Effect of Oxygen on Tumor Cell Vaccine

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Abstract

Gliomas are the most common type of malignant brain tumor. Even with the recent progress in conventional therapies, the prognosis remains poor and development of effective immunotherapy is needed. Tumor vaccines using CpG and tumor lysate have been demonstrated as effective in glioma therapies. We hypothesized that tumor lysate grown in a physiologic 5 % O\textsubscript{2} condition would increase immunogenicity compared to atmospheric 20 % O\textsubscript{2} condition since glioma in situ has been demonstrated hypoxic; and hypoxic conditions can activate the danger signal to induce antitumor immunogenicity in addition to CpG. In this study, we characterized the immunogenicity of tumor lysate derived from 5 % O\textsubscript{2} condition for the first time in comparison with 20 % O\textsubscript{2} condition. Vaccination with lysate from 5 % O\textsubscript{2} condition increased the numbers of several lymphocyte subsets at draining lymph nodes compared to the tumor lysate from 20 % O\textsubscript{2} condition. Vaccination with lysate from 5 % O\textsubscript{2} condition did not change the cytokine levels in the sera compared to the vaccination with lysate from 20 % O\textsubscript{2}. Tumor reactive antibody levels were increased with the amount of lysate used for vaccines; however, there was not a significant difference in antibody levels with 5 % O\textsubscript{2} in comparison with 20 % O\textsubscript{2}. Lastly, using the tumor lysate from 5 % O\textsubscript{2} condition had superior efficacy in inducing cytotoxicity against glioma compared to the tumor lysate from 20 % O\textsubscript{2} condition. CD8\textsuperscript{+} T depletion showed that there were other cells that play a role in this cytotoxicity. Together, these data show that 5 % oxygen tumor lysate has distinct effects on immunogenicity compared to 20 % O\textsubscript{2} lysate. These findings indicate a potential application in cancer treatment.
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### Abbreviations

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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine phosphate guanine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>GAA</td>
<td>Glioma associated antigen</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<tr>
<td>LAKs</td>
<td>Lymphokine activated killer cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor associated antigen</td>
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<td>TLR</td>
<td>Toll like receptor</td>
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Chapter 1  Introduction

1. Brain tumors
   Glioma is the most common type of malignant primary brain tumor in adults\(^1\). Several types of secondary tumors can metastasize into the brain. The most common types are lung cancer, breast cancer, and melanoma, while some types only spread into the brain very rarely such as colon cancer. Malignant gliomas represent a significant class of central nervous system (CNS) tumors from the glial lineage. Most gliomas are characterized by extensive infiltration of brain parenchyma, which can be further classified as astrocytic, oligodendroglial and oligoastrocytic tumors and by the tumor grade\(^1\). Astrocytic, oligodendroglial and ependymal origin of glioma constitute more than 70% of all malignant brain tumors\(^1\). Glioblastoma (GBM) is considered to be the most aggressive glioma, and in this study, we will focus on GBM.

2. Incidence and prognosis
   In the United States in 2008, it was estimated that there were 17,000 new cases of primary brain tumors and more than 13,000 deaths. The median survival rate for patients with GBM is 14.6 months, the overall survival rate is 27.2% at 2 years, and the mortality reaches over 90% after 5 years\(^2\). Children treated with irradiation for acute lymphoblastic leukemia have shown a greater risk of developing gliomas and primitive neuroectodermal tumors within 10 years of therapy\(^3\). Additionally, \(TP53\) mutations are often seen in low-grade gliomas that often progress to secondary GBM\(^3\). In spite of the progress in research, treatment options for the gliomas are limited: chemotherapy with the drug Temozolomide used with radiotherapy modestly prolongs patient survival. One of the limitations of classical chemotherapeutic approaches is the blood brain barrier (BBB). The BBB protects the brain from several bacterial infections and exposures, thus it can also be an obstacle to tumor therapy by blocking the movement of most antitumor drugs from the blood into the brain.

3. Brain tumor immunotherapy
GBM is one of the most aggressive and difficult cancers to treat. In spite of the advances in surgical techniques, radiation therapy, and chemotherapy, sustained tumor regression is rarely achieved and the prognosis for patients remains dismal. Even if current therapies could advance the treatment of GBM, conventional therapies are limited by significant morbidity: nonspecific damage to the healthy cells. In addition, glioma cells often infiltrate into the surrounding brain parenchyma leading to tumor cells that escape surgical resection, radiation and chemotherapy resulting in tumor recurrence\(^4\). Therefore, it is essential to develop novel treatments to target residual tumor cells, and treatment approaches using the body’s own immune system are promising in targeting residual radio- and chemo- resistant tumor cells without harming healthy brain tissues. Studies have focused on immunotherapy from different aspects, such as identifying new tumor associated antigens, understanding how tumors evade anti tumor effect of the immune system, and the translation of preclinical success in brain tumor immunotherapy model into clinical trials.

3.1 Immunotherapy

Immunotherapy of glioma can take several forms. 1) Passive immunotherapy, which involves the administration of antibodies or immunotoxins that are made outside of the body to patients. 2) Adoptive immunotherapy, which involves infusion of the tumor-bearing host with \textit{ex vivo} stimulated effector cells. 3) Active immunotherapy, which involves immunization of the tumor-bearing host to induce or enhance an antitumor immune response \textit{in vivo}.

3.2. Passive immunotherapy

The use of antibody in targeting tumor cells is a potential tool for targeting infiltrating brain tumor cells in the healthy cell population. An ideal scenario is the use of a tumor specific monoclonal antibody to target a cell surface antigen only expressed on the tumor cells. Epidermal growth factor receptor (EGFR) has been an attractive target for the antibody-based immunotherapy. EGFR is a transmembrane receptor tyrosine kinase that binds its ligands EGF and TGF-\(\alpha\)\(^5\). EGFR activation leads to modulation of gene
transcription resulting in enhanced proliferation, angiogenesis, and metastasis of tumor cells and EGFR over expression associated with increased tumor growth and shorter survival rate. EGFR is over expressed in 40 to 50% of malignant gliomas and almost absent in normal parts of the brain\textsuperscript{6}. In addition, a mutant variant EGFR, EGFRvIII is characterized by approximately 45 to 62% of grade III/IV gliomas\textsuperscript{7}. EGFRvIII shows deletion of exons 2-7, producing a truncated protein with constitutively active tyrosine kinase activity\textsuperscript{8,9}. Use of anti-EGFRvIII increased the median survival of mice bearing EGFRvIII expressing tumors in the brain by an average of 286\%\textsuperscript{10}.

Bacterial and plant toxins have been used as effectors when they are conjugated to antibodies or targeting ligands as an agent that can be selectively deliver toxins into tumors. Toxins are in some cases more potent than radiotherapy or chemotherapeutic agents and they are effective against hypoxic cells, which cause the radiation resistant cells in glioma. Thus, it is possible that tumor populations that are resistant to chemo-, and radiotherapy can be targeted by immunotoxins.

Over the past decade, passive immunotherapy has progressed and left reason for optimism. However, this approach has some limitations; it is difficult to identify a prominent cell surface antigen that is only expressed on glioma cells but not on normal cells.

3.3. Adoptive Immunotherapy

Adoptive immunotherapy is the transfer of immune cells manipulated in some way \textit{ex vivo} into patients. In earlier studies, this approach was mostly used to treat melanoma patients, in which tumor infiltrating lymphocytes or peripheral blood leukocytes were isolated and stimulated \textit{in vitro} in the presence of IL-2 and reinfused into the patients\textsuperscript{11,12}. Such lymphokine activated killer cells (LAKs) kill several autologous and allogeneic tumors, including gliomas\textsuperscript{13,14}. For example, Ishikawa et al demonstrated NK cell mediated clinical responses against glioma in six patients with grade III glioma and three patients with grade IV (GBMs)\textsuperscript{15}. They used peripheral blood mononuclear cells (PBMC)
isolated from patients and cultured with human feeder cell line, HFWT in RHAM-a medium with IL-2 and 5% autologous plasma\textsuperscript{15}. The culture was enriched with NK cells with the mean frequency in the cultured lymphocytes of $82.2 \pm 10.5\%$, which were infused back to the patients. This approach was safe and partially effective in these patients with no neurological toxicity observed in any case\textsuperscript{15}. Even though clinical responses were demonstrated in some cases, this approach depends on innate immune cells. In contrast, antigen specific CD8\textsuperscript{+} T cells, also known as cytotoxic T lymphocytes (CTL), can effectively target the tumor cells on a cell-by-cell basis without harming normal cells. This approach involves passive infusion or transfer of autologous CTLs specific for tumor-associated antigens. PBMCs from the patient can be expanded \textit{in vitro} through antigenic stimulation, which eventually generate cells with specificity to tumor-associated antigens (TAA). Yee et al. have shown that CTL clones specific to melanoma-associated antigen-1 (MART-1) localized to melanoma, and destruction of melanocytes and cells expressing MART-1\textsuperscript{16}. In spite of the advances in the approach, adoptive T cell therapy needs to be refined to overcome several issues including their lack of a homing capacity to the tumor site and T cell suppression by Tregs\textsuperscript{17, 18}.

