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Comparison of glioma-associated antigen peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients

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Summary

Dendritic cell (DC) vaccination is emerging as a promising therapeutic option for malignant glioma patients. However, the optimal antigen formulation for loading these cells has yet to be established. The objective of this study was to compare the safety, feasibility, and immune responses of malignant glioma patients on two different DC vaccination protocols. 28 patients were treated with autologous tumor lysate (ATL)-pulsed DC vaccination, while 6 patients were treated with glioma-associated antigen (GAA) peptide-pulsed DCs. Safety, toxicity, feasibility and correlative immune monitoring assay results were compared between patients on each trial. Due to HLA subtype restrictions on the GAA-DC trial, 6/15 screened patients were eligible for treatment, while 28/32 patients passed eligibility screening for the ATL-DC trial. Elevated frequencies of activated natural killer (NK) cells were observed in the peripheral blood from GAA-DC patients

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compared with the ATL-DC patients. In addition, a significant correlation was observed between decreased regulatory T lymphocyte (Treg) ratios (post/pre vaccination) and overall survival (OS; p=0.004) in patients on both trials. In fact, Treg ratios were independently prognostic for OS in these patients, while tumor pathology was not in multivariate analyses. In conclusion, these results suggest that ATL-DC vaccination is associated with wider patient eligibility compared with GAA-DC vaccination. Decreased post/pre-vaccination Treg ratios and decreased frequencies of activated NK cells were associated with prolonged survival in patients from both trials, suggesting that these lymphocyte subsets may be relevant immune monitoring endpoints for immunotherapy protocols in malignant glioma patients.

Keywords

Clinical trial; dendritic cells; glioma-associated antigen; tumor lysate; immunotherapy; glioblastoma; brain tumor vaccine; survival

Introduction

Despite advances in the understanding and treatment of malignant glioma, these primary brain tumors still have a dismal prognosis and few long-term survivors[1,2]. Even with aggressive therapy including surgery, radiation, and chemotherapy, survival is only incrementally improved with a 5-year survival rate of 3%[2]. This poor prognosis for our patients underscores the need to evaluate and develop novel therapies and adjust our treatment paradigms based on our evolving understanding of brain tumor biology and immunology.

Active immunotherapy is an emerging strategy that has the theoretical advantage of a high degree of tumor-specific targeting, while sparing normal brain structures[3]. We and others have utilized dendritic cell (DC)-based vaccine therapies to immunologically target tumors within the central nervous system (CNS). Although prior clinical trials utilizing dendritic cell vaccination in brain tumor patients have demonstrated acceptable safety and toxicity profiles, along with initial clinical promise [4-19], the optimal method for loading dendritic cells with tumor-associated antigens, the ideal dose and regimen for administration, and the selection of patients for which immunotherapy may be beneficial, has yet to be fully elucidated.

In this study, we compared the safety, toxicity, and feasibility of two separate, concurrent DC-based Phase I protocols: one utilizing autologous tumor lysate (ATL) loading of DCs, and the other using DCs loaded with synthetic glioma-associated antigen (GAA) peptides. We also evaluated immune responses, PFS, and OS in the 34 malignant glioma patients enrolled in these two clinical trials. Our results suggest that ATL-pulsed DC vaccination may induce a more heterogeneous and diverse anti-tumor immune response against malignant glioma. The monitoring of post/pre-vaccination ratios of Treg cells and activated NK cell populations may be relevant immune monitoring endpoints in these patients.

Materials and Methods

Patient eligibility

This study reports on 34 patients diagnosed with malignant glioma at our institution and treated with either autologous tumor lysate-pulsed (UCLA IRB #03-04-053, FDA IND #11053, clinical trial registration # NCT00068510; n=28) or glioma-associated antigen (GAA) peptide-pulsed (UCLA IRB #06-01-052, FDA IND #12966, clinical trial registration # NCT00612001; n=6) DC vaccination between 2003 and 2010. All patients provided

written informed consent according to University of California Los Angeles (UCLA) Internal Review Board guidelines prior to treatment. Basic patient inclusion/exclusion criteria can be found at ClinicalTrials.gov for these studies (http://clinicaltrials.gov/).

