Superior Efficacy of Tumor Cell Vaccines Grown in Physiologic Oxygen

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Abstract

**Purpose:** Atmospheric oxygen (∼20% O₂) has been the universal condition employed to culture tumor cells as vaccine antigen. We tested the hypothesis that reducing oxygen tension would increase the efficacy of tumor cell lysate vaccines.

**Experimental Design:** GL261 glioma cells and EMT6 breast carcinoma cells were grown in 5% or 20% O₂. Syngeneic tumor-bearing mice were vaccinated with these tumor cell lysates mixed with CpG oligodeoxynucleotides as an adjuvant. Tumor infiltrating T cells and apoptotic GL261 cells were quantified by immunohistochemistry. Tumor-reactive immunoglobulin was detected by Western blot. Ovalbumin and gp100-derived peptides were mixed with GL261 lysates as marker antigens to detect changes in presentation of exogenous antigen on MHC class I in vitro, and in vivo following adoptive transfer of gp100-specific CD8⁺ T cells.

**Results:** Mice bearing orthotopic glioma and breast carcinoma survived significantly longer when vaccinated with 5% O₂ lysates. Antigen-specific CTL activation was significantly enhanced following stimulation with lysates derived from GL261 cells grown in 5% O₂ versus 20% O₂ through a mechanism that involved enhanced cross-presentation of exogenous antigen on MHC I. Vaccination with 5% O₂ GL261 cell lysates caused a significant increase in CTL proliferation, tumoricidal function, and trafficking into brain tumor sites, whereas 20% O₂ lysate vaccines predominantly evoked an antibody response.

**Conclusions:** Tissue culture oxygen functions as an “immunologic switch” by dictating the cellular and humoral immune responses elicited by tumor cell lysates. These results have profound implications for cancer vaccines that utilize tumor cells as the source of antigen. Clin Cancer Res; 16(19); 4800–8. ©2010 AACR.

Therapeutic cancer vaccines have been administered in over 100 clinical trials in 15 countries (1–3). Immunologic and clinical responses have been documented in patients with numerous cancers, including glioma, and colon and renal cell carcinoma (1, 3–5). However, clinical responses typically occur in a small percentage of patients, and the majority of randomized clinical trials have failed to show a survival advantage in patients receiving cancer vaccines (1, 3). Tumor cells are frequently used as the source of vaccine antigen, functioning as a personalized immunotherapy, targeting multiple, patient-specific tumor antigens.

Vaccines that utilize tumor cells as the source of antigen include tumor lysate-pulsed dendritic cells, dendritic cell/tumor cell fusions, tumor-derived heat shock proteins (HSP), cytokine-secreting tumor cells, and direct administration of tumor cell lysate. In spite of this, tumor cell extracts are typically poorly immunogenic. There is evidence that tumor cell lysates suppress dendritic cell maturation and restrain the priming of T cells (6, 7). Although various strategies have been attempted to increase tumor cell immunogenicity (e.g., heat shock, irradiation, genetic engineering; refs. 1, 7, 8), the limited efficacy of cancer vaccines in randomized clinical trials shows the need for novel approaches.

We hypothesized that tissue culture oxygen would modify the immunogenicity of tumor cells because oxygen is a regulator of many processes, including cell metabolism and gene transcription (8–10). As available oxygen decreases, hypoxia inducible factors accumulate, changing expression of genes such as those encoding angiogenic cytokines, transcription factors, and regulators of stem cell self-renewal (8, 9, 11, 12). Recent studies have shown profound changes in glioma cell gene expression when comparing cells grown in conventional atmospheric oxygen (∼20% O₂) with more physiologically relevant oxygen
tensions that likely exist in the tumor bed, ranging from 1% to 7% O₂ (11–13). Of particular importance to immunotherapy is work showing that hypoxia-associated injury is sufficient to induce inflammation. Ischemia-induced hypoxia is associated with release of alarmins such as heat shock proteins and high-mobility group box 1 (HMGB1; refs. 14, 15). Some HSPs and HMGB1 are recognized as danger signals by cells expressing toll-like receptors (TLR), recently understood to serve a dual function as sensors of infection or physiologic stress (16). TLRs play a pivotal role in shaping innate and adaptive immune responses. For instance, unmethylated CpG oligodeoxynucleotides (ODN) signal through TLR 9 on dendritic cells to increase expression of inflammatory cytokines and costimulatory molecules that promote naïve T-cell activation and differentiation (17). Thus, TLR ligands are often used as vaccine adjuvants in experimental studies and clinical trials (18).