\subsection*{3.4. Active immunotherapy}

Active immunotherapy is a strategy in which antitumor immunity is initiated \textit{in vivo} following vaccination with tumor antigen. Preclinical studies suggest this to be a novel treatment regimen for multiple types of cancers. Whole glioma cell vaccines have an advantage of providing multiple glioma-associated antigens that are naturally expressed by the tumor cells. Use of autologous tumor from the patients would arguably provide the most relevant glioma associated antigen in the patient. Recent clinical studies by Schneider et al. and Steiner et al. have shown that active immunotherapy with autologous tumor lysate combined with Newcastle-Disease virus, which was used as an adjuvant, increased the efficacy of the immunotherapy without major adverse effects\textsuperscript{19, 20}. However, the use of autologous tumor cells in vaccination requires the isolation and maintenance of tumor cells and substantial quality control. In addition, the use of
unfractionated tumor lysate has a concern of inducing autoimmunity, in the form of experimental allergic encephalomyelitis.

In peptide-based vaccination, synthetic glioma associated antigen (GAA) peptides are used to specifically target glioma cells. In a phase I study by Yajima et al. with glioma patients, personalized peptide vaccination was used. From each patient, PBMCs and plasma were isolated and analyzed for cellular and humoral responses to several peptides, and only peptides that can induce positive response were used in preparing vaccinations with Montanide ISA 51 adjuvant. This strategy was well tolerated and demonstrated 622 days of median survival of patients treated with recurrent glioblastoma multiforme.

A dendritic cell (DC) vaccine is the use of DCs loaded with tumor-associated antigens, which are administered to patients to induce an antigen specific immune response. DCs are specialized antigen presenting cells in the immune system. They sample environmental antigens and stimulate T cell activity in a major histocompatibility complex dependent manner in the setting of inflammation. When they encounter an antigen, DCs processes the antigen through an MHC class II mediated pathway that will result in MHC class II molecules stimulating CD4+ T cells. Moreover, antigenic material captured in endosomes can be released into cytosol and digested by the proteasome, which will then be transported onto major histocompatibility complex (MHC) class I molecules and presented to CD8+ T cells. DCs have also shown to recruit NK cells to draining lymph nodes, and NK cells secrete IFN-γ necessary for T_H1 polarization. Through these distinct pathways, DCs can stimulate diverse immune responses. In the early clinical study, Yu et al. used DCs pulsed with peptides eluted with autologous glioma cells and demonstrated an increase in tumor-specific CTLs in four out of seven patients. In the later study, Liau et al. reported a phase I clinical trial in which 12 GBM patients were administered with DCs pulsed with peptides acid-eluted from the surface of the resected autologous tumor. This treatment increased antitumor responses without any adverse affect. The median survival rate was also promising at 23.4 months and the benefit of the vaccinations was more evident in a patient group with slowly progressing tumors expressing low levels of transforming growth factor beta 2 (TGF-β2).
Vaccination with DCs is a very attractive regimen in targeting gliomas. Currently, there is no consensus on the DC subtypes, the optimal conditioning and activation stimuli, loading method, route of administration, dose or frequency of the vaccination. Moreover, regardless of the fact that feasibility and safety have been documented in the studies, clinical efficacy is not yet convincingly proven. Improvement and understanding in these factors will play an important role in influencing the clinical outcomes.

Several immunomodulatory agents that can act on antigen presenting cells and effector cells in the immune system have gained interest recently. Signaling cascade from a family of toll like receptor (TLR) agonists on antigen presenting cells (APCs) can lead to up regulated production of cytokines such as interferon gamma and interleukin 12. In addition, TLR agonists lipopolysaccharide, double stranded RNA, heat shock protein, imiquimod, and cytosine phosphate guanine (CpG) oligonucleotides have been demonstrated to enhance anti glioma immune responses\textsuperscript{25, 26, 27, 28, 29}. The CpG motif is a characteristic of prokaryotic DNA and is unmethylated DNA. CpG induces powerful innate and adaptive immune responses upon its binding to the ligand TLR9 expressed on both human and murine glioma. Thus, local TLR stimulation is an attractive method to induce antitumor immunity. CpG used as a vaccine adjuvant for tumor-associated antigens, has shown a significant antitumor effect in several studies\textsuperscript{29, 30}.

4. Challenges

Tumor immunotherapy for intracranial tumors encounter distinct challenges; the central nervous system has been considered an immunologically privileged site. The limited immune surveillance in the brain was first reported in 1948 showing that allogeneic tissue grafts transplanted into the brains of experimental animals were not rejected\textsuperscript{31}. The later studies support this finding for the presence of unique characteristics to the CNS, such as the BBB and tightly regulated immune cell trafficking into and from the brain, graft acceptance, and lack of conventional lymphatics.
The BBB is capillary endothelial cells of the CNS that can limit passive diffusion and maintain low pinocytotic activity. Since the CNS is extremely sensitive to toxic effects from exogenous sources, it is critical to regulate the homeostasis and restrict entry to the CNS by developing neurovasculature specialized to control both molecular and cellular migration into and out of the CNS parenchyma and cerebral spinal fluid. The limiting of passive diffusion is done by close cell-cell interactions called tight junctions. The lack of fenestrations and the tight junctions prevent the transport of large hydrophilic molecules including peptides, proteins, and cells. Blocking of protein consisting the tight junction by mAb has been shown to inhibit the leukocyte accumulation in cerebrospinal fluid and brain parenchyma, probably due to blocking leukocyte transmigration at the BBB.

Regardless of immune privilege of the CNS, recent studies have shown that immune cells can infiltrate into the brain. Also, specialized microglia, astrocytes, and some types of cells of the choroids plexus epithelium are able to mediate function as surrogate APCs in the CNS.

Tumorigenesis is often associated with immunosuppression in several types of tumors that can result in an unsuccessful immune therapy. In theory, cells such as natural killer cells or cytotoxic T lymphocytes that exhibit cytotoxicity against mutated cancerous cells and foreign cells can clear out transformed neoplastic cells. However, tumors initially escape killing from immune cells, and subsequently develop into established tumors after accumulation of additional mutations, which often induce immuno-evasive qualities. In the case of patients with glioma, many immune defects are apparent and often related with overall lymphopenia, including depressed antibody production, impaired antigen presenting cell functions, and suppressed peripheral T cell responses that can be seen by cutaneous anergy to tumor antigens and T cell receptor mediated intracellular signaling. In glioma patients, defects in T cell subsets are especially well documented; peripheral blood lymphocytes from glioma patients proliferate poorly to T cell mitogens and T-dependent B cell mitogens. Additionally, it seems that the defects are often
seen in the CD4 positive subset of T cells and they fail to elicit helper activity in allogeneic mitogen cultures\textsuperscript{33,37}.

This phenomenon is caused by tumor-secreted cytokines, such as TGF-β, Vascular endothelial growth factor (VEGF), prostaglandin E, and interleukin 10. TGF-β is a homodimer of two disulfide-linked subunits\textsuperscript{38}. Mutated TGF-β has been isolated from supernatant of malignant glioma cells and the gene for TGF-β2 was cloned from a malignant glioma cell line\textsuperscript{38}. TGF-β has been shown to suppress the immune response, such as suppression of the IL-2 induced CTL generation from PBLs and tumor infiltrating lymphocytes, inhibition of IL-2 receptor expression on T cells, down regulation of NK cell cytotoxicity and secretion of IFN-γ, and inhibition of MHC class II dependent antigen presentation\textsuperscript{39,40}. Studies have demonstrated that TGF-β produced by tumors significantly reduced the efficacy of DC/tumor fusion vaccines\textsuperscript{41,42}.

