+, CD4+ TEMRA cells in relationship to survival (p = 0.052) **(Figure 6e, Figures S9c**). Additional changes associated with survival were detected at the Week 11 timepoint, among which ICOS+ CD4+ TEMRA (p = 0.0001), ICOS+NKT (p = 0.002), CD28+CD4+ (p = 0.023), Tregs (p = 0.035), CD28+CD4+CM (p = 0.037), CD137+NKT (p = 0.041) and Tim3+ CD4+ T cells (p = 0.047) **(Figure 6e, Figures S4b, S9d-i).**

Finally, we also characterized T cell diversity metrics: there was a significant difference in TCR richness and clonality at baseline **(Figure 6f)** and at the Week 3 timepoint **(Figure 6g)** between the short- vs. long-term MGMT methylated/ GTR survivors, but not at later timepoints **(data not shown).**

Taken together, these data suggested that immunological changes in terms of cytokines, effector cells and TCR diversity were associated with long term survival in the population of patients undergoing GTR presenting with methylated MGMT promoter.

Discussion

Effective treatments for nHGG/ nGBM are being investigated through several approaches including local intratumoral therapies[20-23] CAN-2409 is a locally delivered gene-based immunotherapy thought to induce *in situ* vaccination against patients' own antigens[24]. Evidence generated both in preclinical models and in clinical trials suggests that CAN-2409 directly injected into the tumor may induce tumor regression via a multimodal mechanism of action that combines direct oncolysis with local and systemic activation of the immune response[11-13,25], and may potentially synergize with PD1 blockade[14]. These findings supported the rationale for exploration of the effects of CAN-2409 plus prodrug in combination with nivolumab in patients with nHGG/ nGBM. In this phase 1 clinical trial, the combination of CAN-2409 plus prodrug with nivolumab was well tolerated, when administered in the context of SOC treatment for nHGG/nGBM patients. DLTs were generally not attributed to CAN-2409 or prodrug; AEs, including immune-related AEs, were as expected in patients treated with ICI and SOC radio chemotherapy and surgery.

While this phase 1 trial was not statistically designed to test efficacy, exploratory immune profiling and scientific analyses unveiled some significant correlations with patient outcomes. There was worst outcome in patients presenting with *MED15/HRC* mutations at baseline. We also observed improved survival in patients presenting with enrichment in B cells, dendritic

cells, HLA-DRhigh macrophages, memory CD4+ T cells and relative scarcity of LAG+, ICOS+ antigen presenting CD163+ mononuclear cells in pre-treatment tumors. These observations suggest that a subpopulation of nHGG/nGBM patients may exhibit increased responsiveness to this combination of immunotherapies, administered in the context of SOC. Correlation of mortality with the *MED15/HRC* mutation profile with other databases was limited: in GLASS[26], only 5 patients had the *MED15* mutation, 6 patients had the *HRC* mutation, and none of these were co-occurring and in TCGA, only 3 patients had the *MED15* mutation, and none had a mutation in *HRC*.

Treatment with CAN-2409 plus prodrug and addition of nivolumab was associated with discrete longitudinal changes in peripheral cytokines, immune cells, and T cell clone diversity, particularly at the early timepoints of 3 and 5 weeks, after patients had been treated with surgery and CAN-2409/VCV but before full courses of radiation, TMZ or nivolumab. The relationship between expansion and diversity of the TCR repertoire with improved prognosis has been previously demonstrated in patients with cancer; low TCR diversity being associated with more aggressive tumor phenotypes and poor prognosis[27-29]. By week 11, cytokine levels, immune cells, and T cell diversity in the periphery were found to be like those at baseline. Interestingly, patients at this timepoint were also treated with nivolumab and TMZ, as well as dexamethasone. This added therapeutic regimen either could not sustain the early immune response elicited by CAN-2409 or was inhibiting the acute stimulatory effect. Based on our preclinical data[14], we would have expected that the addition of nivolumab after the

intratumorally delivered CAN-2409 would sustain an immune activating effect over a much longer period of time, as has been observed in other clinical trials of CAN-2409 in combination with immune checkpoint inhibitors[30]. Several factors might have contributed to the finding in this clinical trial. First, inhibition of the PD-1 pathway may not lead to effective antitumor immunity in nHGG/nGBM, as compared to other indications, perhaps due to epigenetic mechanisms of regulation[31,32] or potentiation of regulatory T-cell function dampening the anti-tumor immune response[33]. Our finding of increased expression of ICOS, CD38 and HLA-DR in the T-reg CyTOF analysis may support this hypothesis. This may also be pertinent to the observation that anti-PD1 monotherapy was unable to improve patient survival in HGG/GBM in a separate clinical trial of immune-based gene therapy[34]. Co-administration of TMZ and dexamethasone (used in this trial to mitigate tumor associated symptoms), and both known as potent immunosuppressors, might have played an additional immunosuppressive role, silencing the immune response and subsequent loss of clinical activity. Finally, in this trial, patients received only a single course of CAN-2409 plus prodrug. Though this yielded some encouraging survival advantages in our previous study without nivolumab[10], in other indications we have demonstrated that a second course of treatment is required to promote the maturation of the antitumoral immune response associated with survival[30]. Therefore, additional courses of CAN-2409 and or VCV may be beneficial. Finally, others have demonstrated that the sequential timing of therapeutic intervention when adding immune checkpoint inhibitors can be critical. In a GBM clinical study comparing neo-adjuvant to adjuvant use of PD-1 blockade, the neoadjuvant group showed a median survival of 13.7 months versus 7.5 months (HR 0.39) for

the adjuvant only group[35]. Further analysis of the relative timing of CAN-2409 and anti-PD-1 may improve the outcome of this combination in a larger subgroup of GBM patients.

In the cohort of patients with GTR and methylation of the MGMT promoter, we observed a small subgroup of patients with mOS >30 months, significantly exceeding the mOS of 24 months, reported for patients receiving optimal SOC[2,36]. Comparing the immune response elicited in these "long survivors" with the response observed in the same group of patients (methylated MGMT undergone GTR) surviving less than 25 months, we demonstrated evidence of significant systemic immune activation in the former, with increase in cytokine levels, immune cell frequency, and T cell clonotype profiling. Despite the small numbers of patients evaluated and the exploratory nature of this analysis, these data suggest that there may be a subgroup of patients with nHGG, methylation of the MGMT promoter and GTR who might benefit from this multimodal therapy.