The purpose of this study was to compare the immunogenicity of tumor lysates derived from cells cultured in conventional atmospheric oxygen (~20% O₂) with a more tumor-physiologic oxygen (5% O₂). We extensively characterized the differential immune response in a murine model of glioma, which led to the identification of oxygen as an "immunologic switch" that affects both cell-mediated and humoral immune responses elicited by tumor cell lysates. Our data suggest that the efficacy of tumor cell vaccines could be markedly improved by reducing tissue culture oxygen tension.

Materials and Methods

Animal models
Tumors were implanted into female mice (6-8 weeks old) that were purchased from Jackson Laboratory and maintained in a specific pathogen-free facility according to the guidelines of the University of Minnesota Animal Care and Use Committee. The GL261 model was established in C57BL/6 (BL6) mice by inoculation with 15,000 GL261-Luc cells in 1 μL PBS. Tumors were implanted stereotactically into the right striatum; coordinates were 2.5 mm lateral and 0.5 mm anterior of bregma, and 3 mm deep from the cortical surface of the brain (19). The EMT6 model was established in BALB/c mice by injection of 1 × 10⁶ EMT6 cells in 50 μL PBS into the left superior mammary fat pad as described (20). Tumor cells used to establish both models were cultured in atmospheric oxygen.

Cell culture
GL261 cells were cultured in media consisting of DMEM/F12 (1:1 with L-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/mL), B27 and N2 supplements, and 0.1-mg/mL normocin. EMT6 cells, splenocytes, and T cells were cultured in RPMI 1640. GL261-Luc cells were cultured in DMEM; both DMEM and RPMI media contained 10% fetal bovine serum, penicillin/streptomycin (100 U/mL), and 0.1 mg/mL normocin. Unless otherwise stated, all cultures were maintained exclusively in atmospheric oxygen. Cultures referred to as "5% O₂" were briefly exposed to atmospheric oxygen one to two times per week for media changes and for cytokine feeding. Cultures were maintained in the indicated oxygen tension uninterrupted for at least 48 hours prior to use in all experiments. A Thermo Scientific Forma Series II multigas incubator set to 5% O₂, or an incubator perfused with atmospheric oxygen was used; both incubators were maintained at 5% CO₂.

Immunization protocol
Tumor cells were dissociated with nonenzymatic cell dissociation buffer (Sigma), washed three times with PBS, resuspended in 500 μL PBS, and frozen initially by placing in −80°C overnight. Cells were further lysed by five cycles of freezing in liquid nitrogen and thawing in a 56°C water bath. Cell debris was pelleted by centrifugation at 14,000 relative centrifugal force, and the protein concentration of the supernatant was determined using a Bradford assay. Pellets were resuspended, and lysates were stored at −80°C until use. Each vaccine was prepared at the time of vaccination and consisted of 65 μg of protein lysate mixed with 50 μg of phosphorothioated type-B CpG ODN 685 (5′-ctgagcgctgctggcttc-3′; SBI Biotech) in a final volume of 100 μL injected intradermally (50 μL in the lower neck and 50 μL in the hind flank on the right thigh). Vaccination was administered weekly starting three days after tumor implantation for a total of six doses.

Flow cytometry
A Becton Dickinson Canto three-laser flow cytometer was used for data acquisition. The following antibodies were used: CD11c-alexafluor700, CD80-PE, CD86-FITC, CD8−, iCOSL-PE, GITRL-PE, Vβ13-FITC, anti-SIFKEK/ H-2Kβ-PE (all from eBioscience), and H-2Dβ-hgp100 tetramer-APC (kind gift from Dr. Robert Prins, UCLA). Antibody staining was conducted by staining 4 to 5 × 10⁵
cells resuspended in 100 μL PBS with specific antibodies according to manufacturer’s protocol, incubating for 20 minutes at 4°C, washing three times, and analyzing. Appropriate isotype controls were used.