VEGF is another immunosuppressive, soluble factor secreted by tumor cells\textsuperscript{43}. VEGF plays an important role in tumor angiogenesis and is also directly responsible for inhibition of DC maturation from progenitor cells, in which VEGF induced inhibition of NFkB signaling in hematopoietic progenitor cells\textsuperscript{44}. A use of anti-VEGF monoclonal antibody (bevacizumab) treatment has been shown to have efficacy in the treatment of glioma, which is due to its anti-angiogenic effect and could possibly be due to inhibition of VEGF mediated immunosuppression\textsuperscript{45}.

Prostaglandin E2 (PGE2) is a product of arachidonic acid metabolism produced at the site of inflammation and exerts several effects including vascular permeability. PGE2 is from gliomas and has modulatory effects on T cell functions\textsuperscript{46}. However, the concentration of PGE2 in the supernatant of malignant glioma cells was not high enough to inhibit T cell proliferation and PGE2 inhibitor did not have an effect on T cell responsiveness\textsuperscript{47}. Further investigation is needed to elucidate the role of PGE2 in glioma-induced immunosuppression.

Increased expression of interleukin 10 is prominent in glioma patients. The expression of IL-10 mRNA is enhanced with increased tumor grade; 87.5% of astrocytomas with WHO grade III-IV express IL-10 whereas 4% of grade II astrocytomas expresses IL-10
mRNA. Also, the invasiveness of glioma correlates with IL-10 mRNA expression levels.

The success of immunotherapy is also associated with certain populations of suppressive leukocytes in patients with malignant glioma. In the late 1990s, the importance of peripheral immune tolerance emerged with a better understanding of a regulatory T cell population (Tregs) characterized by the expression of the transcription factor Foxp3. Tregs represent a CD4+ T cell subset with higher levels of CD25. Tregs inhibit T cell activation and proliferation by many mechanisms including down regulating IL-2 production in responder cells, which has been observed in glioma patients. Also, several studies have shown Tregs are present within the glioma microenvironment and not present in normal brain tissue or in low-grade glioma. Moreover, depletion of Tregs in glioma bearing mice with anti-CD25mAb precedes a T cell dependent destruction of glioma cells and delayed the tumor growth causing longer survival of the mice. In addition, a time-dependent accumulation of CD4+FoxP3+ Tregs is up regulated over a time course in a syngeneic murine glioma GL261 model. These studies illustrate that Tregs play an important role in immunosuppression in a glioma model and in patients.

In a mouse glioma model, microglia and macrophage infiltrations account for 5-20% of the cells within the tumor mass and their abundance positively correlates with the grade of malignancy. In the CNS, macrophage and microglia are postulated as the first line of defense against several infections and stressors, although several reports have shown their innate immune function is compromised in the tumor microenvironment. Glioma infiltrating macrophages (GIMs) do not adequately secrete proinflammatory cytokines such as TNF-a, IL-1, and IL-6. It has also been shown that GIMs failed to induce T cell proliferation. GIMs exhibit decreased expression of MHC class II molecules and co-stimulatory molecules that are critical in T cell activation, such as CD86, CD80, and CD40. Macrophages can release various cytokines and growth factors that may promote immune evasion and proliferation of glioma such as VEGF and
There is currently a debate if the GIMs are due to a failed immune response against the glioma cells or if the GIMs are recruited by the tumor for promoting tumor growth and proliferation.

5. Murine glioblastoma cell line GL261

In order to justify the clinical approach, the use of a suitable animal model is necessary, and the model should reproduce the major characteristics of glioma both \textit{in vivo} and \textit{in vitro}. In 1939, Seligman and Shear induced gliomas by injecting 3-methylcholanthrene into C57BL/6 mice intracranially\textsuperscript{87}. One of the gliomas developed was later designated GL261 and maintained by several intracranial and subcutaneous transplantations of tumor pieces into C57BL/6 mice\textsuperscript{87}. GL261 is one of the most frequently used murine glioma models that mimic the human GBM in several ways such as its invasiveness and angiogenic properties. Traditionally, the U87MG glioma cell has been used as murine model of glioma. However, Hu et al. reported that the leading edge of tumors formed by U87MG clearly divided from the brain adjacent to the tumors\textsuperscript{88}. In contrast, GL261 glioma cells form irregularly shaped borders showing invasive cells at some distance from the main tumor core, which is one of the characteristics of human glioma\textsuperscript{88}. VEGF is an important factor in tumor angiogenesis that is up regulated in human GBM, and the expression of VEGF has been shown in the endothelial cells in the blood vessels of GL261 gliomas\textsuperscript{89, 90}. Several characterizations of the GL261 glioma model show that it represents the relevant tumor biology of human glioma in both immunosuppressed and immunocompetent mouse models. Thus, GL261 glioma cells are used as an important preclinical model in researching therapeutic strategies for glioma therapy.

6. Rationale to determine the effect of oxygen on tumor cell immunogenicity

Heterogeneity in oxygen tension has been demonstrated in several tissues including normal CNS and cancerous tissues. The oxygen tension in the air is 21kPa (21%) and when the air arrives at the lungs, it is 20kPa and keeps decreasing as the distance from the air to 0.5-2.5 kPa in tissues\textsuperscript{57}. In the brain, the partial pressure and condition of oxygen is
low from 0.5 % to 8 %. Low oxygen tension in cancerous tissue is facilitated by tumor cell growth that outpaces the formation of supporting blood vessels. The cancer cells at the tumor core undergo necrosis due to the anoxic environment. The cells in a hypoxic condition deplete their energy source (ATP) and often initiate apoptosis. Previous studies have shown that vaccination with CpG and lysate from tumor cells grown in 20 % O2 condition resulted in greater survival of glioma bearing mice with over 2 times greater median survival time compared to control. Also, splenocytes from animals treated with vaccinations induced potent tumor lysis activity.

We propose that utilizing a physiologic oxygen condition in culturing cells for tumor lysate vaccination is attractive for two reasons; it can imitate the tumor microenvironment, and also induces stress conditions that are linked to increased immunogenicity. The concentration of oxygen has been shown to change the gene expression, which might be more similar to the gene expression at the tumor site. Hypoxia-inducible factor 1 (HIF1) is a heterodimeric transcription factor that mediates the effects of hypoxia. HIF-1α and HIF-2α protein are increased in many types of cancers and their metastasis. HIF activates transcription of enzyme encoding genes that play an important role for glycolysis and angiogenesis such as VEGF. In addition clinical studies have shown that HIF protein levels correlates with cancer mortality. Moreover, recent studies demonstrated that a hypoxic condition is associated with the induction of stress-induced proteins such as Heat Shock Protein (HSP) and High mobility group box 1 (HMGB1) that can be recognized by TLRs as danger signals and thus increase tumor cell immunogenicity by influencing innate and adaptive immune response.

7. Central hypothesis

My central hypothesis is that tumor lysate derived from cells cultured in 5 % O2 condition can induce higher immunogenicity compared to lysate derived from 20 % O2 condition. To test this hypothesis, we assessed the relationship between tissue culture
oxygen (5 % vs. 20 % O₂) and the priming of innate and adaptive antitumor in responses in a murine glioma model.
Chapter 2 Materials and Methods

1. Cell culture
For establishment of tumor, GL261-Luc cells were cultured in DMEM media containing 10% FBS, 1% penicillin/streptomycin, 1µg/ml puromycin, and 0.1 mg/ml normocin and maintained in atmospheric 20% O₂ in a humidified 37°C incubator. Cells were cultured as monolayers and harvested using trypsin-EDTA for detachment when the cells were 70% confluent. For tumor lysate, GL261 tumor cells were passaged in vivo and cultured in DMEM/F12 media containing 1× B27 and 1× N2 supplement (GIBCO Invitrogen), which were used as a serum-free supplement for the culture of tumor cells of neuronal phenotype. 0.1 mg/ml normocin was used as antibiotic solution (GIBCO Invitrogen). Cells were maintained in 5% O₂ using a Thermo Scientific Forma Series II multi-gas incubator or atmospheric 20% O₂ tension in a humidified 37°C incubator. Cells were grown as monolayers and harvested with non-enzymatic cell dissociation buffer (Sigma, St Louis, MO) when they were 70-80% confluent.

