Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein

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Statement of Translational Relevance

Glioblastoma is an aggressive malignant brain tumor for which we have no effective treatment. Standard therapy at initial diagnosis involves maximal surgical resection, followed by radiation and concurrent administration of temozolamide. At recurrence there are few options, and these include administration of avastin or implantation of chemotherapy wafers. For GBM patients there is an unmet medical need for minimally toxic tumor specific therapies. Immunotherapy is an appealing approach to facilitate specific, anti-tumor activity with minimal toxicity. Here we show that specimens derived from patients undergoing resection of recurrent glioblastoma can be used to generate a biologically active and safe polyvalent vaccine consisting of HSPPC-96. These data establish for the first time the specific immunogenicity of peptides associated with tumor derived heat shock protein gp96. Anecdotally, patients exhibiting a tumor specific immune response appeared to survive longer than the one patient who did not.
Abstract:

Background:
Cancer immunotherapy offers hope of a highly specific non-toxic adjuvant treatment. Heat shock protein peptide complexes (HSPPCs) found in cancer cells carry tumor-specific antigenic proteins and can facilitate adaptive and innate immune response. Here we show that peptides bound to a 96 kD chaperone protein (HSP-96) from brain tissue containing glioblastoma multiforme (GBM) can be used to safely immunize patients with recurrent GBM.

Methods:
Multimodality immunomonitoring was completed on 12 patients with recurrent GBM before and after immunization with an autologous HSPPC vaccine derived from surgically resected tumor. Clinical endpoints included safety assessments and overall survival.

Results:
No adverse events attributable to the vaccine were found. Testing of peripheral blood leukocytes (PBLs) before and after vaccination revealed a significant peripheral immune response specific for the peptides bound to HSP-96, in 11 of the 12 patients treated. Brain biopsies of immune responders after vaccination revealed focal CD4, CD8 and CD56 IFN\(\gamma\) positive cell infiltrates, consistent with tumor site specific immune responses. Immune responders had a median survival of 47 weeks after surgery and vaccination, compared to 16 weeks for the single non-responder.

Conclusions:
These data provide the first evidence in humans of individual patient-specific immune responses against autologous tumor derived peptides bound to HSP-96.
Introduction

Primary malignant brain tumors are uniformly fatal, and the 5-year survival rate for the highest grade of malignant glial neoplasm, GBM, is now less than 4%\(^1\). Improvements in conventional treatment modalities have provided some progress, however median survival remains at just over one year from initial diagnosis for patients treated at tertiary care centers\(^2\). Survival data reported for GBM patients from the National Cancer Institute Surveillance Epidemiology and End Results Cancer are significantly worse, possibly reflecting discordance between community based and tertiary practice patterns\(^3\). Currently approved therapy for a newly diagnosed GBM patient in the United States includes maximal surgical resection followed by radiation and temozolomide\(^1\). Upon recurrence there are few approved options and these include surgical implantation of chemotherapy bearing wafers\(^4\) (polifeprosan 20 with carmustine implant, Gliadel Wafer\(^{TM}\)) and systemic administration of the anti-angiogenic agent bevacizumab (Avastin\(^{TM}\))\(^5\),\(^6\). Each of these therapies has shown modest improvement in survival of recurrent GBM patients, with notable treatment related toxicities including wound breakdown after surgical resection\(^7\). Bevacizumab as a single agent in particular may be problematic for recurrent GBM patients because progression patterns after treatment can be multifocal, which is atypical for glioma recurrence\(^8\).

There is an unmet medical need for highly specific and non-toxic adjuvant therapy to treat recurrent GBM patients undergoing surgical resection. Off target effects of adjuvant therapy for brain tumor patients can be particularly disabling, including focal neurological deficits and cognitive dysfunction\(^9\). Immunotherapy is an appealing method to specifically target tumor cells in glioma patients, while minimizing adverse treatment effects\(^10\). Several methods have been used successfully to evoke anti-tumor immunity in GBM patients, with evidence of peripheral and site-specific immune responses\(^11\)-\(^15\). HSPPC-96 consists of the HSP gp-96 and a wide array of chaperoned...
proteins, including antigenic peptides. HSPPC-96 immunization works mechanistically by interacting with APCs via specific receptors, including CD91\textsuperscript{16, 17}. After binding to CD91, the HSPPC-96 complex is internalized and the chaperoned peptides are presented by Class I and Class II Major Histocompatibility Complexes. The highly specific nature of the interaction between HSPPC-96 and APCs is a significant advantage over other cancer vaccine approaches; and has been shown to facilitate robust CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell immune responses\textsuperscript{18, 19}. Clinical grade HSPPC-96 can be easily purified from solid tumor and has been safely tested in hundreds of patients with a variety of solid tumor types\textsuperscript{20-23}.