The mOS observed in the small group of patients with methylated MGMT promoter undergone GTR in the current study is similar to that reported in Checkmate 548, a large phase 3 clinical trial evaluating the effects of adjuvant nivolumab in GBM[7,37]. Despite the clear distinctions between this small, open-label phase 1 clinical trial and the larger phase 3 trial, comparing the two studies may yield interesting insights. In contrast to Checkmate 548, which limited steroid dose at the time of enrollment to $\leq$ 3mg daily, there was no limitation in the use of steroids in the current combination study. On the contrary in the current study, patients often received

high doses of corticosteroids in the perioperative period, potentially attenuating the immune response generated by the combination of CAN-2409 plus prodrug and nivolumab and, as mentioned above, potentially negatively contributing to the efficacy of this therapeutic regimen. Importantly, in the current study there was no preselection for patients with favorable prognostic markers after treatment initiation. Patient enrollment in our clinical trial occurred at the time of surgery, while in Checkmate 548 it occurred after radio chemotherapy, and excluded patients whose imaging or clinical status deteriorated after SOC. In our trial, no post-SOC exclusion was applied. In the present study patients received both chemo (TMZ) and radiation, therapeutic approaches historically associated with poor response to immunotherapy due to induction of lymphopenia. Lymphopenia was observed in seven patients in the current study (2 methylated, 1 partial methylated and 3 unmethylated) without a clear association with survival in either population. Evidence in preclinical models indicate that radiation therapy, that stimulates DNA repair mechanism in tumor cells, can synergize with CAN-2409 mechanism of action that induces the formation of an intercalate that blocks DNA repair and promotes cell death. In support of this preclinical evidence, biomarker analysis and clinical data in pancreatic cancer support the synergistic effect between the two modalities[38]. However, it is not possible to exclude that this modality interferes with the classic immunostimulatory mechanism of action of nivolumab.

In conclusion, the findings of this study indicate that experimental therapeutic approach using CAN-2409 and nivolumab in HGG is both feasible and well tolerated. Although the study was

not designed to explore clinical efficacy, correlative scientific analyses demonstrate the ability of this combination of immunotherapies to induce systemic immune activation, with encouraging outcomes observed in a mall subgroup of patients. Various opportunities to further potentiate this regimen, including sequential timing of the therapies, genetic and protein-based subgroup stratification, and limited administration of immune-suppressant medications are suggested by the data. Of note, recent biological biomarkers and encouraging clinical activity data were presented for a therapeutic regimen consisting of two courses of CAN-2409 plus prodrug in combination with ICI in stage III/IV non-small cell lung cancer with an inadequate response to ICI alone[30]. In that indication, activation of the immune response after the second administration for CAN-2409 was significantly associated with survival, providing the biological rationale for the "booster effect" provided by the second course of CAN-2409[30]. The data presented here along with our previous clinical studies in GBM, provide strong rationale for future work exploring CAN-2409, in particular in the patient population able to undergo GTR.

Abbreviations: **AEs**– adverse events; **ALT** – alanine transaminase; **AST** – aspartate transaminase; **CM** – central memory; **CyTOF** – cytometry by time of flight; **DC** – dendritic cells; **EM** – effector memory**; GBM** – glioblastoma; **GTR** – gross total removal; **HRs** – hazard ratios; **HGG** – high-grade gliomas; **HSV** –herpes simplex virus; **ICI** – immune checkpoint inhibitors; **ICOS** – inducible costimulator; **IDH** – isocitrate dehydrogenase; **ITT** – intent to treat; **IQR** – interquartile range; **MGMT** – methylguanine methyltransferase; **MIBI** – multiplex ion beam imaging; **mOS** – median overall survival; **nHGG** (newly diagnosed high-grade glioma); nGBM (newly diagnosed glioblastoma) **OS** – overall survival; **SoC** – standard of care; **STR** – subtotal resection; **TCR** – T cell receptor; **TME** – tumor microenvironment; **TMZ** – temozolomide; **TTF** – tumor-treating fields; UMAP – Uniform Manifold Approximation and Projection; **VCV** – valacyclovir; **WES** – whole exome sequencing.

Funding. This study was funded by NCI UM1 CA137443 to the Adult Brain Tumor Consortium with additional support from Candel Therapeutics and Bristol Myers Squibb

Scientific and financial support for the Cancer Immune Monitoring and Analysis Centers-Cancer Immunologic Data Center (CIMAC-CIDC) Network are provided through the National Cancer Institute (NCI) Cooperative Agreements, U24CA224319 (to the Icahn School of Medicine at Mount Sinai CIMAC), U24CA224285 (to the MD Anderson Cancer Center CIMAC), U24CA224309 (to the Stanford University CIMAC), and U24CA224316 (to the CIDC at Dana-Farber Cancer Institute), and through NCI contract 140D0421D0007 to the CIDC operated by NCI.

WEX was performed at the Cancer Genomics Laboratory (CGL) in the Department of Genomic Medicine at the University of Texas MD Anderson Cancer Center. Scientific and financial support for the CIMAC-CIDC Network are provided through the National Cancer Institute (NCI) Cooperative Agreements: U24CA224319 (to the Icahn School of Medicine at Mount Sinai CIMAC), U24CA224285 (to the MD Anderson Cancer Center CIMAC), U24CA224309 (to the Stanford University CIMAC), and U24CA224316 (to the former CIDC at Dana-Farber Cancer Institute), and through NCI contract to the CIDC operated by NCI

Conflicts of Interest.

PYW received research support from Astra Zeneca, Black Diamond, Bristol Meyers Squibb, Chimerix, Eli Lily, Erasca, Global Coalition For Adaptive Research, Kazia, MediciNova, Merck, Novartis, Philogen, Quadriga, Servier, VBI Vaccines and served on advisory boards for Anheart, Astra Zeneca, Black Diamond, Celularity, Chimerix, Day One Bio, Genenta, Glaxo Smith Kline, Kintara, Merck, Mundipharma, Novartis, Novocure, Prelude Therapeutics, Sagimet, Sapience, Servier, Symbio, Tango, Telix, VBI Vaccines

SAG is on the Scientific Advisory Board of Myosin Therapeutics and Boston Scientific. He also serves as a consultant to Plus Therapeutics. He has research funding from Boehringer

Ingelheim, Bristol-Myers Squibb, Celgene, Genentech, Regeneron, and Takeda not related to this study.

TW reports honoraria from Prescott Medical Communications and Horizon Presentation

IL reports consulting fees from Medtronic, Monteris, Imvax. and meeting support from Monteris

SJB reports grants from GSK, Novocure, Kite, Eli-Lilly, Incyte and consulting fees from Modifi Bio, Telix, Kiyatec, Bayer, Servier

RS reports grants from NIH; Royalties from Elsevier, Lecturio; consulting fees from Monteris Medical, Novocure, SpingWorks,; DSMB advisor for MGH; Leadership role for ACGME, North Carolina Medical Society Foundation

ST reports grants from Monteris Medical, Azurity Pharmaceuticals.