2. Intracranial glioma model
C57BL/6 female mice, 6-8 weeks of age, were purchased from Jackson Laboratory. Mice were anesthetized with IP injection of 30 µl of ketamine and xylazine cocktail (53.7 mg/ml ketamine and 9.26 mg/ml xylazine) then placed in a stereotactic frame (Figure 1). Mouse head was shaved, cleaned with triiodine and wiped with alcohol. A midline incision was made with a scalpel and 15,000 GL261-Luc cells/1 µl Hanks buffer were inoculated into the right striatum of with coordinates of 2.5 mm lateral, 0.5 mm anterior of bregma, and 3mm deep from the cortical surface of the brain. The cell suspension was inoculated at 0.2 µl/min. Hamilton syringe was used as catheter to deliver the cells. Mice were maintained in a specific pathogen-free facility according to the guidelines of the University of Minnesota Animal Care and Use Committee. Mice were imaged before the vaccinations to ensure the establishment of tumor. For imaging, mice were anesthetized with isoflurane and injected with 100 µl of luciferin, the substrate for luciferase, intraperitoneally (IP) and imaged by using Xenogen IVIS 100 system (Caliper Life Sciences, Hopkinton, MA)
Figure 1. Stereotactic apparatus
(1) The mouse is positioned on the stereotactic apparatus by using the right and left ear bars, and nose clamp. (2) The syringe holder is controlled in the x, y, and z planes by the stereotactic arm. (3) Injection speed is controlled by Quintessential stereotactic injector (Stoelting, Co., Wood Dale, IL).
Figure 2. Imaging of mice three days after the inoculation, before the vaccination. 10 min after the luciferase injection, GL261-luc cells inoculated intra-cranially in the mice were visualized with 5 min of exposure.
3. Preparation of tumor lysate
After harvesting, GL261 cells were washed extensively with PBS. $2 \times 10^6$ Cells were re-suspended in 500 µl of PBS and lysates were made by 5 cycles of quickly freezing by incubating the tube in liquid nitrogen and thawing by incubating the tube in a 56°C water bath. Between the freezing and thawing, the tube was mixed by vortexing extensively. The protein concentration was quantified using a Bradford assay. The tumor lysates (2-4 mg / ml) were aliquoted in small tubes stored at -80°C until use. Once thawed, the lysates left in the tubes were discarded and were not frozen again.

4. Preparation of vaccines
For a development of glioma specific immune response, tumor vaccination using tumor lysate (0.65, 6.5, 65, or 650µg) and CpG 685 (5’-tcgtagctcttcgcttctc-3’; SBI Biotech, Japan) adjuvant were used. Vaccines were made with 50 µg CpG 685 and tumor lysate cultured in 5 % O₂ condition or 20 % O₂ condition. Saline was used as a negative control. The total volume was brought to 100 µl with PBS. Vaccines were made freshly right before use.

5. Vaccination of mice
Glioma bearing mice were anesthetized by isoflurane. The area above shoulders were shaved and swabbed with triiodine and alcohol. Vaccines were injected intradermally three and ten days after intracranial tumor inoculation (Figure 3).
Figure 3

Figure 3. Design of vaccination on tumor bearing mice. On day 0, mice were inoculated with 15,000 GL261-luc cells. Three and ten days after the inoculation, mice were vaccinated with 5% or 20% O$_2$ tumor lysate and 50 µg CpG 685 above the shoulder blade intradermally. Thirteen days after the tumor inoculation, mice were sacrificed and blood, draining lymph nodes, and spleen were isolated from each mouse.
6. Collection of sera
Three days after the second vaccination, mice were euthanized with IP injection of 70 µl of ketamine and xylazine cocktail (53.7 mg / ml ketamine and 9.26 mg / ml xylazine). Blood was collected by cardiac puncture using an insulin syringe in 1.5 ml microcentrifuge tube and centrifuged at 22,000RPM for 20 min. The supernatant was collected and kept in -80°C for western blot assay.

7. Isolation of lymphocytes from lymph nodes and spleen
Three days after the second vaccination, mice were euthanized with IP injection of 70 µl of ketamine and xylazine cocktail. Four to six of tumor-draining cervical lymph nodes and the spleen were isolated from each mouse and rendered to a single cell suspension: a cell strainer was put on the top of 50 ml conical tube and lymph nodes or spleen were placed and squashed using the plunge of 3 ml syringe as complete RPMI was added. Red blood cells were lysed with Ammonium-Chloride-Potassium buffer: ddH₂O with 0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1mM EDTA, and pH was adjusted to 7.2-7.4. Cells were resuspended in PBS and cell number was counted manually after staining the cells by using trypan blue.

8. Flow cytometry analysis
Single cell suspension of lymphocytes from draining lymph nodes was stained using following antibodies (eBioscience): CD4-FITC, CD8-Alexa Fluor 700, CD69-APC, NK1.1-APC, CD3-Alexa Fluor 700, FoxP3-PE, CD45-FITC and CD19-APC. 5 x 10⁵ cells in 100 µl PBS were incubated with specific antibodies for 30 min at 4°C followed by washing three times. For intracellular staining of cells with FoxP3 antibody, fixation and permeabilization buffers (eBioscience) were used. After the last wash of surface antibody, cells were fixed by adding 100 µl of fixation buffer to each tube while vortexing, followed by the incubation in the dark at room temperature for 20 minutes. 1 ml of permeabilization buffer was added to each tube, the tubes were centrifuged for 5 minutes and supernatant was aspirated. This wash with permeabilization buffer was repeated once. Cells were resuspended in 100 µl of permeabilization buffer. FoxP3
antibody was diluted in 20 µl permeabilization buffer/ tube and added to each tube (concentration of antibody was 0.5µg/10^6 cells). Tubes were incubated in the dark at room temperature for 20 minutes. 1ml of permeabilization buffer was added to each tube, tubes were centrifuged for 5 minutes and supernatant was aspirated. The cell pellet was resuspended in 0.5 ml of flow cytometry staining buffer. Cell populations were measured by using FACScanto and data were analyzed by FlowJo software. To define a lymphocyte population and exclude debris and dead cells, data from unstained cells were used for gating with FSC and SSC. Single antibody stained cells were used for compensation controls and setting the gating. Appropriate isotype controls were used.

9. Measurement of cytokines in sera
Cytokine levels in sera were measured using Raybio Mouse Cytokine Antibody Array (RayBiotec, inc.). Measurement was done according to the manufacturer’s protocol. Briefly, 100 µl of 1× blocking buffer was added into each well and incubated at room temperature for 30 min. Blocking buffer was removed from the wells, 50 µl of sera was added to each well without dilution and incubated for two hours at room temperature. Sera were removed from the wells. Each well was washed with 1× wash buffer I on rocker with gentle rocking at room temperature. Wells were further washed two times by putting the glass chip with frame into a beaker with 1× wash buffer I at room temperature. Wash buffer was completely removed from each well, 70 µl of diluted biotin-conjugated antibodies were added to each well and incubated at room temperature for two hours. Wells were washed by putting the glass chip with frame into a beaker with 1× wash buffer I at room temperature, then washed with 1×wash buffer II on rocker with gentle rocking at room temperature. 70 µl of 1000 fold diluted fluorescent dye conjugated streptavidin was added and incubated for 2 hours. Wells were washed with 1× wash buffer I, 1× wash buffer II, and distilled H_2O. Fluorescent signals from the arrays were detected using laser scanner. Subtracting the negative control data from the sample data normalized Cytokine levels.

10. Western blot
The level of glioma reactive antibody was measured by western blot. Sera isolated from the mice were used as the primary antibody, which was blotted against GL261 cells. After harvesting, the GL261 cells were centrifuged at 400g for 5 min in 50 ml conical tube. Supernatant was discarded. The cells were washed for three to five times with 40-50 ml of 4°C PBS and centrifuged at 400g for 5 min. After the final wash, the supernatant was discarded. Cell pellets were re-suspended in 400 µl in 4°C PBS and transferred into micro-centrifuge tube. The 50 ml conical tube was rinsed one more time in 400 µl 4°C PBS and the PBS was transferred to the same micro-centrifuge tube. The cells were centrifuged at 400g for 5 min. To make lysate, lysis buffer containing 1× Protease Inhibitor Cocktail and 1× phosphatase inhibitor was added and cells were physically disrupted by using syringe and needle, passing the lysate through more than ten times. Lysate was centrifuged in micro-centrifuge tubes at maximum speed for 10 min. Supernatant was collected and the concentration was determined with Bradford assay. The concentration was adjusted to 2 mg / ml with SDS sample buffer.