**Methods**

**Patient Population:** All twelve patients treated with vaccine were enrolled at the University of California, San Francisco. Pre-surgery eligibility included the presence of resectable recurrent glioma tumor tissue of grade 3 or 4. Patients with a Karnofsky Performance Status of $\geq 60$ and a life expectancy greater than 8 weeks were eligible for the study. All eligible patients with $\geq 4$ vaccines available for clinical use received investigational therapy. All patients enrolled (N=28) were surgical candidates based on presumed radiographic progression of intracranial tumor who were not being treated with corticosteroids at the time of surgical resection. Intraoperative tissue was confirmed to be recurrent glioma prior to sending for vaccine manufacturing at Agenus Incorporated. Patients underwent biopsy after vaccine administration as part of standard care, and not exclusively for the purposes of the present study. Patients who underwent biopsy after vaccine administration were consented for surgery in the standard fashion. The clinical trial is registered at www.clinicaltrials.gov.
**Exclusion Criteria:** Pre-surgery exclusion criteria included a history of immunodeficiency or immunosuppressive drug use excluding corticosteroids, current malignancies at other sites or other cancers within 5 years, active uncontrolled infection, and other serious medical conditions. Between 2 and 4 weeks after surgery, patients were evaluated to confirm eligibility, which included the availability of 7g of viable tumor tissue from which to generate vaccine. The first cohort of 6 patients received 25 micrograms of HSSPC-96 every 2 weeks for the first 4 vaccinations followed by 2 week dosing. The second cohort of 6 patients received 25 micrograms of HSSPC-96 every week for the first 4 vaccinations followed by 2 week dosing. The trial was approved by the University of California, San Francisco Institutional review board and was done in accordance with the Declaration of Helsinki and guidelines for Good Clinical Practice.

During treatment, patients were evaluated for safety, toxicity, autoimmunity, and injection site reactions. Patients underwent tumor assessments clinically every 4 weeks and radiographically every 8 weeks per standard of care protocol. Immune response was evaluated at time points obtained prior to surgery, baseline (prior to the first treatment), and a post-treatment time point. The primary endpoint was safety and feasibility. Secondary endpoints were overall survival and immune response to therapy (treatment #1 blood sample compared to later vaccination time points).

**Radiographic Assessments:** Standard MRI scans with contrast were obtained at 8-week intervals on each patient or at shorter intervals if clinically indicated (i.e. new neurological deficit or deterioration). Patients with evidence of a moderate increase in contrast enhancement after vaccination (i.e. up to 20% increase in any residual enhancement after surgical resection) were not declared as tumor progressions unless there was an associated clinical decline. As indicated above, if medically indicated patients underwent biopsy or surgical resection after treatment with vaccine.
**Vaccine Preparation:** Macroscopically non-necrotic tumor was surgically resected and shipped on dry ice to Agenus Incorporated and processed under current good manufacturing practice conditions. HSPPC-96 preparations were dispensed into individually identified, 25-microgram aliquots and shipped on dry ice to the pharmacy, where they were stored at -80°C.

**Peripheral Blood Leukocyte isolation:** Approximately 90mls of blood was obtained prior to surgery, baseline (prior to the first treatment), and a post-treatment time point, stored at room temperature, and processed within 2 hours of the blood draw for the isolation of peripheral blood leukocytes (PBLs). PBLs were isolated by Ficoll centrifugation and stored in liquid nitrogen until the time of analysis.

**Tumor Infiltrating Leukocyte Isolation:** Tumors were Collagenase D treated for 45 minutes at 37°C and mechanically dissociated. Tumor infiltrating lymphocytes (TIL) were isolated with a three-step Percoll density gradient as described (Ford and Sedgwick, 1995).