JD reports grants from Novartis, Astra Zeneca, Servier, Philogen, Curis, Ono; royalties/licenses from UptoDate, Elsevier; Consulting fees from Servier, Novocure; Advisory Board participation for Hemerion and University of Pittsburgh; Stock options in Gilead, Pfizer.

CH reports grants from Sanofi, Avenge, Iovance, KSQ, Theolytics, BTG, Novartis, 280Bio/Yingli, 4D Pharma, Medimmune/AZ, EMD Serono, Merck, Takeda, Obsidian, Genentech, BMS, Summit Thereptuics, Artidis, Immunogenesis; Consulting fees from Regeneron; Stock options from Briacell; Travel support from SITC; and patents

IIW reports grants from Genentech, Merck, BMS, Medimmune, Adaptive, Adaptimmune, EMD Serono, Pfizer, Takeda, Amgen, Karus, Johnson and Johnson, Bayer, Iovance, 4D, Novartis, Akoya; Consulting fees for Genentech/Roche, Bayer, BMS, Astra-Zeneca, Pfizer, Merck, Guardant health, Flame, Novartis, Sanofi, Daiichi Sankyo, Amgen, Jansen, Merus, G1 Therapeutics, Abbvie, Catalyst Therapeutics, Johnson and Johnson, Boehringer-Ingelheim, Regeneron, and Oncocyte; Honoraria from Medscape, Genentech/Roche, Platform Health, Pfizer, Merus, AstraZeneca, Merck, Physician Education Resource (PER), Dava Oncology, Suzhou Liangyihui Network Technology, Touchpoint Group Holdings; and support for meeting attendance from WOG, IASLC, and ECOG-ACRIN

SG reports grants from Boehringer Ingelheim, Bristol-Myers Squibb, Celgene, Genentech, Regeneron, and Takeda, NIH/NCI; Consulting fees from Taiho Pharmaceuticals.

ES participated in an advisory board for Mallinckrodt Pharmaceuticals and served as a consultant on one occasion for D.E. Shaw Research.

EA-C and LKA are shareholders of Candel Therapeutics, Inc.

LKA is a consultant for Diakonos Oncology and board member of the American Brain Tumors Association

AM, LL, DL, GN, PPT, JD, FB are employees and shareholders of Candel Therapeutics, Inc.

HM reports grants from the Bill and Melinda Gates Foundation and NIH

PP reports equity from Ternalys Therapeutics

EAC is an advisor to Bionaut Labs, Genenta, inc., Insightec, Inc., Seneca Therapeutics, Theravir. He has equity options in Bionaut Laboratories. Seneca Therapeutics, Ternalys Therapeutics. He is co-founder and on Board of Directors of Ternalys Therapeutics. Patents related to oncolytic viruses are under the possession of Brigham and Women's Hospital (BWH) with EAC as co-

inventors. These patents have been licensed to Candel Therapeutics, Inc. Present and future milestone license fees and future royalty fees are distributed to BWH from Candel.

EGK, XY, JF, JS, AD, CK, AL, NA, FL, MM, DT, Ga-A, JMP, AF, LDL, MDHI, DD, PJ, AR, SEL, MP, AG, SB, AK, CC, DDV, SKS, MON, report no disclosures.

Authorship:

Conceptualization: PYW, SAG, EC, LKA, FB, EAC

Methodology: PYW, CD, EGK, SAG, XY, JF, IL, TW, JS, AD, SJB, CK, RS, ST, AL, GL, NA, FL, ID, MM, DT, CH, IIW, Ga-A, JMP, AF, LDL, MDHI, DD,PJ, AR, SEL, MP, AG, SB, AK, CC, DDV, SKS, SG, ES, MON, KT, LS, PP, DL

EC, LKA, HM, JD, FB, EAC

Investigation: PYW, AM, CD, EGK, LL, SAG, XY, IL, TW, JS, AD, SJB, SJB, CK, RS, ST, AL, GL, NA, FL, ID, MM, DT, CH, IIW, Ga-A, JMP, AF, LDL, MDHI, DD, PJ, AR, SEL, MP, AG, SB, AK, CC, DDV, SKS, SG, ES, MON, KT, LS, PP, DL, JD, FB, EAC

Visualization: PYW, AM, EGK, LL, SAG, PJ, AR, JD, FB, EAC

Funding acquisition: PYW, SAG, SG, MP, HM, SB, EA-C, PPT, EAC

Project administration: PYW, SAG, SB, JF, SG, MP, LKA, HM, JD, FB, EAC

Supervision: PYW, SAG, SG, MP, HM, SB, PPT, FB, EAC

Writing – original draft: PYW, AM, CD, LL, CCF, AK, JD, PPT, FB, EAC

Writing – review & editing: PYW, AM, CD, EGK, SAG, XY, JF, SB, PJ, AR, SG, EA-C, MP, GN, JD, PPT, HM, FB, EAC

Data availability: Data used to support the findings of this study will be deposited under controlled access in the database of Genotypes and Phenotypes (dbGaP) under accession number phs003753.

Acknowledgments: The authors would like to acknowledge all the patients and families that participated in the trial. Additionally, the authors would like to thank the CIMAC-CIDC project managers and National Cancer Institute project managers (EMMES) for their support of the correlative studies. Candel Therapeutics provided CAN-2409 and Bristol Myers Squibb provided nivolumab for this study.

References

1. Ostrom QT, Price M, Neff C, et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2016-2020. *Neuro Oncol.* 2023; 25(12 Suppl 2):iv1-iv99.
2. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* 2005; 352(10):997-1003.
3. Stupp R, Taillibert S, Kanner A, et al. Effect of Tumor-Treating Fields Plus Maintenance Temozolomide vs Maintenance Temozolomide Alone on Survival in Patients With Glioblastoma: A Randomized Clinical Trial. *JAMA.* 2017; 318(23):2306-2316.
4. Thorsson V, Gibbs DL, Brown SD, et al. The Immune Landscape of Cancer. *Immunity.* 2018; 48(4):812-830 e814.
5. Weller M, Butowski N, Tran DD, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol.* 2017; 18(10):1373-1385.
6. Omuro A, Brandes AA, Carpentier AF, et al. Radiotherapy combined with nivolumab or temozolomide for newly diagnosed glioblastoma with unmethylated MGMT promoter: An international randomized phase III trial. *Neuro Oncol.* 2023; 25(1):123-134.
7. Lim M, Weller M, Idbaih A, et al. Phase III trial of chemoradiotherapy with temozolomide plus nivolumab or placebo for newly diagnosed glioblastoma with methylated MGMT promoter. *Neuro Oncol.* 2022; 24(11):1935-1949.
8. Reardon DA, Brandes AA, Omuro A, et al. Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol.* 2020; 6(7):1003-1010.