For costimulatory molecule analysis splenocytes were harvested from BL6 mice and exposed to 156 mmol/L ammonium chloride to eliminate erythrocytes. Remaining splenocytes were then washed three times and cultured in complete RPMI media with 10 μg of GL261 lysate from cells grown in 5% or 20% O2. Following 48 hours of incubation, splenocytes were analyzed by flow cytometry.

**CTL-mediated INF-γ secretion and cross-presentation**

For measurement of IFNγ BL6 mouse splenocytes were plated at a concentration of 4 × 10^5 cells/well in a 96-well plate in complete RPMI 1640 media. Splenocytes were pulsed with 10 μg of the indicated tumor lysate with or without 10 μg of human (h) gp100_{25-33} peptide (KPVPRNQDWL) and incubated for 24 hours. CD8+ T lymphocytes were purified from Pmel mouse (21) splenocytes using negative immunomagnetic selection according to the manufacturer’s protocol (Miltenyi Biotec). Purity was confirmed to be >95% by flow cytometry; 2 × 10^5 purified Pmel CTLs were cocultured with splenocytes. Forty-eight hours after coculture, IFNγ was quantified in the tissue culture supernatant using a flow cytometric bead array according to the manufacturer’s protocol (BD Biosciences).

For measurement of presented chicken ovalbumin (OVA) peptide, BL6 mouse splenocytes were plated at a concentration of 4 × 10^5 cells/well in a 96-well plate in complete RPMI 1640 media. Splenocytes were pulsed with 10 μg of the indicated tumor lysate with or without 10 μg of a peptide containing the core OVA-derived SIINFEKL/H-2Kb epitope (EVSQLQLEQLESIINFEKLTELWSSSNVM) and incubated for 24 hours. Cells were harvested, stained, and analyzed by flow cytometry as described above.

**Immunohistochemistry**

Mice were perfused with 30 mL PBS followed by 30 mL of 10% formalin. Standard processing, embedding, and dehydration methods were employed to generate tissue sections (5 μm thick) for staining. Following dehydration, sections are put into ReVeal, 6.0 pH citrate buffer (Biocare Medical) and placed in a steamer for 30 minutes followed by a 20-minute cool-down. Slides were rinsed well in tap water and placed into a PBS bath for 5 minutes. Sections were incubated with Sniper blocking reagent (Biocare Medical) for 30 minutes followed by incubation at 4°C overnight with the first primary antibody, rabbit monoclonal CD3 (Lab Vision; 1:400). The following day a biotinylated rabbit link (Covance) was applied for 30 minutes at room temperature followed by a tap water rinse and a 5-minute PBS bath. This was followed with a 4+ streptavidin AP label (Biocare Medical) for 30 minutes at room temperature and rinsing with PBS. Vulcan Fast Red (Biocare Medical) was applied for 5 to 15 minutes at room temperature. The samples were counterstained with Harris hematoxylin for 1 minute, decolorized, rinsed in tap water for 5 minutes followed by routine dehydration, and cover slipped using permount. Seventy-two hours following the first vaccination, draining inguinal and cervical lymph nodes were harvested, dissociated, and analyzed by flow cytometry. For the CTL assay, 72 hours following the second vaccination, draining inguinal and cervical lymph nodes were harvested, dissociated, and incubated with CFSE-labeled GL261 cells for 4 hours, and analyzed for cytotoxicity according to the manufacturer’s protocol (Immunocytometry, LLC). Briefly, following incubation, the percentage of CFSE-labeled target cells that incorporated 7-AAD was determined by flow cytometry and plotted as the percent lysis.