Preparation of Autologous Dendritic Cells and pulsing with glioma antigen

Monocyte-derived DCs were established from adherent peripheral blood mononuclear cells (PBMC) obtained via leukapheresis, as we have recently described [11,12]. All *ex vivo* DC preparations were performed in the UCLA-Jonsson Comprehensive Cancer Center GMP facility under sterile and monitored conditions.

Treatment Schema and Vaccine Administration

Newly diagnosed glioblastoma patients underwent surgery and a standard course of external beam radiotherapy (to 60 Gy) with concurrent temozolomide chemotherapy prior to DC vaccination[2]. These patients were then given three biweekly DC vaccinations prior to adjuvant temozolomide treatment. Recurrent malignant glioma patients had previous radiation therapy and chemotherapy prior to presenting with tumor recurrence, so they underwent surgical resection of their tumors followed by DC immunotherapy after they had recovered from surgery and were tapered off peri-operative steroids. Eligible patients initially received three (3) intradermal injections at biweekly intervals, and then booster vaccinations every 3 months until the autologous vaccine ran out or until tumor recurrence, whichever came first.

Collection of PBMC for immune monitoring and flow cytometric analysis of PBMC

Peripheral blood was drawn from subjects at several designated time points pre- and post-DC vaccination (pre-tx, post 1^{st}, 2^{nd}, 3^{rd} vaccination, 6 month follow-up). Antibody cocktails were prepared according to manufacturer's specifications, and used as we have recently published[20]. The lymphocyte subsets that were gated include: $CD3^+CD4^+$ helper T cells, $CD3^+CD8^+$ cytotoxic T cells, $CD3^-CD16^+$ classical natural killer (NK) cells, $CD3^+CD16^+$ NKT cells, CD3-$CD19^+$ B cells, $CD3^+CD25^+CD127^{low}$ Treg cells.

Results

Patient and tumor characteristics

Patient and tumor data are provided in **Table 1**. The median age for the ATL-DC patients was 49 years, while that of patients on the GAA-DC trial was 44. The age of patients on the two trials was not significantly different (p=0.27). At the time of DC vaccination, the median KPS score was 90 for the ATL-DC patients and 80 for the GAA-DC patients. This difference in KPS scores was also not statistically significant (p=0.19).

Of the patients treated on the ATL-DC clinical trial (n=28), 23 were histologically classified as glioblastoma (WHO Grade IV; 82.1%; 15 newly diagnosed and 8 recurrent), and 5 with anaplastic glioma (WHO grade III; 17.9%). Of the patients treated on the GAA-DC clinical trial (n=6), 4 were classified as glioblastoma (66%; 2 newly diagnosed and 2 recurrent) and 2 with anaplastic tumors (33%). 17% of tumors from the ATL-DC trial had evidence of IDH1 mutations, while 50% of tumors from the GAA-DC trial were IDH1 mutated (**Table 1**). Mutant IDH1 alleles were almost exclusively found in tumors histologically characterized as WHO Grade III in this series of patients, suggesting that the majority of glioblastoma patients in these two vaccine trials were primary glioblastomas[21].

Safety and Feasibility

The incidence of adverse events (AE) related to DC vaccination was similar between the two clinical trials. The frequency and severity of AE was also similar between the two protocols, with predominantly NCI CTC grade I-II sequelae (CTCAE, v.4), directly or probably related to the vaccination procedure. The most common grade I-II AE were flu-like symptoms (headache, low-grade fever, nausea/vomiting, fatigue), injection site reactions, lymphadenopathy, and rashes developing 24-48 hours after vaccination (**Supplementary Table 1**). Grade III SAE were rare (i.e., seizures) and likely related to tumor progression.

Both clinical trials utilized a Phase 1, dose-escalation scheme with identical numbers of DC for vaccination (1, 5, and 10×10^6 DC/injection). The ATL-DC trial utilized 7-day, monocyte-derived DC, but without a dedicated maturation step [9,11,12]. The GAA-DC trial similarly used 7-day monocyte-derived DC, but added a maturational step for 24-48 hours to upregulate MHC and co-stimulatory molecules, as previously demonstrated by other investigators [22]. A comparison of the typical flow cytometric profiles for DC produced for the ATL and GAA DC clinical trials is shown in **Supplementary Figure 1**. We generated adequate numbers of viable loaded DC for all dose cohorts, with all of the appropriate lot release requirements, for 100% of patients on each clinical trial. In addition, there were no differences in the time delay after surgical resection until the first DC vaccination between patients on each clinical trial (p=0.75; **Table 1**). Thus, there were no feasibility and time delay differences in our ability to produce clinical-grade DC and initiate vaccination between the two clinical trials for this patient population.