9. Chiocca EA, Aguilar LK, Bell SD, et al. Phase IB study of gene-mediated cytotoxic immunotherapy adjuvant to up-front surgery and intensive timing radiation for malignant glioma. *J Clin Oncol.* 2011; 29(27):3611-3619.

10. Wheeler LA, Manzanera AG, Bell SD, et al. Phase II multicenter study of gene-mediated cytotoxic immunotherapy as adjuvant to surgical resection for newly diagnosed malignant glioma. *Neuro Oncol.* 2016; 18(8):1137-1145.

11. van der Linden RR, Haagmans BL, Mongiat-Artus P, et al. Virus specific immune responses after human neoadjuvant adenovirus-mediated suicide gene therapy for prostate cancer. *Eur Urol.* 2005; 48(1):153-161.

12. Ayala G, Satoh T, Li R, et al. Biological response determinants in HSV-tk + ganciclovir gene therapy for prostate cancer. *Mol Ther.* 2006; 13(4):716-728.

13. Fujita T, Teh BS, Timme TL, et al. Sustained long-term immune responses after in situ gene therapy combined with radiotherapy and hormonal therapy in prostate cancer patients. *Int J Radiat Oncol Biol Phys.* 2006; 65(1):84-90.

14. Speranza MC, Passaro C, Ricklefs F, et al. Preclinical investigation of combined gene-mediated cytotoxic immunotherapy and immune checkpoint blockade in glioblastoma. *Neuro Oncol.* 2018; 20(2):225-235.

15. Okada H, Weller M, Huang R, et al. Immunotherapy response assessment in neuro-oncology: a report of the RANO working group. *Lancet Oncol.* 2015; 16(15):e534-e542.

16. Wen PY, Macdonald DR, Reardon DA, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. *J Clin Oncol.* 2010; 28(11):1963-1972.

17. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* 2016; 131(6):803-820.

18. Louis DN, Perry A, Wesseling P, et al. The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. *Neuro Oncol.* 2021; 23(8):1231-1251.

19. Zou H, Hastie T. Regularization and Variable Selection Via the Elastic Net. *Journal of the Royal Statistical Society Series B: Statistical Methodology.* 2005; 67(2):301-320.

20. van Solinge TS, Nieland L, Chiocca EA, Broekman MLD. Advances in local therapy for glioblastoma - taking the fight to the tumour. *Nat Rev Neurol.* 2022; 18(4):221-236.

21. Umemura Y, Orringer D, Junck L, et al. Combined cytotoxic and immune-stimulatory gene therapy for primary adult high-grade glioma: a phase 1, first-in-human trial. *Lancet Oncol.* 2023; 24(9):1042-1052.

22. Nassiri F, Patil V, Yefet LS, et al. Oncolytic DNX-2401 virotherapy plus pembrolizumab in recurrent glioblastoma: a phase 1/2 trial. *Nat Med.* 2023; 29(6):1370-1378.

23. Ling AL, Solomon IH, Landivar AM, et al. Clinical trial links oncolytic immunoactivation to survival in glioblastoma. *Nature.* 2023; 623(7985):157-166.

24. Abazeed ME, Adams DJ, Hurov KE, et al. Integrative radiogenomic profiling of squamous cell lung cancer. *Cancer Res.* 2013; 73(20):6289-6298.

25. Nestler U, Wakimoto H, Siller-Lopez F, et al. The combination of adenoviral HSV TK gene therapy and radiation is effective in athymic mouse glioblastoma xenografts without increasing toxic side effects. *J Neurooncol.* 2004; 67(1-2):177-188.

26. Consortium G. Glioma through the looking GLASS: molecular evolution of diffuse gliomas and the Glioma Longitudinal Analysis Consortium. *Neuro Oncol.* 2018; 20(7):873-884.

27. Hopkins AC, Yarchoan M, Durham JN, et al. T cell receptor repertoire features associated with survival in immunotherapy-treated pancreatic ductal adenocarcinoma. *JCI Insight.* 2018; 3(13).

28. Kato T, Kiyotani K, Tomiyama E, et al. Peripheral T cell receptor repertoire features predict durable responses to anti-PD-1 inhibitor monotherapy in advanced renal cell carcinoma. *Oncoimmunology.* 2021; 10(1):1862948.

29. Robert L, Harview C, Emerson R, et al. Distinct immunological mechanisms of CTLA-4 and PD-1 blockade revealed by analyzing TCR usage in blood lymphocytes. *Oncoimmunology.* 2014; 3:e29244.

30. Aggarwal C, Sterman D, Alesi ER, et al. Overall survival after treatment with CAN-2409 plus valacyclovir in combination with continued ICI in patients with stage III/IV NSCLC with an inadequate response to ICI. *Journal of Clinical Oncology.* 2024; 42(16_suppl):8634-8634.

31. Mineo M, Lyons SM, Zdioruk M, et al. Tumor Interferon Signaling Is Regulated by a lncRNA INCR1 Transcribed from the PD-L1 Locus. *Mol Cell.* 2020; 78(6):1207-1223 e1208.

32. Toker J, Iorgulescu JB, Ling AL, et al. Clinical Importance of the lncRNA NEAT1 in Cancer Patients Treated with Immune Checkpoint Inhibitors. *Clin Cancer Res.* 2023; 29(12):2226-2238.

33. Kumagai S, Togashi Y, Kamada T, et al. The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. *Nat Immunol.* 2020; 21(11):1346-1358.

34. Chiocca EA, Gelb AB, Chen CC, et al. Combined immunotherapy with controlled interleukin-12 gene therapy and immune checkpoint blockade in recurrent glioblastoma: An open-label, multi-institutional phase I trial. *Neuro Oncol.* 2022; 24(6):951-963.

35. Cloughesy TF, Mochizuki AY, Orpilla JR, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med.* 2019; 25(3):477-486.

36. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 2009; 10(5):459-466.

37. Omuro A, Reardon DA, Sampson JH, et al. Nivolumab plus radiotherapy with or without temozolomide in newly diagnosed glioblastoma: Results from exploratory phase I cohorts of CheckMate 143. *Neurooncol Adv.* 2022; 4(1):vdac025.

38. Aguilar LK, Shirley LA, Chung VM, et al. Gene-mediated cytotoxic immunotherapy as adjuvant to surgery or chemoradiation for pancreatic adenocarcinoma. *Cancer Immunol Immunother.* 2015; 64(6):727-736.

Figure Legends

Figure 1- Trial schematic and patient survival. **(a)** CONSORT diagram. Trial safety population (n=41) represented by methylation status. **(b)** Representation of time of survival (months) since treatment started for each evaluable patient (n=35). **(c)** Kaplan-Meier survival analysis for all evaluable patients (n=35) by months. **(d)** Kaplan-Meier survival analysis for evaluable patients (n=35) split by resection and methylation status.