**Western blot**

GL261 tumor cells cultured in 5% O2 were washed, pelleted, and lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (Pierce). Protein concentration was determined using the bichinchoninic acid colorimetric method (Pierce). GL261 lysates were diluted in reducing sample buffer and 40 μg were loaded per lane on a 4% to 12% SDS-PAGE gel and run at 160 volts. Gels were then transferred to nitrocellulose at 5 volts overnight (BioRad), blocked using 5% NFDM/TBS/0.1% Tween-20 for 1 hour, incubated in 1:1000 serum in blocking buffer for 1 hour, and washed six times over 1 hour in Tween-TBS. Blots were then incubated in 1:50,000 antimouse IgG HRP (Jackson ImmunoResearch) in blocking buffer for 1 hour and washed six times over 1 hour in Tween-TBS. Nitrocellulose was then incubated in ECL Plus chemiluminescent substrate (GE) for 5 minutes, drained, and exposed to HyBlot CL Autoradiography film (Denville Scientific) for 30 seconds.
Statistical analysis

Statistical comparisons were made by ANOVA, followed by post hoc comparisons using a two-tailed t-test. Differences in animal survival were evaluated by log-rank test. All tests were done with Prism 4 software (Graph Pad Software, Inc). P values <0.05 were considered significant.

Results

Superior efficacy of 5% O2 lysate/CpG ODN vaccine at clinically relevant dose

We had previously shown that vaccination of glioma-bearing mice with 20% O2 lysates mixed with CpG ODN doubled median survival by a mechanism that required CTLs and the combination of lysate mixed with CpG ODN (24). Consistent with our previous study, glioma-bearing mice treated with lysate or CpG ODN alone did not survive significantly longer than saline controls (Supplementary Fig. S1). However, our previous study employed a clinically unachievable dose of protein lysate; we dosed mice at 50 mg/kg (1 mg protein from 4 x 10^6 cells), which would require over a billion tumor cells to generate enough lysate for a single vaccination in a human. We therefore compared the potency of tumor lysate vaccination using 65 μg of protein lysate (3 mg/kg), which would require 10^7 to 10^8 tumor cells per vaccine in a human (routinely achieved in clinical trials; reviewed in ref. 1). Mice bearing intracranial GL261 glioma were treated by vaccination with GL261 cell lysates from 5% or 20% O2 cultures mixed with CpG ODN as an adjuvant. The group treated with 5% O2 lysate exhibited a 41% increase in median survival relative to saline controls (55 versus 39 days, respectively). Twenty-seven percent of the 5% O2 lysate–treated mice survived beyond 100 days (Fig. 1A). In contrast, mice treated with 20% O2 lysate did not survive significantly longer than saline controls. Thus, tumor lysate vaccination was efficacious at a clinically relevant antigen dose only when the cells were cultured in 5% O2.

Vaccination with 5% O2 lysates increased the number of tumor infiltrating T cells and apoptotic tumor cells

To determine the potential mechanisms responsible for the improved survival after GL261 challenge, T-cell infiltration and tumor cell apoptosis were quantified at the brain tumor site. Immunohistochemistry for CD3
revealed significantly more T cells in the tumors of mice treated with 5% O₂ lysate relative to other groups (Fig. 1B). Accordingly, immunohistochemistry for activated caspase 3 showed greater numbers of apoptotic tumor cells in the 5% O₂ lysate group (Fig. 1C). Closer examination by double labeling revealed T cells forming putative immunologic synapses with tumor cells undergoing various stages of apoptosis in the 5% O₂ lysate–treated mice (Fig. 1D). There were more putative immunologic synapses between T cells and apoptotic tumor cells in mice treated by 5% O₂ lysate, although this trend did not reach statistical significance \( (P = 0.16; \text{data not shown}) \). Flow cytometry on brain infiltrating lymphocytes was done to further characterize T-cell subsets at the tumor site. Glioma-bearing mice were adoptively transferred with Pmel CD8⁺ T cells that express a T-cell receptor that reacts with both human and murine gp10025-33 epitopes (21), the latter of which is expressed in GL261 cells (25). There was a 4-fold increase in the number of hgp10025,33-H²-D² tetramer⁺CD8⁺ T cells in the brains of mice that were vaccinated with 5% O₂ lysate relative to other groups (Supplementary Fig. S2).