Using our documentation of all eligible patients screened and enrolled, we compared the percentage of patients eventually treated relative to the intent-to-treat population on these two distinct DC-based protocols. On the ATL-DC trial, 28 out of 32 screened patients received DC vaccination, resulting in a 12.5% screen failure rate. The GAA-DC trial required an additional HLA typing requirement because the synthetic glioma-associated antigen peptides utilized were restricted to HLA-A0201$^+$ MHC haplotypes. On the GAA-DC trial, we screened 15 patients and eventually treated only 6 patients, resulting in a 60% screen failure rate. Since the only difference in eligibility criteria between these two clinical trials was the HLA-A0201 requirement, at least twice as many of the intent-to-treat population could be treated on our ATL-DC vaccination protocol compared to a more HLA-restrictive immunotherapy trial.

Immune Monitoring

The source of tumor antigen used to load DC was the main distinction between these two trials. The ATL-DC trial utilized autologous, patient-specific proteins derived from primary, digested tumor cells after freeze-thaw cycles. The GAA-DC trial utilized synthetic peptide antigens (TRP-2, gp100, her-2/neu, survivin) known to be expressed by gliomas [23,24]. While expression of these GAA was not an eligibility criterion for enrollment onto the GAA-DC trial, post-hoc IHC staining confirmed that survivin was expressed uniformly by all tumor samples, while her-2 expression was patchy and variable. Gp100 was not easily detectable by IHC when compared with melanoma (**Fig. 1**), which is consistent with other recent studies[10]. TRP-2 was only detectable at the mRNA level, and previously shown to be variably expressed[24].

Because different tumor antigen preparations were loaded onto DC for these two clinical trials, a direct comparison of discrete tumor antigen-specific T lymphocyte responses was not possible. Increased tetramer positive CD8$^+$ T cells were observed in GAA-DC patients (**Supplementary Figure 2**). However, as with other recent glioma-associated antigen peptide-pulsed DC trials, no association was found between tumor antigen-specific T

lymphocyte induction and survival[10]. Thus, we elected to compare lymphocyte subsets, activation markers, and regulatory T cell (Treg) frequencies obtained from peripheral blood lymphocytes post/pre DC vaccination.

Using flow cytometry, we stained PBL from patients pre- and post-DC vaccination using a multi-color panel of antibodies designed to evaluate lymphocyte populations (T cells, B cells, NK cells) and the expression of activation markers (CD69, CD25) on each sub-population. No differences in the frequency of helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), regulatory T cells (Treg; $CD3^+CD4^+CD25^+CD127^{low}$), NK cells ($CD3^-CD16^+$) or B cell populations ($CD3^-CD19^+$) were observed between PBL samples from both clinical trials (**Table 2**). Interestingly, a significantly elevated population of activated NK cells ($CD3^-CD16^+CD25^+$) was observed in the samples from the GAA-DC trial (**Table 2**, **Fig. 2**). No other differences in activated lymphocyte populations were observed.

To account for the heterogeneity in PBL populations between patients, comparisons were made between pre- and post-DC vaccination for each patient, in order to calculate fold changes. We examined these fold changes in each lymphocyte subset and looked for associations with overall survival in these patients. Using a Cox proportional hazards model stratifying on each trial, we discovered a significant relationship between Treg cell fold changes and survival in both the GAA-DC and ATL-DC trials (hazard ratio=7.19; 95% C.I. (1.87, 27.73); **Table 3**). Based on this statistical assessment, every unit increase in the Treg cell ratio is associated with an increased risk of death by 6.19 times. This association is statistically significant (p=0.004). A non-significant trend (p=0.08) was also observed between the activated NK cell ratio (post/pre DC vaccination) and overall survival (**Table 3**). These findings suggest that extended survival is observed in patients whose Treg and activated NK cell frequencies significantly decreased after DC vaccination.

We then utilized univariate and multivariate stratified Cox models to examine the association of various clinical and immune monitoring factors with overall survival. KPS, tumor pathology, and the Treg cell ratio were all significantly correlated with survival for each clinical trial (**Table 3**). When adjusted for each other in a multivariate model, tumor pathology no longer was significant (p=0.485), while the Treg ratio was still borderline significant (**Table 4**; p=0.057). These data suggest that the Treg ratio (post/pre-DC vaccination) may be a prognostic biomarker for overall survival in glioblastoma patients that received DC vaccination, even after controlling for tumor pathology.