Figure 2- WES analysis reveals combinations of mutated genes associated with mortality. (a) Double mutation status assessed in baseline tumor tissue of the 24 evaluable patients with available samples reveal presence of gene pairs significantly negatively associated with survival**. (b)** Identified mutation pairs are more frequently present in patients with MGMT unmethylation than those with methylated MGMT. Fisher's exact test showed the proportions of the two patient groups that do not have the mutation pair not to be significantly different from those that had the mutation pair (p=0.118 for *MED15/HRC*, p=0.052 for SKIDA1/CACNA1A, p=0.351 for SKIDA1/ARID3A and PAXIP1/ARID3A). **(c)** When patients with methylated MGMT were removed from the analysis, only mutation pair (*MED15, HRC*) was still negatively associated with survival (p=0.036).

Figure 3- MIBI Analysis of baseline tumors. (a) Cell clusters whose frequencies were shown by the Elastic Net model to be predictive of increased (blue) or decreased (red) survival. Coefficients and the optimistic c coefficient are shown. **(b)** Example of cellular infiltrate

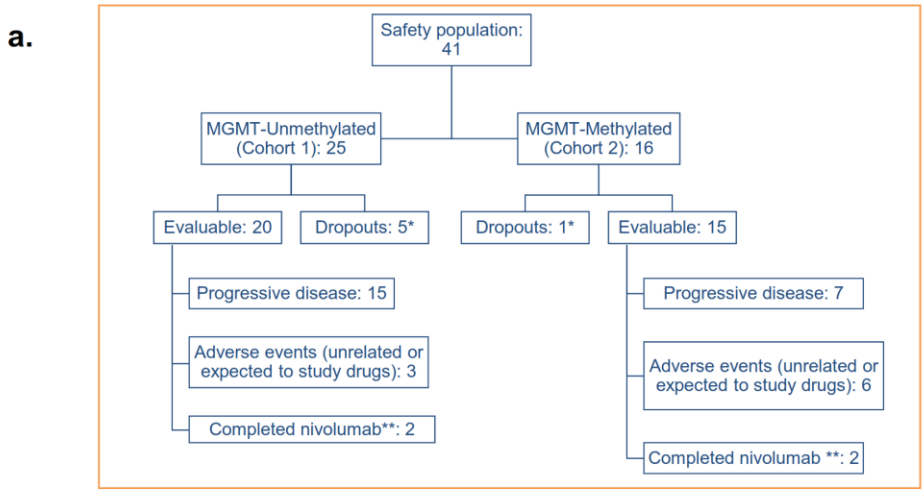
staining for patients in high and low survival group, and corresponding high vs. low coefficients for the B cell/dendritic cell cluster. **(c)** Survival curves based on frequencies of individual clusters from the Elastic Net model of panel A, comparing patients above and below the median frequency. No individual cluster frequencies were statistically significantly predictive of OS.

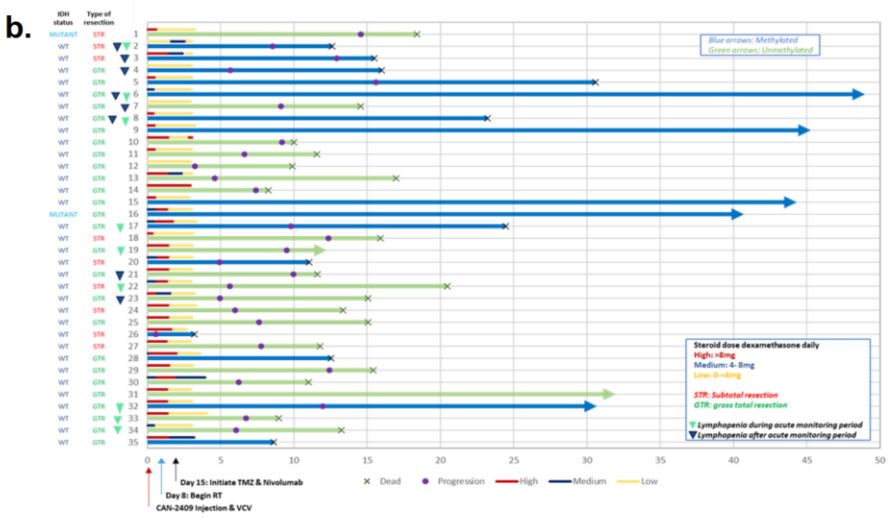
Figure 4- Longitudinal cytokine analyses, n=41. (a) Circulating proteins that showed associations with survival at each of the timepoints assessed for all patients. High hazard ratios (HR) with significant p values (circled and marked by asterisks) include PDCD1 and KLRD1 at baseline. Low hazard ratios at week 3 and week 5 include TNFRSF21, CD5, and IL13. **(b)** Kaplan Meier survival curves revealed cytokines that were significantly associated with survival at baseline, week 3**,** and week 5; **(c)** Volcano plots of immune subsets frequency comparisons between week 3 and Baseline (CAN-2409+VCV only) and week 5 and week 3.

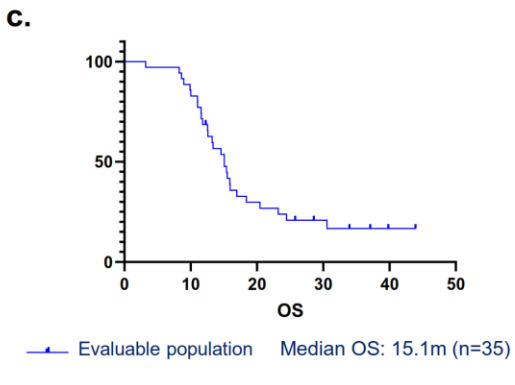
Figure 5- Longitudinal peripheral T cell clonotype analyses. (a) Density, richness and clonality of the TCR frequencies among the T cells from the PBMC at the indicated timepoint. P values < 0.05 are indicated. **(b)** Survival probability depending on Density, Richness and Clonality evaluated by TCR sequencing at Week 3 by Cox regression analysis. The high/low cut-off was determined using the median of the total values for each parameter. Nominal p-values are shown without any multiplicity adjustment and nominal p-value less than 0.05 indicates meaningful difference.