**In vitro restimulation:** Restimulations of bulk PBL were done using 10 micrograms/ml of autologous vaccine (agp96) remaining in the UCSF pharmacy following patient removal from the trial. If all vaccine was used, filtered excess was obtained from Agenus, Inc (Lexington, MA). 10 micrograms/ml recombinant human gp96 TRA-1 (rgp96, AbNova, Inc.) was used as a negative control. Cells were stimulated for 48 hours for qPCR analysis, or 72 hours for intracellular cytokine staining or proliferation. All assays were done in triplicate. Negative controls included isotype control staining for each antibody, unstimulated cells from all time points and rgp96 stimulated cells for all time points. In some cases, due to limited supply of the residual vaccine, restimulation assays were not done (ND).

**Intracellular Cytokine Staining:** Positive controls were single color staining for CD45 in each channel and PMA/Ionomycin (Sigma) stimulation to induce IFNγ production. Intracellular staining
(ICS) was used to examine T-cell (defined as CD3 positive, CD4 or CD8 positive), and NK cell (defined as CD3 negative, CD56 or NKG2D positive) IFNγ production following restimulation. Regulatory T-cell percentages were also examined (defined as CD3 positive, CD4 positive, CD25 positive and FoxP3 positive). Fluorescently conjugated antibodies were acquired from Becton Dickinson (San Diego, CA) or eBioscience (San Diego, CA). Gates were set based on the population of interest relative to positive and negative controls. Positive cells were determined to be those above the threshold fluorescence intensity based on negative controls listed above for each patient.

**Reverse Transcription and Quantitative PCR (qPCR):** Cells were stimulated as described above for 48 hours and whole cell mRNA was isolated using the Qiagen RNeasy MicroKit (Qiagen Inc., Valencia, CA). 250 ng mRNA was reverse transcribed using Superscript III First Strand Synthesis System (InVitrogen, Carlsbad, CA). cDNA was amplified using target specific primers and the Sybr Green Master Mix (Applied Biosystems, Foster City, CA) and analyzed using a BioRad iQ5 thermocycler (Bio-Rad, Hercules, CA). The primer sets used were HPRT forward 5' GAC CAG TCA ACA GGG GAC AT 3', HPRT reverse 5' CCT GAC CAAGGA AAG CAA AG 3', IFNγ forward 5' CGA GAT GAC TTC GAA AAG CTG 3', IFNγ reverse 5' ATA TTG CAG GCA GGA CAA CC3'. All qPCR samples were first analyzed using the Livak method and expressed as mRNA units relative to the housekeeping gene HPRT, which was selected because it is present in low copy number and varies minimally following stimulation. All expression levels were displayed as relative to the level expressed by the pre-surgery unstimulated PBMCs for each patient using the Pfaffl method.

**Proliferation:** PBL were enriched for T-cells by negative magnetic selection using Easy Sep CD3 enrichment kit according to the manufacturer’s instructions (Stem Cell Technologies, Inc. Vancouver, BC). Proliferation was assessed in T-cells by carboxyfluorescein succinimidyl ester
(CFSE) dilution detected by flow cytometry using the CellTrace CFSE Cell Proliferation kit (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Enriched T-cells were incubated with 10 micromolar CFSE for 10 minutes, quenched with five volumes of complete media and washed three times. CFSE stained cells were then subjected to \textit{in vitro} restimulation described above for 72 hours, and analyzed by flow cytometry for CFSE dilution.

**PBL Phenotyping:** Phenotypes of circulating cells was assessed prior to surgery, prior to administration one, and just prior to administration three. Cells were stained for 25 minutes on ice with the following antibody combinations and washed three times: a) CD3 FITC, CD8 PE, CD4 PerCP, NKG2D APC, b) FoxP3 FITC, CD4 PE, CD3 PerCP, FoxP3, and CD25 APC, and c) CD3 FITC, CD8 PE, CD56 PeCy7, NKG2D APC. All antibodies for surface staining were obtained from BD Pharmingen or eBioscience. PBL were then analyzed for protein expression by flow cytometry.