Discussion

In this study, we compared the safety, feasibility, immune responses and survival of malignant glioma patients treated with two distinct methods of dendritic cell vaccination. One cohort of patients received autologous tumor lysate-pulsed DC vaccination, while the other patient cohort received glioma-associated antigen peptide-pulsed DC vaccination. There were no dose-limiting toxicities and no detectable differences in safety or toxicity between the two trials. Due to the requirement for a particular HLA type (HLA-A0201), the feasibility of treating patients with GAA peptide-pulsed DC vaccination was more limited than tumor lysate-pulsed DC vaccination, as only 40% of the intent-to-treat population was eligible for treatment on the GAA peptide DC vaccination regimen compared to 88% of screened patients on the ATL-DC trial.

The ATL-DC trial utilized DC without a dedicated maturational step in vitro so that tumor lysate proteins could be efficiently uptaken, processed, and presented; a process known to be downregulated upon final maturation[25]. We included an in vitro cytokine maturation step

(TNFα, IL-6, IL-1β, PGE_2) for DC on the GAA-DC trial because previous data had suggested that such a cytokine cocktail upregulated MHC and costimulatory molecules advantageous for class I peptide binding[22,26], and promoted enhanced lymph node trafficking dependent on chemokine responsiveness[27,28]. However, PGE_2 has recently been shown to facilitate DC interactions with regulatory T cells [29] and even directly promote Treg cell development[30]. It is possible that the PGE_2 included in the DC maturational cocktail for the GAA-DC trial may have induced regulatory T cell or NK cell populations that inhibited anti-tumor immune responses.

This may have contributed to the shorter survival observed in these patients. In contrast, we administered the Toll-like receptor (TLR) agonists, imiquimod or poly ICLC, following intradermal injections of ATL-DC to induce DC maturation in vivo. We previously demonstrated in pre-clinical models that the utilization of TLR agonists could enhance the survival and trafficking of DC in situ and enhance the priming of tumor antigen-specific T lymphocytes [31]. The findings from this current study suggest that the induction of patient-specific anti-tumor immunity using ATL-DC vaccination and in situ maturation with TLR agonists may represent a preferred formulation for DC-based therapies.

No obvious differences in any lymphocyte population were evident before or after DC vaccination between these two clinical trials, suggesting that baseline T cell populations were similar between the two groups. We designed these trials to focus our immune monitoring at two time points because they represent lymphocyte populations before and after the completion of vaccination cycles. While such discrete time points cannot rule out some inherent bias, the number of samples tested at these points may have minimized the variability. A significantly elevated population of activated NK cells ($CD3^-CD16^+CD25^+$) was found in the peripheral blood of GAA-DC patients. A recent study demonstrated that this population of activated NK cells was a negative prognostic biomarker for non-small cell lung cancer patients treated with a MUC1 vaccine and chemotherapy[32]. Such data are representative of a new, emerging understanding of how activation and inhibitory receptor stimulation by NK cells may influence adaptive immune responses [33] and impact clinical outcomes in cancer patients [34].

When we evaluated the ratios of post-vaccination vs. pre-vaccination lymphocyte population frequencies, we found a striking, independent association between Treg cell ratios (post/pre-DC vaccination) and overall survival, which was independent of tumor pathology. Decreased post-vaccination frequencies of T-reg cell populations, relative to pre-vaccination, were associated with longer overall survival in patients from both clinical trials. These findings are consistent with the current understanding that Treg cells may play a significant role in down regulating anti-tumor immune responses. In support of this, Mitchell et al. recently demonstrated that immune responses were dramatically enhanced after dendritic cell vaccination in glioblastoma patients that received CD25 mAb blockade (daclizumab, Roche Pharmaceuticals) and temozolomide chemotherapy[35]. The observations of Treg changes seen in our study are intriguing and warrant further, detailed analysis and validation in prospectively designed immunotherapy clinical trials.