Figure 6- Analyses of the MGMT methylated, GTR patients (a) Selected cytokines that showed significant fold increase in peripheral blood at the 3 (before start of SOC TMZ and nivolumab), 5 (after CAN-2409, SOC, and first Nivolumab cycle), and 11 (after CAN-2409, SOC, and third Nivolumab cycle) week timepoints. Cytokines in bold (PDCD1, PGF, CD40-L, ANGPT1, PDGF subunit B) were characterized by a significant change at week 3 or 5 from previous timepoint (**see also Figure S8a-d**). Cytokines in blue (PDCD1, MMP12, EGF) were also identified as significant predictors of survival outcome at the 3 for PDCD1 and MMP12 **(b, c)** or 5 **(d)** week timepoints. **(e**) CyTOF profiles of significant immune cell sub-population changes in MGMT promoter methylated /GTR long vs. short term survivors **(see also Figure S8a-i)**. **(f, g)** T cell metrics at baseline **(f)** and 3 week timepoint **(g)** between the short vs. long-term MGMT methylated GTR patients.

Figure 1







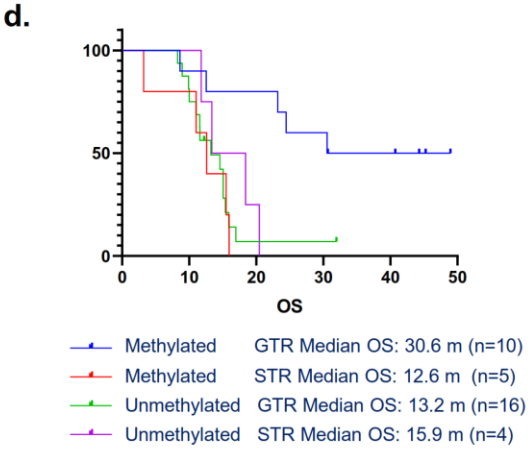


Figure 2

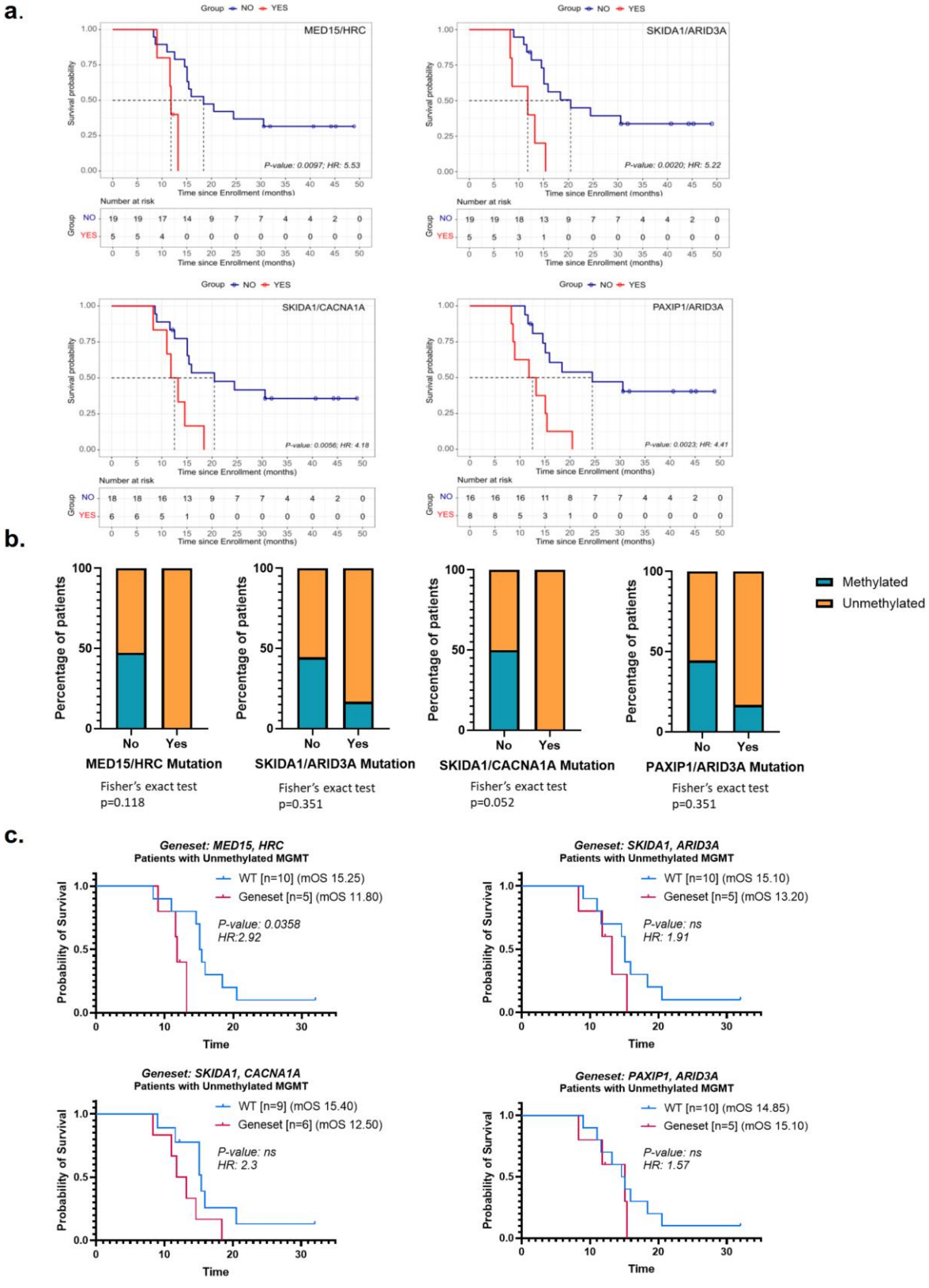
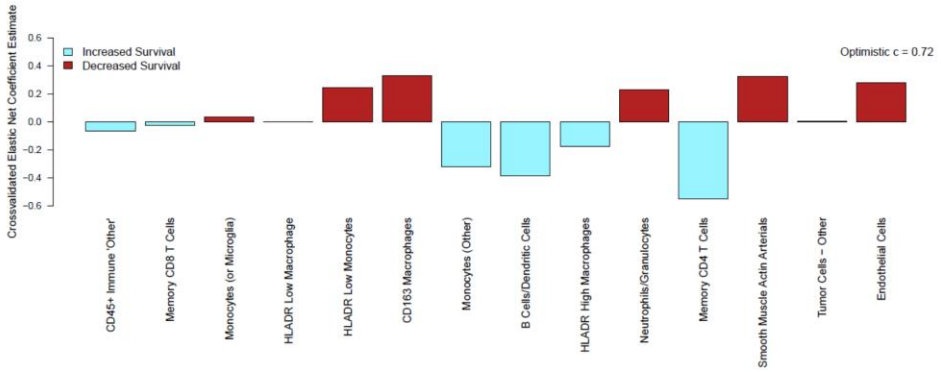
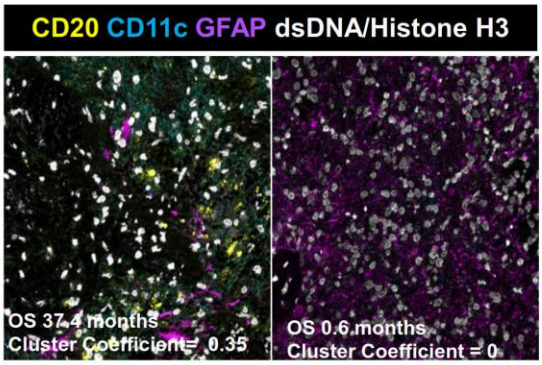


Figure 3

a.



b.



c.