**5% O₂ lysates enhanced antigen-specific CD8⁺ T-cell activation**

To directly measure the effect of cell lysates on CD8⁺ T-cell activation, we developed a cell culture assay whereby GL261 cell lysates were or were not mixed with hgp100 25-33. Lysate/hgp100 25-33 combinations were pulsed onto BL6 mouse splenocytes, and purified Pmel CD8⁺ T cells were cocultured with the splenocytes for 48 hours. CD8⁺ T-cell activation was quantified by IFNγ release in the tissue culture supernatant. There was no appreciable IFNγ measured when splenocytes were not pulsed with hgp10025-33 (Fig. 2A). Lysates alone did not result in significant IFNγ production above background, revealing minimal reactivity to endogenous mouse gp10025-33 in the lysate (Fig. 2A). When pulsed in concert with hgp10025-33, 5% O₂ lysates more than doubled IFNγ secretion compared with hgp10025-33 or lysate alone. In contrast, the 20% O₂ cell lysates significantly suppressed antigen-specific IFNγ secretion (Fig. 2A). Thus, 5% O₂ lysates significantly increased antigen-specific CD8⁺ T-cell activation but had no intrinsic ability to induce IFNγ secretion from the cultured antigen-presenting cells.

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**Fig. 2.** CD8⁺ T-cell activation is increased by 5% O₂ lysates via cross-presentation. Splenocytes were pulsed with tumor lysates derived from GL261 cells cultured in 5% or 20% O₂ with or without hgp10025-33. Purified Pmel CD8⁺ T cells were added and cocultured for an additional 48 hours. A, IFNγ in the tissue culture supernatant was quantified by bead array. Control labeled as “No pulse” indicates unpulsed splenocytes that were cocultured with Pmel CD8⁺ T cells. B, splenocytes were pulsed with tumor lysates derived from GL261 cells cultured in 5% or 20% O₂ and analyzed 48 hours later by flow cytometry for the indicated markers. Data shown represent cells within a CD11c-positive gate. C, splenocytes were or were not pulsed with OVA268-274 mixed with 5% or 20% O₂ lysates. The OVA268-274–derived SIINFEKL epitope was detected 24 hours later by flow cytometry using an antibody against the SIINFEKL/K⁺ complex. Data shown represent cells within a CD11c-positive gate. D, aggregate data from C showing relative cell surface levels of SIINFEKL/K⁺. Error bars, ± SE. MFI, mean fluorescent intensity \( (n = 3/\text{group}; \,* P < 0.05; \,** P < 0.01) \).
The adjuvant property of 5% O2 lysates was associated with an increase in cross-presentation of exogenous antigen on MHC I

We investigated if the adjuvant properties of the 5% O2 lysates could be attributed to increasing expression of costimulatory molecules involved in T-cell activation. Splenocytes from BL6 mice were pulsed with GL261 cell lysates from 5% or 20% O2 cultures. Forty-eight hours later CD11c+ cells (dendritic cell marker) were analyzed by flow cytometry for coexpression of CD80, CD86, iCOSL, and GITRL. Relative to unpulsed controls, the only significant change was an increase in CD86 expression in cells pulsed with 20% O2 cell lysates (Fig. 2B). Therefore, the adjuvant properties of the 5% O2 lysates were not attributable to changes in expression of these costimulatory molecules, implying other mechanisms.

We hypothesized that the 5% O2 lysates could enhance cross-presentation of exogenous antigen on MHC I. To test this directly, splenocytes were pulsed with a 26-amino-acid peptide derived from OVA (OVA248-274) that must be cleaved for the SIINFEKL epitope to be presented on H-2Kb; the H-2Kb/SIINFEKL complex is then detectable by a specific antibody using flow cytometry (26). Splenocytes were pulsed with OVA248-274 alone or in combination with GL261 cell lysates from 5% or 20% O2 cultures. Cells were analyzed by flow cytometry 24 hours later. CD11c+ splenocytes that were pulsed with OVA248-274 mixed 5% O2 lysates exhibited an appreciable increase in cell surface levels of the H-2Kb/SIINFEKL complex (Fig. 2C). The mean fluorescent intensity quantifying the amount of the H-2Kb/SIINFEKL complex on CD11c+ cells was significantly increased in the 5% O2 lysate group relative to all other groups (Fig. 2D). These data revealed that one mechanism by which 5% O2 lysates enhance antigen-specific CD8+ T-cell activation was by increasing cross-presentation of exogenous antigens on MHC I.