The number of patients treated in this comparative study does not allow for meaningful comparisons in survival. However, the patient characteristics (age, KPS, extent of resection) and tumor histopathologies suggest that these two patient cohorts were comparable. In addition, the patients on each trial were enrolled during the same time period (2003-2010), had similar eligibility criteria and similar other treatments. The median survival of patients on the ATL-DC trial was 34.4 months, while that of patients on the GAA-DC trial was 14.5 months. It is possible that our choice of antigenic targets (survivin, her-2/neu, gp100, TRP-2), or inclusion of PGE_2 in the DC maturation cocktail, may have negatively impacted

effective anti-tumor immune responses elicited by our GAA-DC vaccination. It is also possible that the diversity of patient-specific anti-tumor immune responses induced by tumor lysate-pulsed DC vaccination may be more important than the small number of well characterized, tumor-specific antigens targeted by GAA peptide-pulsed DC vaccination. Such conjecture is supported by recent clinical investigations using ipilumimab, with and without gp100 peptide vaccination, for metastatic melanoma patients. The addition of a gp100 peptide vaccine with ipilumimab did not extend survival beyond ipilumimab alone, and in fact, was associated with a worse outcome [36]. Although enhanced vaccine-elicited gp100-specific immune responses were observed when followed by ipilumimab[37], patient survival was not similarly extended, suggesting that it may be more important to induce heterogeneous immune responses rather than to drive single antigen responses.

In conclusion, our studies demonstrate that two distinct modes of tumor antigen-loaded dendritic cell vaccination are safe and without any dose-limiting toxicity in malignant glioma patients. In our patient population, ATL-pulsed DC vaccination was associated with wider feasibility for treatment of the intent-to-treat population and decreased fractions of activated NK cell populations, compared with GAA peptide-pulsed DC vaccination. Multivariate analyses suggest that the monitoring of regulatory T cell ratios (post-vaccination/pre-vaccination) may be an independent prognostic indicator of survival for glioma patients treated with immunotherapy. Our results also suggest that the induction of a diverse, patient-specific anti-tumor immune response may be an important factor in the efficacy of DC vaccination strategies for malignant glioma patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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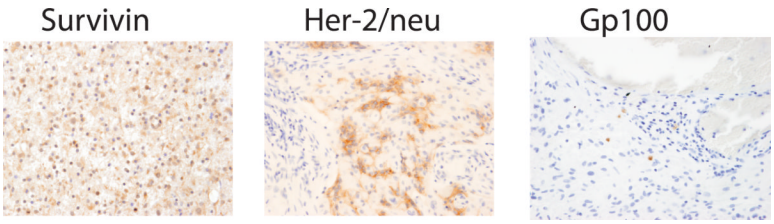

Figure 1. Immunhistochemical detection of GAA in malignant glioma patient tumor tissue
Representative IHC staining of survivin, her-2/neu, and gp100 from a patient (GAA-03).

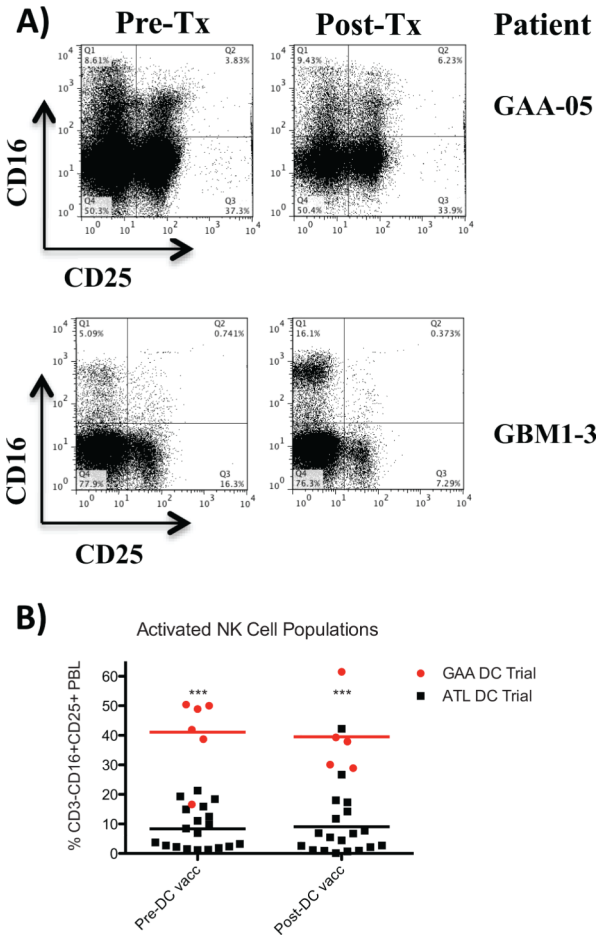

Figure 2. Increased frequencies of activated NK cells in peripheral blood from patients on the GAA peptide-pulsed DC trial