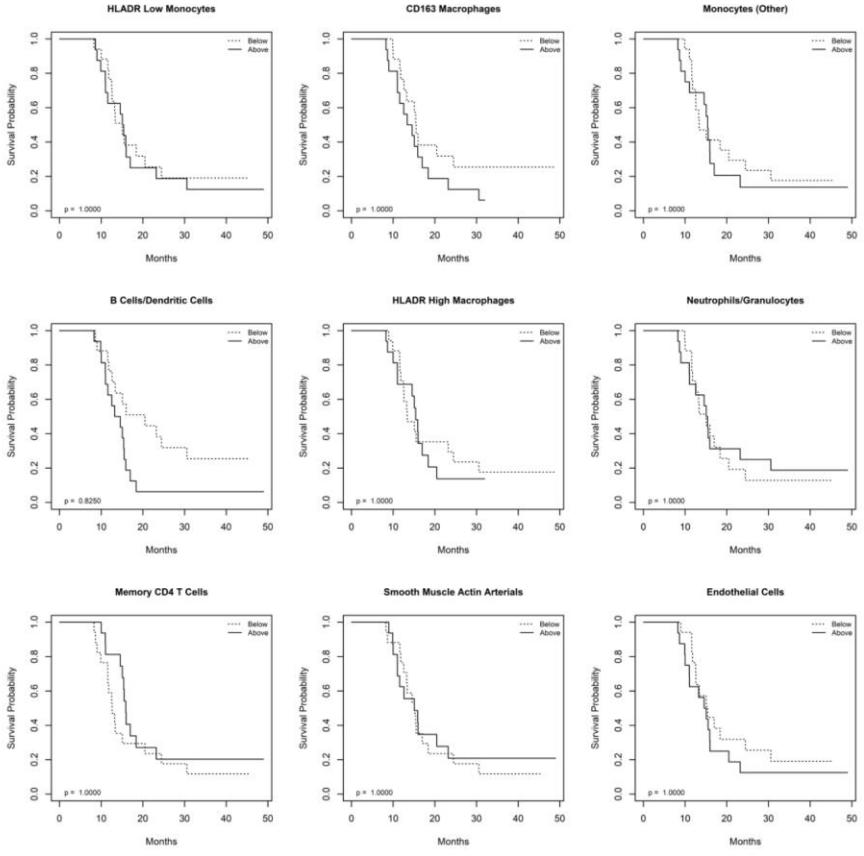


Figure 4

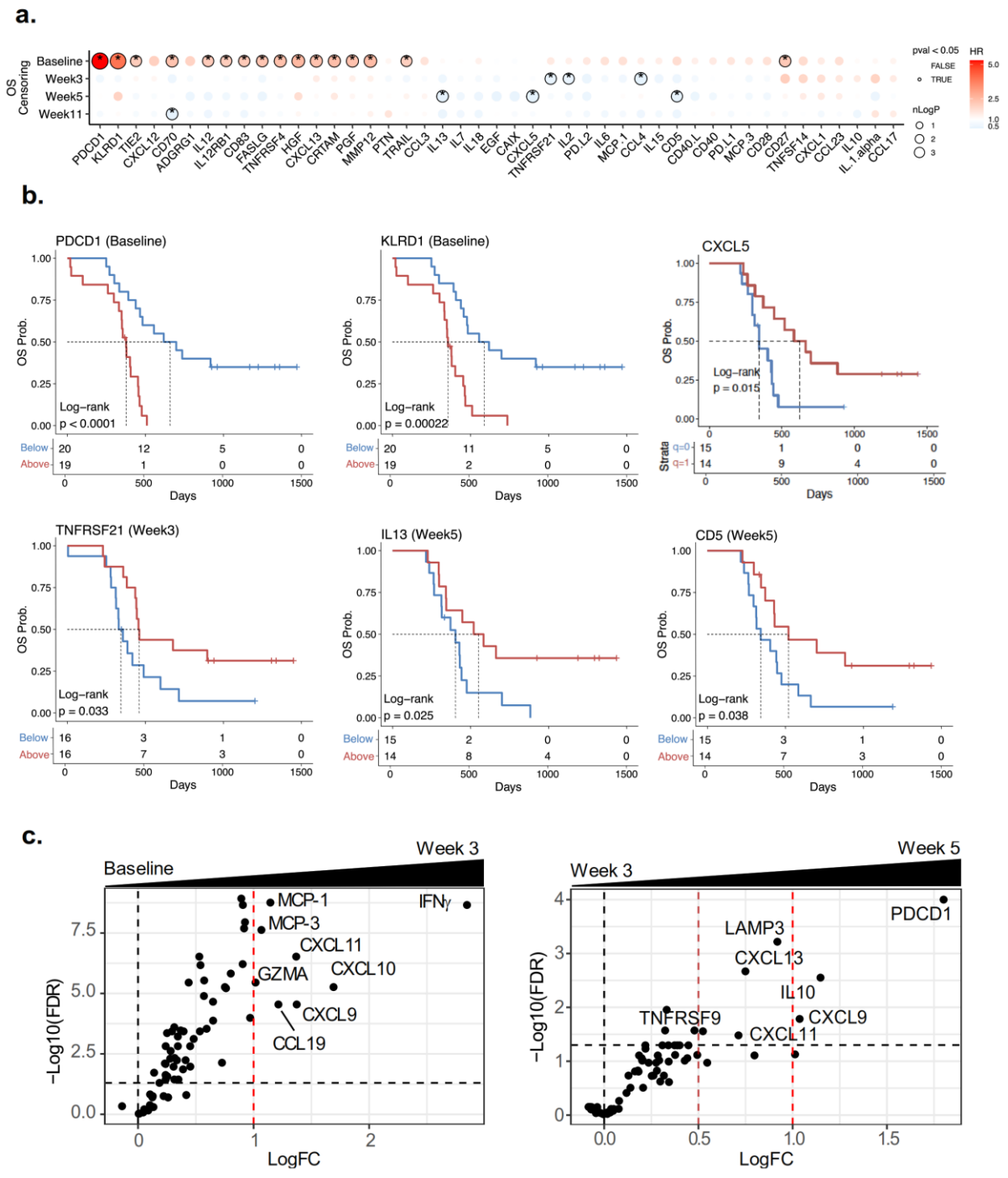


Figure 5

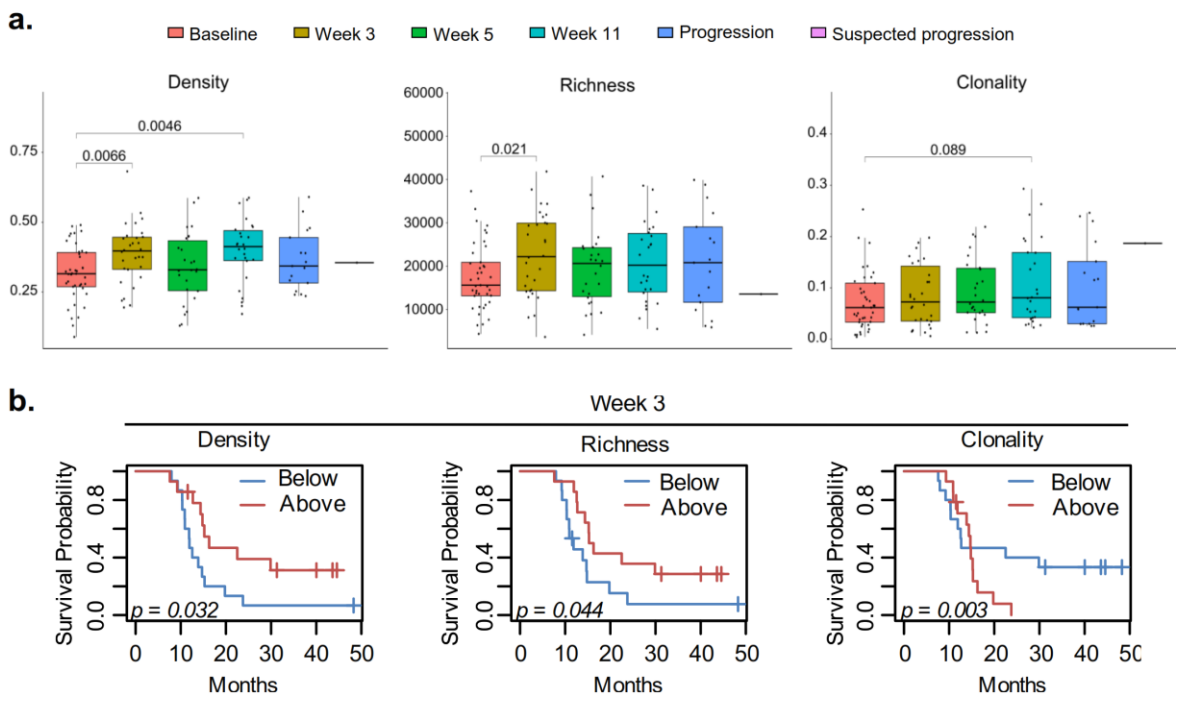


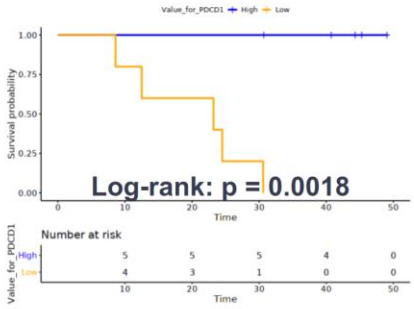
Figure 6

a.

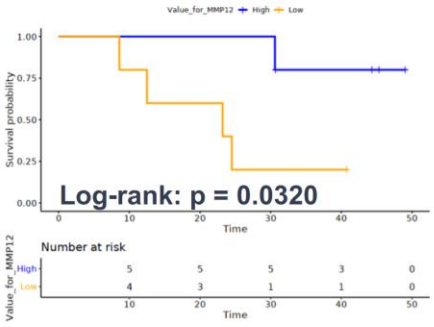
Average fold change – p values

	Week 3	Week 5	Week 11
PDCD1	**0.012**	**0.019**	0.059
MMP12	**0.023**	0.079	0.123
PGF	**0.047**	0.376	0.441
CD40-L	0.114	**0.021**	0.552
EGF	0.431	**0.017**	0.770
ANGPT1	0.793	**0.037**	0.967
PDGF subunit B	0.836	**0.032**	0.889

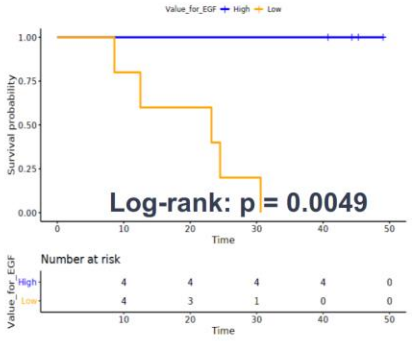
b.



c.



d.



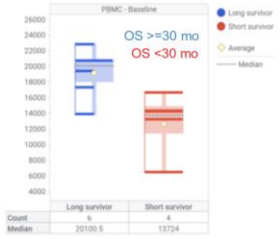
e.

Fold change – p values