A Bis/Tris 4-12 % 1.5mm 10well gels were used. After the transferring, membrane was blocked in 5% Non Fat Dry Milk (NFDM): 5gm NFDM per 100ml Tris-Tween Buffered Saline, with gentle rocking for one hour at room temperature. Primary antibody (sera isolated from mice) was diluted with a ratio of 1:1000 in the NFDM in which the membrane was placed and incubated for one hour with gentle rocking. Membrane was washed in TTBS for 60 min with gentle rocking for four to six times. Secondary antibody (Jackson Immunoresearch anti-mouse HRP) was diluted with the ratio of 1:50,000 in NFDM and the membrane was incubated for 60 min with gentle rocking. Membrane was washed in TTBS for 60 min with gentle rocking for four to six times. After washing with TTBS, membrane was washed with TBS followed by developing using 3,3,5,5’-Tetramethylbenzidine (TMB) substrate.

11. CD8+ T cell depletion
CD8+ T cells were depleted directly from single suspension of lymphocytes with magnetic bead selection (miltenyibiotec).
Magnetic labeling: Cell number was determined, and cell suspension was spun down at 300 g for 10 min, supernatant was completely removed. Cell pellet was resuspended in isolation buffer (40 µl / 10^7 cells). Biotin-antibody cocktail was added (10 µl / 10^7 cells), cell suspension was mixed well and incubated in 4°C for 10 min. Isolation buffer (30 µl / 10^7 cells) and anti-biotin micro beads (20 µl / 10^7 cells) were added. The mixture was mixed well and incubated in 4°C for 15 min. Cells were washed with isolation buffer (1-2 ml / 10^7 cells) by centrifuging at 300g for 10 min. Supernatant was completely removed and cells were resuspended in isolation buffer (500 µl /10^8 cells).

Magnetic separation: CD8+ T cells were depleted using autoMACS separator. The autoMACS separator was primed. Magnetically labeled cells were applied to the auto MACS separator apparatus, and positive fraction of cells representing cells depleted with CD8+ T cells, were collected. After counting the cell number, cells were resuspended in complete RPMI and used for cytotoxicity assay.

12. Cytotoxicity assay
Total Cytotoxicity and Detection Kit from Immunochemistry Technology Llc. was used to measure the cytotoxic effect of lymphocytes isolated from draining lymph nodes. The target GL261 cells were cultured in 20 % O₂ condition in DMEM containing 10 % FBS and 1 % penicillin/streptomycin. The GL261 cells were stained with CFDA (carboxyfluorescein diacetate), which enters cells and gets cleaved by intracellular esterase enzymes to form an amine reactive product producing detectable fluorescence. 7-aminoactinomycin D (7-AAD) was used to stain the dead cells by passing through the permeabilized or disrupted cell membrane only and intercalating into the double stranded DNA. Three days after the second vaccination, cervical lymph nodes were isolated and used as effector cells in cytotoxicity assay. 5×10⁶ GL261 cells were re-suspended in 1 ml of PBS. 0.8 µl of CFDA was added and incubated for 5 min at room temperature. Cells were washed two times with complete RPMI and incubated at 37°C for 30 min. 2×10⁴ target cells were used. Unstained lymphocyte effector cells isolated from mice and the CFDA stained GL261 cells were co-cultured for 4 hours with ratio of 50 : 1 = number of effector cells : number of target cells. 210X 7AAD stock was diluted with 1:10
in PBS and kept on ice. 20 µl of 7AAD was added to the samples after the incubation time, the sample tubes were gently vortexed, then incubated on ice for 10 min and analyzed by the flow cytometry. The control tubes used to set the gating were, CFSE single stained GL261 cells which were incubated with the samples, and CFDA stained GL261 cells which were frozen and thawed for five times and stained with 7AAD to be CFDA/7AAD positive control. The cytotoxicity was determined by measuring CFDA/7AAD double positive population by flow cytometry. The following equation was used: % cytotoxicity = % CFDA/7AAD double positive cells / % CFDA positive cells × 100 %. Syngeneic EL4 cell lines were used for negative target control to examine if the specificity of cytotoxicity is due to the tumor cells used for the lysate.

13. Statistical analysis
Statistical analysis was performed by ANOVA, followed by ad hoc comparisons using 2-tailed t-test. All tests were performed by using Prism 4 software (Graph Pad Software, Inc, San Diego, CA)
Chapter 3 Results: Analysis of draining lymph nodes

5 % O₂ tumor lysate increases the number of lymphocytes at tumor draining lymph node but not at spleen.

Oxygen condition on culturing condition of the tumor cells for the lysate might have a profound effect on immunogenicity of tumor vaccines. To compare the physiologic oxygen condition (5 % O₂) to atmospheric oxygen condition (20 % O₂) in induction of a glioblastoma lysate for vaccination, GL261 tumor cells for lysate were cultured in 5 % O₂ and 20 % O₂ condition and then compared for their effects on lymphocyte population.

Figure 4a shows that when groups of animals were vaccinated with 5 % O₂ lysate, there was a significant increase compared to the saline controls in the lymphocyte number in the draining lymph node (6.3 × 10⁷ cells/mouse vs. 2.3 × 10⁷ cells/mouse, p=0.04). Animals immunized with the 20 % O₂ lysate had an increase that was not significantly greater then saline controls. The increase in 5 % O₂ lysate treated group was also significant compared to the 20 % O₂ lysate treated group (6.3 × 10⁷ cells/mouse vs. 3.2 × 10⁷ cells/mouse, P=0.02).

Next, groups of animals were vaccinated and lymphocytes were isolated from the spleen. In this case, animals immunized with 5 % O₂ lysate vaccination did not have a significant increase in the lymphocyte number in the spleen compared to the saline controls (6.8 × 10⁷ cells/mouse vs. 8.2 × 10⁷ cells/mouse, p=0.99, (Figure 4b)). Likewise, there was not a significant increase in the lymphocyte number in the spleen from 20 % O₂ lysate treated group compared to the controls (6.8 × 10⁷ cells/mouse vs. 8.2 × 10⁷ cells/mouse, p=0.51(Figure 4b)).

Taken together these data show that in draining lymph nodes, lysate from cells cultured in 5% O₂ induced a increase of lymphocyte population compared to lysates from cells cultured in the 20% O₂ condition. As one might expect, this was not the case in the spleen possibly due to the distance from the vaccination site.
Figure 4a. **Effect of oxygen tension used in cell culture for lysate on the number of lymphocytes in draining lymph nodes.**

5% \( \text{O}_2 \) lysate increases the number of lymphocytes in the draining lymph nodes. GL261 tumor bearing mice were vaccinated as described in Materials and Methods. Three days after the second vaccination, four to six lymph nodes were isolated from each mouse. After the RBC lysis, cells were counted by trypan blue staining. Cell numbers were compared between each group. The saline only vaccinated group was used as the control. Statistical significance was indicated by *P<0.05. The standard error of the mean is shown by error bars. N=3/group.
Figure 4b. **Effect of oxygen tension used in cell culture for lysate on the number of lymphocyte in spleen**

Oxygen condition does not influence the lymphocyte number in the spleen. GL261 tumor bearing mice were vaccinated as described in Materials and Methods. Three days after the second vaccination, a spleen was isolated from each mouse. After the RBC lysis, cells were counted by trypan blue staining. Cell numbers were compared between each group. The saline only vaccinated group was used as the control. The standard error of the mean is shown by error bars. N=3/group
5 % O₂ tumor lysate changes the number of CD8⁺CD69⁺ cells in draining lymph nodes and spleen.

To further characterize the effect of the oxygen conditions in culturing tumor cells for lysate on the number of lymphocytes, cells isolated from draining lymph nodes and spleens were stained with CD8⁺CD69⁺ cells to measure activated CD8 positive cells.

Figure 5a shows when animals were treated with 5 % O₂ lysate, the CD8⁺CD69⁺ cell number at the tumor draining lymph node increased significantly compared to the saline treated control (2 × 10⁶ /mouse vs. 0.3 × 10⁶ /mouse, p=0.001). This was an increase of 6-fold. 20 % O₂ lysate also increased the number at the tumor draining lymph nodes compared to the saline treated control group (1.2 × 10⁶ cells/mouse vs. 0.3 × 10⁶ /mouse, p=0.046). Although this increase was not as great, a significant difference was seen in the number of CD8⁺CD69⁺ cells from tumor draining lymph nodes between 5% and 20% O₂ lysate treated groups (2 × 10⁶ / mouse vs. 1.2 × 10⁶ cells / mouse, p=0.0351). Together, these data indicate that the tumor lysate treatment has a significant effect on the CD8⁺CD69⁺ cell number for both spleen and tumor draining lymph node. But the increase was greater for 5 % lysate.