5% O2 lysate vaccination enhanced CTL proliferation and tumoricidal function

We next investigated the effects of 5% O2 lysate on in vivo proliferation and ex vivo tumoricidal function of CTLs. Glioma-bearing mice were adoptively transferred with CFSE-labeled Pmel splenocytes. Mice were vaccinated with 5% or 20% O2 GL261 lysates mixed with hgp10025-33 and CpG ODN one day after adoptive transfer. Lymphocytes were isolated from the draining lymph nodes (DLN) 72 hours following vaccination and analyzed for proliferation by flow cytometry. Pmel CTLs exhibited significantly increased proliferation in 5% O2 lysate–treated mice compared with other treatment groups (Fig. 3A and B). To measure tumoricidal function, vaccinations were repeated and lymphocytes from the DLN were harvested 72 hours following the second vaccination and used as effector cells in a CTL assay. Significantly greater GL261 cell lysis was documented in the 5% O2 lysate group (Fig. 4A). In contrast, Western blot analysis revealed a tumor-reactive antibody response of greater breadth and magnitude in the serum from mice...
treated with 20% O2 lysate relative to 5% O2 lysate (Fig. 4B). These data show that 5% O2 lysates significantly enhanced CTL responses whereas 20% O2 lysates predominately evoked tumor-reactive antibody responses.

Efficacy of 5% O2 lysate vaccination was not dependent on a specific cell line or mouse strain

The broader reproducibility of the survival data obtained using the GL261 glioma model was tested in another tumor model. BALB/c mice were inoculated with syngenic EMT6 breast carcinoma in the mammary fat pad. Tumor-bearing mice were vaccinated with EMT6 cell lysates from 5% or 20% O2 cultures mixed with CpG ODN as an adjuvant. Vaccination with 5% O2 lysates was even more efficacious than the GL261 model, rendering 60% of the mice long-term survivors with no apparent evidence of residual tumor (Fig. 5 and data not shown). Similarly to the GL261 studies, mice treated with 20% O2 EMT6 lysates did not survive significantly longer than saline controls. These data suggest that the effect of oxygen on the therapeutic efficacy of tumor lysate vaccination is not restricted to a specific cell line, tumor type, or genetic background.

Discussion

The results of this study identify oxygen as a master immunologic switch that can be manipulated to augment the efficacy of cancer vaccines. There are several possible mechanisms by which tissue culture oxygen changes tumor cell lysate immunogenicity. Such mechanisms could include altering levels of immunomodulatory adenosine (27), expression of target antigens, endogenous alarmins (e.g., TLR ligands, HSPs), or proteins that modulate antigen presentation by novel means. We were unable to find evidence that 5% O2 lysates activated CD11c+ dendritic cells as assessed by upregulation of costimulatory molecules (Fig. 2B), or increasing secretion of IFNγ, interleukin-4 (IL-4), IL-6, IL-10, or IL-12 (Fig. 2A and data not shown). Although target antigen expression could change as a function of oxygen tension, the differences measured in Pmel CD8+ T-cell activation were not attributable to changes in

![Fig. 4.](image1.png) Vaccination with 5% O2 induced increased tumoricidal function of CTL and diminished tumor-reactive antibody responses. A, CTL assay using freshly isolated lymph node cells harvested 72 hours after two vaccinations as effectors against GL261 target cells. Error bars, ± SE (n = 3 mice/group; *, P < 0.05). B, GL261 glioma-bearing mice were vaccinated weekly for six consecutive weeks with GL261 cell lysates derived in 5% O2 or 20% O2 and CpG ODN. Serum was collected upon morbidity, and serum from a non–tumor-bearing mouse was used as a control. GL261 cell lysates derived from 5% O2 were run on a 4% to 12% SDS-PAGE gel, transferred to nitrocellulose, and blotted with pooled serum (n = 5/group) from the different treatment mice, followed by an anti-IgG-HRP to visualize tumor-reactive antibody.