**Flow Cytometry:** Samples were stained with antibody cocktails as described above and analyzed using a BD FACSCalibur. Samples were acquired using CellQuest Pro software and analyzed using FlowJo software (Treestar).

**Statistical Analysis:** Comparison of samples to determine statistical significance was performed using a students two tailed t test. Statistical significance was determined by Prism graphing software with a p value <0.05 required to be statistically significant (Graphpad Inc). In patients lacking sufficient residual vaccine for restimulation across all assays, restimulation was analyzed with the assay using the least amount of patient vaccine, quantitative PCR. The positive response was based on the statistically significant induction of HSPPC-96 response over background. Determination of patient overall responsiveness to vaccine was based on qPCR data as no patient who was analyzed for all assays was positive for qPCR anf negative for flow cytometry.
Results

To assess the feasibility, safety, and anti-tumor immunogenicity of HSPPC-96 in patients with recurrent high-grade glioma we initiated a Phase I dose escalation trial. Twelve patients met inclusion criteria for post-surgical enrollment and safety assessments. The mean age of the treated patient population was 52 years (range: 36y – 73y), and patients were treated with a variety of adjuvant therapies prior to recurrence (patient demographics are shown in Supplementary Tables 1A-1C). In terms of feasibility, twenty-eight patients were enrolled initially but only 12 met the final inclusion criteria for treatment and safety assessments. Reasons for exclusion from analysis included the following: processing error during surgical resection (1 patient), resected tumor was predominantly treatment effect with minimal viable tumor for vaccine and therefore inadequate for vaccine production (9 patients), consent was withdrawn after sending tumor for production but prior to vaccination (4 patients), clinical deterioration or progression prior to administering 4 vaccines with requisite follow-up (2 patients).

Consistent with previous reports, no toxicity attributable to HSPPC-96 was observed in any of the 12 patients treated, with the exception of mild injection site erythema and/or induration (<5mm). There were no serious adverse events associated with vaccine administration (Supplementary Table 2). To document peripheral immune responses we developed a novel assay that takes advantage of HSP’s unique specificity for binding of APCs. Pre and post vaccine blood from all patients was evaluated by an antigen re-presentation assay using autologous HSPPC-96 (agp96) in parallel with recombinant (non-antigen bearing) HSP-96 (rgp96), as a control for non-specific responses attributable to HSP-96. IFNγ production was then measured using quantitative RTPCR. Further analysis was performed on a subset of patients including: 1) intracellular cytokine staining (ICS) to detect which immune effector cells were secreting pro-inflammatory cytokines, 2)
CFSE dilution to assay T-cell proliferation in response to tumor specific antigen, and 3) phenotyping of PBL surface markers to characterize the peripheral immune compartment. When feasible, post vaccination, site directed brain tumor biopsies were performed, with analysis of infiltrating immune effector cells. A summary of the assays and description of implementation for each patient with specific time points are shown in Supplementary Figures 2A-2C, and Table 1.

Eleven of the twelve patients had a significant response to HSPCC-96 when controlling for the non-specific adjuvant effect of HSP (Figure 1A); indicating specific immunization against chaperoned antigenic peptides. An example of an immune responder compared to the single non-responder is shown in Figure 1B. Patient 009 experienced a 14.1 fold increase in IFNγ transcription by total PBLs in response to antigenic gp96 (agp96) following vaccination as compared to patient 003 who had only a 3.28 fold increase which was not significant when compared to the effect of recombinant gp96 (rgp96). In order to identify the populations of immune effector cells responsible for the observed increase in IFNγ production, we performed flow cytometry based ICS. Following restimulation with agp96, we found that innate NK and adaptive T-cells contribute to the IFNγ production observed by qPCR (Figure 1C). An increase in IFNγ producing T-cells suggests that peptides chaperoned by gp96 induced peptide specific T-cell expansion following repeated vaccination.