Next, the splenic lymphocytes were stained with CD8 and CD69 antibodies to characterize the effect of oxygen culturing condition.

Figure 5b shows that when animals were immunized with 5 % O₂ lysate, the CD8⁺CD69⁺ cell number in the spleen increased compared to the saline control group (1×10⁶ cells/mouse vs. 0.55×10⁶ cells/mouse, P=0.03). The 20 % O₂ lysate increased the number compared to the saline control group, however, the increase was not as remarkable as those observed in the lymph node (P=0.23).
Figure 5a. **Effect of oxygen tension used in cell culture for lysate on the number of CD8^+CD69^+ cells in draining lymph node**

20 % O_2 condition increases the number of CD8^+CD69^+ cells in the draining lymph nodes and results were even higher when mice were treated with 5 % O_2 lysate. GL261 tumor bearing mice were vaccinated as described in Materials and Methods. Three days after the second vaccination, four to six lymph nodes were isolated from each mouse. After the RBC lysis, a percentage of CD8^+CD69^+ population in cervical lymph nodes was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4a. The saline only vaccinated group was used as the control. Statistical significance was indicated by * \( P<0.05 \). The standard error of the mean is shown by error bars. N=3/group
Figure 5b. Effect of oxygen tension used in cell culture for lysate on the number of CD8^+CD69^+ cells in spleen
5 % O_2 condition increases the number of CD8^+CD69^+ cells in the spleen. GL261 tumor bearing mice were vaccinated as described in Materials and Methods. Three days after the second vaccination, a spleen was isolated from each mouse. After the RBC lysis, a percentage of CD8^+CD69^+ population in the spleen was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4b. The saline only vaccinated group was used as the control. Statistical significance was indicated by ^*P<0.05. The standard error of the mean is shown by error bars. N=3/group
5% O₂ tumor lysate changes the number of CD4⁺CD69⁺ cells in draining lymph nodes and spleen.

To further characterize the effect of the oxygen conditions in culturing tumor cells for lysate on the number of lymphocytes, cells isolated from draining lymph nodes and spleens were stained with CD4⁺CD69⁺ cells to measure activated CD4 positive cells.

Figure 6a shows that when animals were treated with 5% O₂ lysate, the CD4⁺CD69⁺ cell number at the tumor draining lymph node significantly increased compared to the saline treated control (3.5×10⁶/mouse vs. 0.9×10⁶/mouse p=0.008). This was an increase of three fold. 20% O₂ lysate also increased the number at the tumor draining lymph nodes compared to saline treated control, although this increase was not significant (2.2×10⁶ cells/mouse vs. 0.9×10⁶/mouse, P=0.14). Significant difference was not seen in the number of CD4⁺CD69⁺ cells from tumor draining lymph nodes between 5% and 20% O₂ lysate treated groups (P=0.07)).

Next, splenic lymphocytes were stained with CD4 and CD69 antibodies to measure the CD4⁺CD69⁺ population. Figure 6b shows that when animals were treated with 5% O₂ lysate the cell number was seen and the significant difference was not seen compared to the saline control (1.85×10⁶ cells/mouse vs. 1.9×10⁶ cells/mouse P=0.90). 20% O₂ treated group also did not have a significant increase compared to the saline control (2.4×10⁶ cells/mouse vs. 1.9×10⁶ cells/mouse P=0.41) Figure 6b). Together, the 20% O₂ lysate increased the population of CD4⁺CD69⁺ cell population at the draining lymph nodes, and an even higher increase was seen with animals immunized with 5% O₂ lysate. However, these increases were not seen in the spleen.
Figure 6a. Effect of oxygen tension used in cell culture for lysate on the number of CD4⁺CD69⁺ cells in draining lymph node

5 % O₂ condition increases the number of CD4⁺CD69⁺ cells in the draining lymph nodes. GL261 tumor bearing mice were vaccinated as described in Materials and Methods three days after the second vaccination. Four to six lymph nodes were isolated from each mouse. After the RBC lysis, a percentage of CD4⁺CD69⁺ population in cervical lymph nodes was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4a. The saline only vaccinated group was used as the control. Statistical significance was indicated by *P<0.05. The standard error of the mean is shown by error bars. N=3/group

* is p<0.05
Figure 6b. **Effect of oxygen tension used in cell culture for lysate on the number of CD4$^+$CD69$^+$ cells in spleen**

Oxygen condition does not influence the number of CD4$^+$CD69$^+$ cells in the spleen. GL261 tumor bearing mice were vaccinated as described in Materials and Methods. Three days after the second vaccination, a spleen was isolated from each mouse. After the RBC lysis, a percentage of CD4$^+$CD69$^+$ population in the spleen was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4b. The saline only vaccinated group was used as the control. The standard error of the mean is shown by error bars. N=3/group
5 % O_2 tumor lysate changes the populations of CD4^+Foxp3^+ subsets in draining lymph nodes.

Cells isolated from draining lymph nodes and spleens were stained with CD4 and Foxp3 antibodies to measure regulatory T cells to further characterize the accumulation of lymphocytes in response to the tumor lysates.

Figure 5a shows when animals were treated with 5 % O_2 lysate, the CD4^+Foxp3^+ cell number at the tumor draining lymph node significantly increased compared to the saline controls (2.1×10^6 /mouse vs. 0.6×10^6 /mouse, p=0.01) with an increase of three fold. 20 % O_2 lysate also increased the number at the tumor draining lymph nodes compared to the saline controls although this increase was not profound (1.0×10^6 cells/mouse vs. 0.6×10^6 /mouse, p=0.34) Significant difference was seen when 5 % O_2 lysate treated group was compared with 20 % O_2 lysate group (2.1×10^6 /mouse vs. 1.0×10^6 cells/mouse, p=0.01). This was not expected as regulatory T cell has inhibitory effects to the pro-immune response.

Next, splenic lymphocytes were stained with CD4 and FoxP3 antibodies to measure the regulatory T cell population (Figure 5b). When animals were treated with 5 % O_2 lysate the cell number was seen and the significant increase was not seen compared to the saline control (1.4×10^6 cells/mouse vs.1.5×10^6 cells/mouse, p=0.64). Although 20 % O_2 treated group had a significant increase compared to 5% treated group (2.7×10^6 cells/mouse vs. 1.4×10^6 cells/mouse, p=0.048). Thus, 20 % O_2 lysate increased the CD4^+Foxp3^+ population compared to the saline or 5 % O_2 lysate treated group.
Figure 7a Effect of oxygen tension used in cell culture for lysate on the number of CD4⁺FoxP3⁺ cells in draining lymph node

5 % O₂ condition increases the number of CD4⁺FoxP3⁺ cells in the draining lymph nodes. GL261 tumor bearing mice were vaccinated as described in Materials and Methods, three days after the second vaccination. Four to six lymph nodes were isolated from each mouse. After the RBC lysis, a percentage of CD4⁺FoxP3⁺ population in cervical lymph nodes was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4a. The saline only vaccinated group was used as the control. Statistical significance was indicated by *P<0.05. The standard error of the mean is shown by error bars. N=3/group.
Figure 7b. Effect of oxygen tension used in cell culture for lysate on the number of CD4\(^+\)FoxP3\(^+\) cells in spleen

20 % O\(_2\) condition increased the number of CD4\(^-\)FoxP3\(^+\) cells compared to 5 % in the spleen. GL261 tumor bearing mice were vaccinated as described in Materials and Methods, and three days after the second vaccination, a spleen was isolated from each mouse. After the RBC lysis, a percentage of CD4\(^+\)FoxP3\(^+\) cells population in the spleen was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4b. The saline only vaccinated group was used as the control. Statistical significance was indicated by *P<0.05. The standard error of the mean is shown by error bars. N=3/group

* is p<0.05
5 % $O_2$ tumor lysate changes the populations of $CD45^+CD19^+$ subsets in draining lymph nodes.

Next, cells isolated from draining lymph nodes and spleens were stained with $CD45^+CD19^+$ antibodies to measure B cells to further characterize the accumulation of lymphocytes in response to the tumor lysates.