Survival Groups	Week3 fc	Week5 fc	Week11 fc
ICOS+ CD4+ TEMRA	**0.017**	0.229	**<0.001**
CD137+CD4+TEMRA	**0.057**	0.591	0.757
OX40+CD4+TEMRA	0.336	**0.052**	0.505
ICOS+NKT	0.203	0.066	**0.002**
CD28+CD4	0.843	0.714	**0.023**
Tregs	0.477	0.795	**0.035**
CD28+CD4+CM	0.845	0.799	**0.037**
CD137+NKT	0.933	0.121	**0.041**
Tim3+CD4	0.722	0.350	**0.047**

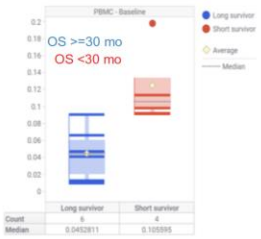
f.

Richness



Mann-Whitney U: 0.038

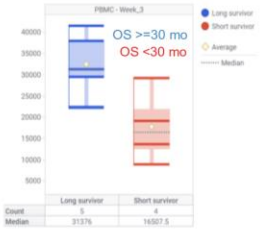
Clonality



Mann-Whitney U: 0.019

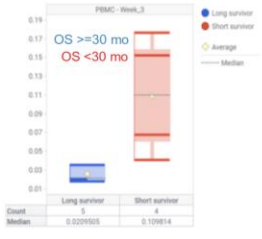
g.

Richness



Mann-Whitney U: 0.063

Clonality



Mann-Whitney U: 0.016