Because of reagent limitations (i.e. not all patients had sufficient vaccine for both clinical use and immunomonitoring), exploratory assays including ICS, CFSE and phenotypic analysis could only be performed on a subset of patients. IFNγ production was associated with T-cell proliferation in response to agp96 restimulation. An example of this is shown in Figure 3A, with 12.4% of T-cells proliferating following vaccination and restimulation in patient #007 after 19 vaccinations. The data for patient 007 indicate that T-cell division is driven by agp96, consistent with tumor peptide
specific responses. This is in contrast to the data from the non-responder patient 003, with only 1.1% of T-cells proliferating in response to re-stimulation with agp96. In patients who underwent surgery after vaccination, a post-vaccine biopsy at the original tumor site was performed. An increase in IFN-γ positive CD3+CD8-, CD3+CD8+ and CD3-CD56+ cells (Figure 4, 5) was seen in all patients biopsied, indicating localization of immune effector cells to the tumor site after vaccination. Phenotypic analysis of circulating T-cells shown in Supplementary Figure 4 provides insights into how vaccination with HSPPC-96 can impact the peripheral immune compartment. Following three HSPPC-96 vaccinations, CD4 and CD8+ T-cell frequencies remain stable, but were associated with an expansion of a CD3+, CD4/CD8 double positive population, which was not seen in the non-responder (Supplementary Figure 3). Vaccination in responders was also associated with an increased frequency of NK cells (Supplementary Figure 2A-B), and a decrease in CD4+, CD25+, Fox P3+ regulatory T-cells in circulation (Supplementary Figure 2C). Interestingly the single non-responder had significantly elevated regulatory T-cells, as compared to the other treated patients (Supplementary Figure 3).

Results of immunomonitoring data are summarized in Table 1 and reveal that patients vaccinated with HSPPC-96 show an immune response peripherally and suggest that this correlates with local pro-inflammatory responses. Collectively our results suggest that surgically resected tumor from recurrent glioma patients contain adequate amounts of HSPPC-96 that can be safely administered as a source of adjuvant immunotherapy. Ex vivo, restimulation following such vaccination resulted in improved pro-inflammatory immune responses as indicated by NK and T-cell cytokine production and T-cell proliferation, suggesting that tumor specific T-cells may clonally expand and release Th1 cytokines in vivo following vaccination. Long-term individual specific
cellular immunity against a solid tumor after vaccination with autologous HSPPC-96 in humans has not been previously shown, appropriately controlled for the non-specific adjuvant effects of HSPs.

Brain MRI scans with contrast were obtained at 8-week intervals on each patient or at shorter intervals if clinically indicated. Scans were reviewed for signs of tumor progression based on an increase in contrast enhancement. We used parameters similar to the recently published RANO criteria\textsuperscript{24} and allowed for up to 20\% increase in focal enhancement provided that there was no associated clinical decline. An example of a true progressor, as compared to a patient with treatment effect after vaccination, is shown in Figure #6.

**Discussion**

How might the results of our study impact the treatment of patients with GBM or other solid tumors? Successful cancer immunotherapy requires immunization with relevant antigen, but simply identifying the correct antigens is not sufficient. The tumor antigens must be delivered in a manner that can facilitate antigen presentation, typically by APCs at the site of vaccination. A number of other strategies have been devised to facilitate antigen delivery to APCs including \textit{ex vivo} loading of dendritic cells followed by immunization, as well as simultaneous administration of cytokines and adjuvants with recombinant peptides to promote APC activation\textsuperscript{14, 15, 26, 27}. Cancer vaccines composed of HSPPCs have the distinct advantage of a specific mechanism to engage APCs.

Although the documentation of a polyvalent immune response is an important step, it is only the first of many necessary to facilitate efficacy. HSPPC-96 has been tested in large multicenter trials with modest evidence of clinical efficacy. However a closer examination of the clinical data published to date using HSPPC-96 would suggest that sufficient consideration to optimizing the patient population for vaccination was not put into the study designs. It is clear now that patients with minimal tumor burden are the best candidates for immunotherapy\textsuperscript{28}. Several groups have
shown a correlation with tumor burden and an elevated level of regulatory T-cells in circulation; which in turn act to suppress immunity\textsuperscript{22, 29}. A closer examination post- hoc of both the renal cell carcinoma and melanoma trials using HSPCC-96 shows a statistically significant benefit to vaccination of patients with minimal or stable residual disease\textsuperscript{23, 25}. These trials lacked rigorous immunomonitoring necessary to document immune responses. Anecdotally, data from our study would seem to support the premise of treating patients with minimal tumor burden as well. The only patient to not show a peripheral immune response had intracranial tumor at time of initial vaccination, and relatively high levels of circulating regulatory T-cells (patient #003). Another hurdle to effective immunotherapy can arise at the molecular level where genetic features of tumor can make them suboptimal targets for immunotherapy. For example, tumor cells with loss of the tumor suppressor gene PTEN have increased expression of the immunosuppressive protein B7-H1, which in turn inhibits T-cell mediated killing of tumor cells\textsuperscript{30, 31}. The previously published reports on HSPPC-96 included trials with patients containing tumor burden in some cases, as well as no formal characterization of the immunoresistant phenotype of the tumors being treated.