Figure 8a shows when animals were treated with 5 % $O_2$ lysate, the $CD45^+CD19^+$ cell number at the tumor draining lymph node significantly increased compared to the saline control ($34 \times 10^6$ /mouse vs. $13 \times 10^6$ /mouse, $p=0.03$). 20 % $O_2$ lysate also increased the number at the tumor draining lymph nodes compared to the saline control although this increase was not significant ($18 \times 10^6$ cells/mouse vs. $13 \times 10^6$ /mouse, $p=0.53$). In addition, significant difference was seen when 5% $O_2$ lysate treated group was compared with 20% $O_2$ lysate group ($34 \times 10^6$ /mouse vs. $18 \times 10^6$ cells/mouse, $p=0.02$).

Next, splenic lymphocytes were stained with CD45 and CD19 antibodies to measure the B cell population (Figure 8b). When animals were treated 5 % $O_2$ lysate the cell number was seen and the significant difference was not seen compared to the saline control ($41 \times 10^6$ cells/mouse vs. $44 \times 10^6$ cells/mouse, $p=0.79$) or 20 % $O_2$ treated group ($46 \times 10^6$ cells/mouse, $p=0.64$). Thus, the immunization with tumor lysate did not have an effect on the B cell population in the spleen. Thus, the oxygen tension for the tumor cell lysate did not influence the splenic B cell population.
Figure 8a. Effect of oxygen tension used in cell culture for lysate on the number of CD45⁺CD19⁺ cells in draining lymph nodes

5 % O₂ condition increases the number of CD45⁺CD19⁺ cells in the draining lymph nodes. GL261 tumor bearing mice were vaccinated as described in Materials and Methods, three days after the second vaccination. Four to six lymph nodes were isolated from each mouse. After the RBC lysis, a percentage of CD45⁻CD19⁻ population was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4a. The saline only vaccinated group was used as the control. Statistical significance was indicated by *P<0.05. The standard error of the mean is shown by error bars. N=3/group
Figure 8b. Effect of oxygen tension used in cell culture for lysate on the number of CD45^+CD19^+ cells in spleen
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5 % O₂ tumor lysate changes the populations of CD3\(^{-}\)NK1.1\(^{+}\) subsets in draining lymph nodes.

To further characterize the accumulation of lymphocytes in response to the tumor lysates, cells isolated from draining lymph nodes and spleens were stained with CD3 and NK1.1 antibodies to measure the NK cell population.

Figure 9a shows that when animals were treated with 5 % O₂ lysate, the CD3\(^{-}\)NK1.1\(^{+}\) cell number at the tumor draining lymph node did not increase compared to the saline control (0.13\(\times\)10\(^6\) /mouse vs. 0.17\(\times\)10\(^6\) /mouse, p=0.67). However, treatment with 20 % O₂ lysate significantly decreased the CD3\(^{-}\)NK1.1\(^{+}\) population compared to 5 % O₂ treated group (0.022\(\times\)10\(^6\) cells/mouse vs. 0.13\(\times\)10\(^6\) /mouse, p=0.0005). NK cells are lymphocyte subset of innate immune response. This data suggests that tumor lysate vaccination does not increase NK cell numbers at draining lymph nodes, and surprisingly it even has suppressive effect when the lysate was grown in 20% oxygen condition.

Figure 9b shows that when animals were treated 20 % O₂ lysate, the NK cell number in the spleen was significantly decreased compared to the saline treated group (1633 cells/mouse vs. 1.4\(\times\)10\(^6\) cells/mouse, p=0.007) or 5 % O₂ treated group (0.6\(\times\)10\(^6\) cells/mouse, p=0.0002). Together, immunization with tumor lysates has significant suppressive effect on the innate lymphocyte subset at both draining lymph nodes and spleen.
Figure 9a. **Effect of oxygen tension used in cell culture for lysate on the number of CD3\(^+\)NK1.1\(^+\) cells in draining lymph nodes**

20 \% O\(_2\) condition decreases the number of CD3\(^+\)NK1.1\(^+\) cells in the draining lymph nodes. GL261 tumor bearing mice were vaccinated as described in the Materials and Methods, three days after the second vaccination. Four to six lymph nodes were isolated from each mouse. After the RBC lysis, a percentage of CD3\(^+\)NK1.1\(^+\) population was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4a. The saline only vaccinated group was used as the control. Statistical significance was indicated by *P<0.05. The standard error of the mean is shown by error bars. N=3/group
Figure 9b. Effect of oxygen tension used in cell culture for lysate on the number of CD3\(^{-}\)NK1.1\(^{+}\) cells in spleen

20 % O\(_2\) condition decreases the number of CD3\(^{-}\)NK1.1\(^{+}\) cells in the spleen. GL261 tumor bearing mice were vaccinated as described in the Materials and Methods. Three days after the second vaccination, a spleen was isolated from each mouse. After the RBC lysis, a percentage of CD3\(^{-}\)NK1.1\(^{+}\) population was measured by flow cytometry. The total cell number per mouse was calculated by multiplying the percentage of double positive population and the lymphocyte cells number measured in Figure 4b. The saline only vaccinated group was used as the control. The standard error of the mean is shown by error bars. N=3/group
Chapter 4 Results: Analysis of cytokine levels in sera

5 % lysate does not induce a change in systemic cytokine levels
To compare the effect of 5 % and 20 % tumor lysate on several cytokine levels, serum from vaccinated mice were isolated three days after the second vaccination and measured for cytokines.

IL-2 is a cytokine that influences T cell proliferation. In Figure 5 and Figure 6, it was shown that there was an increase in the CD4^+CD69^+ and CD8^+CD69^+ cell population of 20 % O_2 lysate vaccinated animals and even higher increase in the 5 % O_2 lysate treated animals. The effect of oxygen conditions to culture tumor cells for lysate on the amount of IL-2 was measured. IL-2 cytokine level in the 5 % oxygen treated animals was not different from 20% O_2 treated animals (Figure 10a). Also, 5 % O_2 lysate treated animals did not have a significant difference compared to the saline treated groups (Figure 10a). Together, these data show that tumor lysate vaccination does not have an effect on the level of IL-2 in the sera at this time point. In addition, oxygen-culturing condition of cells for the tumor lysate does not influence the IL-2 cytokine level.

IL-4 activates B cells and the Th2 T cell population. Figure 8a showed that B cell proliferation were seen in the tumor draining lymph nodes and spleen in the 20 % treated animals and even higher increase in the 5 % O_2 lysate treated animals. Thus, IL-4 levels in the sera were examined to determine if there is a correlation between proliferation and the amount of the cytokine. When animals were treated with 20 % O_2 lysate, the IL-4 levels were not significantly different compared to the saline treated group (Figure 10b). Also, a significant difference was not seen when animals were treated with 5 % O_2 lysate compared to the saline treated group (Figure 10b).

Together, the tumor lysate vaccination did not change the IL-4 levels in the vaccinated animals. In addition, the oxygen conditions to culture tumor cells for lysate did not affect the IL-4 levels in the sera and the correlation between the B cell proliferation and the IL-4 was not seen.
IL-10 is a potent suppressant of macrophage, Th1 cytokines and MHC class II antigens. Figure 7 showed that immune suppressive cell population CD4$^+$FoxP3$^+$ cell population in 5 % O$_2$ lysate treated animals was increased in tumor draining lymph nodes, and decreased in the spleen compared to 20 % O$_2$ lysate treated animals. To further examine the effects of oxygen conditions to culture tumor cells for lysate induced immunosuppressive effect, the effect of oxygen lysate on IL-10 levels in the sera was examined. When animals were treated with 20 % O$_2$ lysate, the IL-10 levels decreased, and it decreased even more in 20 % O$_2$ lysate treated group, but not significantly so. (Figure 10c). This trend correlates with the previous figures in which the lymphocyte numbers were accumulated when animals were treated with 20 % O$_2$ lysate, and even more accumulation in 5 % O$_2$ lysate treated group.

IL-12 is a NK stimulatory factor and also induces CD4 T cell differentiation into Th1 like cells. Figure 9 showed that animals vaccinated with 20 % O$_2$ lysate decreased CD3$^-$NK1.1$^+$ cell population significantly compared to 5 % O$_2$ lysate vaccinated group. The levels of IL-12 in the sera were examined to see if the oxygen culturing condition also influenced IL-12 levels. When animals were treated with 20 % O$_2$ lysate, the IL-12 levels were not significantly different compared to the saline treated group (Figure 10d). Also, no difference was seen when animals were treated with 5 % O$_2$ lysate compared to the saline treated group (Figure 10d). Together, the tumor lysate vaccination did not change the IL-12 levels in the vaccinated animals. In addition, the oxygen culturing condition did not affect the IL-12 levels in the sera and inhibitory effect of 20 % O$_2$ lysate on NK cells were due to factors other than the IL-12 in sera.