![Fig. 5.](image2.png) Superior efficacy of 5% O2 lysate vaccination against orthotopic breast carcinoma. BALB/c mice bearing established EMT6 breast carcinoma were vaccinated weekly for six consecutive weeks with EMT6 cell lysates derived in 5% O2 or 20% O2 and CpG ODN. Mice were sacrificed due to tumor ulceration, tumor volume ≥1,000 mm3, or at a moribund state. *, statistical significance at P < 0.05.
endogenous mouse gp100 expression (Supplementary Fig. S3). Nevertheless, our data show that 5% O$_2$ lysates significantly increased T-cell activation as shown by antigen-dependent IFN-γ secretion from highly purified CD8+ Pmel T cells. Pulsation of splenocytes with OVA248–274 confirmed that 5% O$_2$ lysates increased presentation of exogenous protein antigens on MHC class I relative to 20% O$_2$ lysates. It was therefore not surprising that 5% O$_2$ lysates enhanced T-cell priming. This is the first study to show that tumor lysates from low O$_2$ environments enhance antigen-specific CD8+ T-cell proliferation, trafficking to tumor sites, and tumor-specific cytolytic responses, resulting in increased survival in two mouse tumor models. Identification and purification of the molecule(s) that is(are) responsible for the enhancement in cross-presentation could yield a valuable new reagent to increase the efficacy of cancer vaccines. Although our study focused on CTL responses, the effect of 5% O$_2$ lysates on the presentation of MHC II-restricted peptides and effector CD4+ T-cell responses should be addressed in future studies.

Western blot analysis showed that vaccination with CpG/lysate primed a polyclonal tumor-reactive antibody response in both the 5% O$_2$ and 20% O$_2$ groups; each band revealed an antibody reactive to an antigen in the GL261 cell lysate (Fig. 4B). Relative to the 20% O$_2$ group, the antibody response in the 5% O$_2$ group was diminished as illustrated by a reduction in the number and intensity of bands. This could reflect an intrinsic humoral immune response bias to vaccination with 20% O$_2$ lysates or recognition of different antigens present in each lysate. Oxygen concentration exhibits intertumoral and intratumoral heterogeneity in the glioma microenvironment, ranging from 0.1% to 10% and trending towards lower oxygen as tumor grade increases (28). Thus, lowering oxygen in cell culture may select for expression of antigens that are in greater abundance on the tumor in situ. If true, vaccination with lysates from a more tumor-physiologic oxygen tension should increase the “signal to noise” ratio by focusing the adaptive immune response against antigens relevant at the tumor site. Further studies will be required to understand if tumor-physiologic oxygen tension selects for antigens that are more abundantly expressed in the tumor.

Although it was beyond the scope of this study to conduct immune monitoring in the breast carcinoma model, the survival data in this model illustrate that the translational significance of our findings are not limited to gliomas. An additional consideration with translational significance is the dose of tumor cell lysate used in vaccination. Many investigators, including our group, have shown effective immunotherapy using an excess of one million tumor cells grown in 20% O$_2$ as vaccine antigen in the mouse (24). However, our analysis of the protein content in GL261 cells suggests that this corresponds to a clinically unfeasible cell dose (50 mg/kg dose of protein lysate requiring over a billion tumor cells in a human).

When reduced to a protein lysate dose of 3 mg/kg, requiring $\sim$10$^7$ to 10$^8$ cells in human, tumor cells grown in 20% O$_2$ were not effective as vaccine antigen. This observation illustrates the importance of the dose of tumor antigen relative to the size of the animal. We speculate that the discordant doses of vaccine antigen used on a mg/kg basis constitute one factor contributing to the poor predictability of murine tumor models.

It is possible that our results with CpG/lysate vaccination are relevant to other approaches that utilize tumor cells as the source of antigen (e.g., lysate-pulsed dendritic cells, irradiated tumor cells). This is an ongoing area of investigation in our laboratory. Further study of the effect of oxygen on tumor cell immunogenicity using clinically scalable lysate doses is warranted. It will be important to determine if reducing oxygen below the 5% threshold used in these studies can further enhance CTL responses against tumors. We propose that exploitation of oxygen as a master immunologic switch could improve the efficacy of cancer vaccines in the foreseeable future.

**Disclosure of Potential Conflicts of Interest**

J. Ohlfest: Ownership interest/patent, University of Minnesota.

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