There are limitations to our study worth mentioning, in particular the inability to perform all of our assays for each patient. Because autologous HSPPC-96 was first and foremost used as a clinical agent in the trial, some patients did not have excess HSPPC-96 to use in multiple restimulation assays including ICS and CFSE proliferation assays. However this limitation is mitigated in part by restimulation experiments uniformly performed on all the patients, using qPCR assays of pro-inflammatory transcripts with autologous HSPPC-96. The overall responses were determined based on all assays when available, but if unavailable, were based on significant responses to HSPPC-96 by qPCR. The rationale for this analysis was that patients who had a significant response as analyzed by qPCR also were statistically positive when analyzed by flow
cytometry. However, given the small number of patients available for this study, patients were not analyzed for predictive time to recurrence or association with treatment effect following vaccination. This binary assignment will be refined on the analysis of the remainder of Phase II patients, as a larger patient population will allow further stratification of patients. These limitations also apply to our post-treatment site directed biopsies, which were performed only on patients undergoing post-treatment surgery. In addition, in many cases the amount of tissue available for vaccine limited the utilization of tissue for exploratory analysis. Accordingly not all patients had pre-treatment tissue evaluated for TIL assessments shown in Figure 4 and Figure 5. Our study also highlights the limitations associated with applying a phase I surgically based study to a multiply recurrent GBM patient population that requires acquisition of viable tumor, with minimal treatment effect. Of the 28 patients enrolled in the study, only 12 received 4 vaccines with requisite follow-up for safety. We believe that this is more related to the patient population than the technology, as multiple recurrent GBM patients are more likely to have a mixture of treatment effect with tumor than patients who have their first recurrent after standard radiation and temozolomide. Recently a Phase II trial evaluating this technology for GBM patients at first recurrence completed accrual and is currently in follow-up phase of analysis for overall survival determination. Our experience was substantially better with this patient population with vaccine production success approaching 90% of patients who underwent surgical resection. Another limitation to this study is that we can not accurately predict or project the possible cost of the vaccine, beyond the standard costs associated with surgery and hospitalization. Because the product is not yet formally commercialized in the United States, and because much of the technology is proprietary, it is impossible to accurately predict what a patient could potentially be charged.
In conclusion, we have shown for the first time in humans that a tumor specific immune response to peptides bound to gp96 can be generated with autologous HSPPC-96 derived from a surgically resected solid tumor. There have been other reports describing immune responses after treatment with HSPPC-96 for patients with a variety of cancer types \(^{20, 33-37}\). These studies collectively used a combination of phenotypic marker analysis of PBLs after vaccination or restimulation assays with a variety of antigens, including autologous tumor cell components and allogeneic cancer cell lines to assess immune responses. These important studies documented that HSPPC-96 can evoke an immune response, but lacked the recombinant gp96 control utilized in our study.

There is abundant evidence of immune responses to human cancers and these responses may be individual patient specific, or shared among patients. Immune responses shared between patients have shown inconsistent correlation with clinical outcomes\(^{19, 38}\), in contrast individual patient specific immune responses have shown excellent correlation with clinical outcomes\(^{21, 39-42}\). Our observations are significant because they demonstrate a general mechanism to elicit individual patient-specific immune responses that appear to correlate with clinical outcome: Immune responders had a median survival of 47 weeks, as compared to 16 weeks for the non-responder. Our study also affirms the safety and feasibility of an autologous HSPPC-96 approach for recurrent glioma patients. Our results, combined with the need for biologically active non-toxic adjuvant therapies, provide the impetus for further testing of autologous HSPPC-96 in recurrent glioma patients undergoing surgical resection. The documentation of a robust immune response suggests that re-evaluation of HSPPC-96 in optimally selected patients with other types of solid tumors is warranted. Finally, the advent of clinical grade antibodies targeting inhibitor pathways such as
CTLA-4$^{43}$ and PD-1$^{44}$ provide practical approaches to amplifying the tumor specific immunity we describe $^{45,46}$. 
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Authorship:

Immunomonitoring data collection and analysis: CC, SH, BA, JO. Patient enrollment and survival analysis: VK, AF, NB, SC, JC MP. Operative surgical sample collection for local immunesurveillance data: MSB, MWD, ATP. Study design: ATP

Disclosure of Conflict of Interest:

The authors have no conflict of interests or disclosures
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Figure Legends:

**Figure 1.** IFNγ transcription increases following autologous heat shock protein restimulation ex vivo, and correlates with clinical response. Total patient PBL isolated prior to tumor resection (Pre-Op) or at the time of the final vaccine administration (Final Vacc) were restimulated with 10 micrograms/ml of autologous, tumor derived HSPPC-96 (agp96), recombinant HSP-96 (rgp96) or left unstimulated. 48 hours later, whole cell mRNA was isolated and reverse transcribed. cDNA was subjected to quantitative PCR for IFNγ and expressed relative to the housekeeping gene HPRT. Relative expression was then expressed as a fold induction relative to unstimulated cells. A) Representative qPCR of a clinical responder (Patient #009) and a clinical non-responder (Patient #003). B) Induction of IFNγ in negative control unstimulated (grey bars) and positive control Phorbol myristate/ Ionomycin stimulated PBL in patients C) Summary of qPCR data following restimulation shown relative to unstimulated controls graphed in Figure 1B, *= p<0.05.

**Figure 2.** Intracellular IFNγ protein secretion increases following autologous heat shock protein restimulation ex vivo, and correlates with clinical response. Total patient PBL were cultured for 72 hours alone, with rgp96 or with agp96 (10 micrograms/ml). 66 hours after stimulation, BFA was added to wells, and cells were surface stained for CD3, NKG2D (or CD56), permeabilized and stained for IFNγ. PMA/Ionomycin stimulated cells were also stained as a positive control. A) Representative flow cytometry for patient # 009 of CD3+ cell production of IFNγ, B) Patient #009 flow cytometry of CD3- gated, NKG2D positive IFNγ production. C) Comparison % IFNγ producing T cells (top panels) and NK cells (bottom panels) of a clinical responder (patient #009) and a clinical non-responder (patient #003).

**Figure 3.** T cell proliferation following autologous heat shock protein restimulation ex vivo correlates with clinical response. CFSE labeled patient PBL were cultured for 72 hours alone, with rgp96 or with agp96 (10 micrograms/ml) and analyzed by flow cytometry for proliferation by CFSE dilution. A) Representative comparison of a clinical responder (patient #007) and a clinical non-responder (patient #003). Histograms represent CD3 gated populations. B) Summary of % CD3+ cells with dilute CFSE for patient #007 and patient #003.
Figure 4. HSPPC-96 vaccination increases IFN\(\gamma\) producing T cells found infiltrating the tumor. Tumor infiltrating lymphocytes were isolated from clinical trial patients opting for a repeat surgery following vaccination and recurrence. TILs were cultured for 6 hours in the presence of BFA, stained for CD3, CD45. TILs were then permeabilized, stained for IFN\(\gamma\) and analyzed by flow cytometry. A) Top panels show gating of TILs for CD45 +, CD3 + cells, bottom panels show % of CD3+, CD8+ cell producing IFN\(\gamma\). B) Summary of CD3+ CD8+ cell IFN\(\gamma\) production in all Phase I/II patients following surgery prior to and following HSPPC-96 vaccination. C) Summary of CD3+ CD4+ cell IFN\(\gamma\) production in all Phase I/II patients following surgery prior to and following HSPPC-96 vaccination.