PBL from pre and post-DC vaccination timepoints were stained with an antibody cocktail that identifies activated NK cells populations ($CD3^{-}CD4^{+}CD16^{+}CD25^{+}$). (A) Representative FACS plots of activated NK cell populations from a representative patient on the GAA DC trial (Top) and ATL DC trial (Bottom). (B) Quantitative analysis of activated NK cell frequencies from peripheral blood. ***$p<0.0001$ by 2-way ANOVA testing.

Table 1

Demographic and baseline clinical characteristics

Characteristic	**ATL-DC**	**GAA-DC**
	(N=28)	**(N=6)**
AGE – yr	49	44
Gender		
• Male	20	6
• Female	8	0
KPS (@ DC vacc.)	90	80
Tumor Pathology		
• Glioblastoma (WHO Grade IV)	23 (82.1)	4 (66)
• Anaplastic glioma (WHO Grade III)	5 (17.9)	2 (33)
Tumor Characteristics		
• IDH1 (% mutated)	17	50
Time to Treatment* **(months)**	4.9+/-4.1	4.4+/-1.8
Survival Characteristics		
• OS (months)	34.4	14.5
• PFS (months)	18.1	9.6

* Time interval from the date of surgery until date of 1st DC vaccination in months +/- standard deviation.

Table 2

Lymphocyte Subset Changes Following DC Vaccination

	ATL-DC Trial	GAA-DC Trial
Lymphocyte Subset*	**Post-Tx (Avg %)**	**Post-Tx (Avg %)**
$CD3^+CD4^+$ Helper T cells	37+/-3.0	43.1+/-4.4
$CD3^+CD8^+$ CTL	23.5+/-2.5	27.7+/-4.5
$CD3^+CD16^+$ NK T cells	2.6+/-0.9	4.7+/-1.8
$CD3^-CD16^+$ NK cells	15.6+/-1.5	13.22+/-3.7
$CD3^-CD16^+CD25^+$ activ. NK	9.1+/-2.5	39.5+/-5.9
$CD3^-CD19^+$ B cells	10.2+/-1.6	9.5+/-0.6
$CD3^+CD4^+CD25^+CD127^{low}$ Treg	17.1+/-3.1	23.3+/-3.9

*Percent of cells stained from ficoll-isolated PBMC at each timepoint.

Table 3

Stratified Cox proportional hazards model for survival with clinical endpoints and immune monitoring ratios

Covariate*	Hazard Ratio	95% C.I. for Hazard Ratio	p-value
Age (1 unit increase in years)	1.03	(0.99, 1.08)	0.187
Gender (Female vs Male)	1.77	(0.51, 6.10)	0.368
KPS	0.92	(0.86, 0.98)	0.010
Overall Tumor Path Effects			0.023
Recurrent Grade IV vs. newly dx Grade IV	4.42	(1.46, 13.38)	0.009
Recurrent Grade IV vs. Grade III	6.86	(0.62, 75.91)	0.116
Grade IV vs. Grade III	1.55	(0.15, 15.56)	0.709
Treg cell fold change**	7.19	(1.87, 27.73)	0.004
Activated NK cell fold change**	1.99	(0.92, 4.31)	0.081

*Each model includes a single covariate. Stratification is on trials.

**Refers to frequency of cells (%) at post-DC vaccination/pre-DC vaccination

Table 4

Multivariate stratified Cox model for Overall Survival.

Covariate	Hazard Ratio	95% C.I. for Hazard Ratio	p-value
Treg-fold change (1 unit increase)	4.56	(0.96, 21.73)	0.057
Overall Tumor Path Effects			0.485
Recurrent Grade IV vs. newly dx Grade IV	2.08	(0.53, 8.16)	0.293
Recurrent Grade IV vs Grade III	3.57	(0.26, 48.00)	0.338
Grade IV vs. Grade III	1.71	(0.16, 18.92)	0.661

*Stratification is on trials. Covariates included are tumor pathology and Treg cell fold change.