Figure 5. HSPPC-96 vaccination increases IFN\(\gamma\) producing T cells and NK cells found infiltrating the tumor. Tumor infiltrating lymphocytes were isolated from clinical trial patients opting for a repeat surgery following vaccination and recurrence. TILs were cultured for 6 hours in the presence of BFA, stained for CD3, NKG2D (or CD56). TILs were then permeabilized, stained for IFN\(\gamma\) and analyzed by flow cytometry. A) Representative staining of TILs (top panels) and IFN\(\gamma\) production by NK cells (bottom panels). Plots show CD45+, CD3-, CD56+ gated cells. B) Summary of CD3- CD56+ lymphocyte production of IFN\(\gamma\) in all Phase I/II patients following surgery prior to and following HSPPC-96 vaccination.

Figure 6. Representative MRI images of patient at enrollment, immediately post-operatively, and at true recurrence or treatment effect. T1-gadolinium enhanced images of a treated patient at enrollment (A), then immediately after surgical resection (B), and at recurrence (C). T1-gadolinium enhanced images of patient treated with the HSP vaccine at enrollment (D), immediately post-resection (E), and at pseudo-progression, which was proven on pathology to be treatment effect (F)

Table Legends

Table 1: Summary of patient study #, assays performed, immune monitoring time point and overall survival after tumor resection. Ex vivo restimulation assays were performed to determine specific immune responses to HSPPC-96. The time points for each patient included a) pre-surgery,
b) post-surgery, pre-vaccination and c) at the time of the final vaccination. Restimulation was performed using 10μg/ml HSPPC-96 or recombinant gp96 for 48 hours. Statistical significance of responses was determined by comparing cytokine production 1) relative to pre-stimulation with HSPPC-96 and 2) relative to recombinant gp96 protein (tumor peptide negative). Patient responses were evaluated as + if p<0.05 for both for a given assay using a two tailed student’s t-test. All patients in the Phase I/II cohort were analyzed by whole cell reverse transcription and quantitative PCR for IFNγ production following restimulation. When sufficient residual vaccine was available, additional assays were performed, including intracellular cytokine staining and analysis of proliferation using CFSE dilution. Patients were classified as overall responders if they were + for at least one assay. ND: not determined due to lack of residual vaccine.
Figure 1

A. Representative IFNγ qPCR

Patient 009: Responder

- recombinant gp96
- autologous HSPPC-96

Fold induction vs. media alone
Pre-Op | Final Vacc
--- | ---
0 | 10

Patient 003: Non-Responder

- recombinant gp96
- autologous HSPPC-96

Fold induction vs. media alone
Pre-Op | Final Vacc
--- | ---
0 | 5

B. Restimulation controls, pre-vaccination

Unstimulated | PMA/Iono
--- | ---
002 | 40
003 | 30
005 | 20
007 | 10
009 | 0
011 | 10
013 | 20
017 | 30
023 | 40
024 | 50
025 | 60
028 | 70

C. Summary of patient IFNγ transcription following autologous HSPPC-96 restimulation

Expression relative to unstimulated PBL
Pre-Op | Last Vaccination
--- | ---
002 | 0
003 | 5
005 | 10
007 | 15
009 | 20
011 | 25
013 | 30
017 | 35
023 | 40
024 | 45
025 | 50
028 | 55
Figure 3

A. Patient #007: Responder vs. Patient #003: Non-Responder
   - No Stim
   - + r gp96
   - + a gp96

B. Graph showing % CD8+ Cells Proliferating
   - No Stim
   - r gp96
   - a gp96

#007: Responder vs. #003: Non-Responder
Figure 5

A. Patient #005: Pre-Vaccine

Patient #005: Post-Vaccine

B. % CD3 - CD86+ IFN+ for each patient:

- Pre-Vaccination
- Post-Vaccination
<table>
<thead>
<tr>
<th>Patient #</th>
<th>Survival (weeks)</th>
<th>qPCR</th>
<th>ICS</th>
<th>CFSE</th>
<th>Overall Immune Responder</th>
<th>Immune monitoring Time point (weeks)</th>
<th>Post Vaccination tumor resection? (TIL analysis available)</th>
<th>Residual Tumor or Recurrence (RTR) or Treatment Effect (TE) at re-op?</th>
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Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein

Courtney A. Crane, Seunggu J. Han, Brian J. Ahn, et al.

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