TNF-$\alpha$ is secreted by macrophages, NK cells, and T cells and promotes inflammation. In the previous figures the proliferation of the lymphocyte subsets were seen when animals were treated with 20 % O$_2$ lysate or 5 % O$_2$ lysate, thus the effect of oxygen condition on the TNF-alpha was measured. When animals were treated with 20 % O$_2$ lysate, TNF-$\alpha$ levels were not significantly different compared to the saline treated group (Figure 10e). Also, differences were not seen when animals were treated with 5 % O$_2$ lysate compared to the saline treated group (Figure 10e). Together, the tumor lysate vaccination did not
change the TNF-α levels in the vaccinated animals. In addition, the oxygen culturing condition did not affect the TNF-α levels in the sera.

VEGF is an important inducer of angiogenesis. In order to see the effect of oxygen condition of the lysate on the tumor formation, VEGF levels in the sera were measured. When animals were treated with 20% O₂ lysate, VEGF levels were not significantly different compared to the saline treated group (Figure 10f). Also, no difference was seen when animals were treated with 5% O₂ lysate compared to the saline treated group (Figure 10f). Together, the tumor lysate vaccination did not change the VEGF levels in the vaccinated animals. In addition, the oxygen culturing condition did not affect the VEGF levels in the sera.
Figure 10a to 10f Effect of oxygen tension used in cell culture for lysate on the amount of cytokines in the serum

GL261 tumor bearing mice were vaccinated as described in the Materials and Methods. Three days after the second vaccination, blood was collected by cardiac puncture and the sera were isolated. Cytokine levels were measured by Raybio Mouse Cytokine Antibody Array. Units are arbitrary. The means are shown by bars. N=3/group.
Chapter 5 Results: Analysis of antibody levels and cytotoxicity

650 µg lysate induces high antibody response.
CD4+ T plays an important role in the immunogenicity by establishing and maximizing the immune response through stimulating CD8+ T cells and antibody class switching. Previous studies have shown that CD4+ T cells have a profound effect on glioma immunotherapy using lysate vaccines: depletion of CD4+ T cell results in less effective responses resulting in shorter survival compared to CD8+ T cell or NK cell depletion. Thus it is expected that antibody levels have an important effect on the immunogenicity against glioma. To examine the effect of 5 % O₂ lysate on the antibody production compared to 20 % O₂, sera were isolated from mice three days after the second vaccination and used for western blotting to see the levels of tumor reactive antibodies. The darkness of the band (amount of anti GL261 antibody) and lysate amount directly correlated, while the oxygen condition did not. Together, vaccination induces antibody levels in which antibody levels and the lysate amount have a positive correlation.
Figure 11 Effect of oxygen tension used in cell culture for lysate on the level of tumor reactive antibody
GL261 tumor bearing mice were vaccinated as described in the Materials and Methods. Three days after the second vaccination, sera were isolated from blood, which was collected by cardiac puncture. Antibody levels in the sera were measured with western blotting assays. Sera were used as primary antibody and blotted against GL261 cells. Each strip shows one mouse. N=3/group.
5 % oxygen tension used in cell culture for lysate enhances the tumoricidal effect of lymphocytes.

To assess the influence of oxygen condition on tumoricidal function of lymphocytes, mice were vaccinated with 5 % or 20 % O₂ lysate. Furthermore, the optimal lysate dose was measured by varying the amount, 650 µg, 65 µg, 6.5 µg, and 0.65 µg per vaccination. Tumor draining lymph nodes were isolated on day13 and used as effector cells in cytotoxicity assays.

Figure 12 shows that when animals were vaccinated with 0.65 µg of lysate, lymphocytes from the 5 % O₂ lysate treated group had 5.97 % cytotoxicity and there was no significant difference compared to the 4.37 % cytotoxicity of lymphocytes from 20% O₂ lysate treated group against GL261 cells. When animals were vaccinated with 6.5 µg of lysate, lymphocytes from the 5 % O₂ lysate treated group had 30.2 % cytotoxicity and there was no significant difference compared to the 23.5 % cytotoxicity of lymphocytes from 20 % O₂ lysate treated groups against GL261 cells. When animals were vaccinated with 65 µg of lysate, lymphocytes from the 5 % O₂ lysate treated group had 33.6 % cytotoxicity and there was a significant difference compared to the 15.7 % cytotoxicity of lymphocytes from 20 % O₂ lysate treated groups against GL261 cells (p=0.02). However, when animals were vaccinated with 650 µg of lysate, lymphocytes from the 5% O₂ lysate treated group decreased to 21.5 % cytotoxicity and there was no significant difference compared to the 16.5 % cytotoxicity of lymphocytes from 20 % O₂ lysate treated groups against GL261 cells.

In addition, cytotoxicity against EL4 (negative control cells) remained constant across the various lysate amounts, which indicates the specificity of the antitumor immunity. Thus, cytotoxicity was the most enhanced when animals were vaccinated with 65 µg of 5 % O₂ lysate.
Figure 12. **Effect of oxygen tension used in cell culture for lysate on the cytotoxicity of lymphocytes**

Five percent oxygen lysate have a profound effect on enhancing the cytotoxicity at 65 µg. GL261 tumor bearing mice were vaccinated as described in the Materials and Methods. Three days after the second vaccination, tumors draining cervical lymph nodes were isolated from mice and used as effector cells. GL261 cells were used as target cells, with EL4 cells as target control. The error bars are shown by bars. N=3/group.
The cytotoxicity partially depends on CD8$^+$ T cells

CD8$^+$ T cells are classically thought of as mediating antitumor responses. To test the role of CD8$^+$ T cells in vaccine specific immune responses, CD8$^+$ T cells were depleted from lymphocytes of a group treated with 65 µg 5 % O$_2$ lysate (Figure 13). The depletion decreased CD8$^+$ T cell population from 15.1 % to 1.4 % (91 % depletion, Figure 13).

The cytotoxicity assay was repeated against GL261 cells. Figure 14 shows that when lymphocytes with CD8$^+$ T cell depletion were used to target the GL261 cells, the cytotoxicity decreased to 45.6 % compared to 56.4 % in lymphocytes without depletion (p=0.007) at E/T ratio of 50. Thus, the cytotoxicity does not completely depend on the CD8$^+$ T cell and there are other lymphocyte populations playing a role.
Figure 13. **Depletion of CD8^+ T cells**
Mice were vaccinated with 65ug of 5 % O_2 lysate. Three days after the second vaccination, tumor-draining lymph nodes were isolated. CD8^+ T cells were depleted using magnetic beads.
Figure 14. Cytotoxicity of draining lymph nodes after CD8\(^+\) T cell depletion

CD8\(^+\) T cells are not the only population that plays a role in tumor cytotoxicity. GL261 tumor bearing mice were vaccinated with 65 µg of 5 % O\(_2\) lysate as described in the Materials and Methods. Three days after the second vaccination, tumors draining cervical lymph nodes were isolated from mice and used as effector cells. CD8\(^+\) T cells were depleted using the magnetic bead selection (Figure 13). GL261 cells were used as target cells. The means are shown by bars.
Taken together, when animals were vaccinated with the tumor lysate, the tumor draining lymph node could induce cytotoxicity against the tumor cells. 5% O_2 lysate induced higher cytotoxicity than 20% O_2 lysate at 65 µg tumor lysate. Although the cytotoxicity from 5% O_2 tumor lysate vaccinated animals decreased when the lysate amount increased to 650 µg.
Chapter 6 Discussion

A successful vaccine requires the presence of a tumor-associated antigen that will raise a selective immune response against glioblastoma. In order to have successful vaccination, it is necessary to have a quality vaccine that is easy to prepare.

A wide range of approaches for glioma immunotherapies have been previously reported. For example, Crombet et al. used monoclonal antibody targeting EGFR only expressed on the glioma cells. The restorative approach was also used to stimulate the immunity passively with cytokines such as rIL-2 injected intracavitally or intracerebroventricularly, and recombinant human interferon beta injected intravenously. Some groups reported active specific immunotherapy using glioma lysates, and showed the safety and clinical response of tumor-lysate pulsed dendritic cell therapy. The approach we chose was a production of tumor lysates. We chose this approach because it is a fairly simple procedure that keeps GAA intact.

Activating TLR pathways for the cancer immunotherapy is based on the hypothesis that activation of innate immune response in the presence of tumor antigens might have innate antitumor effects and enhanced tumor antigen presentation with Th1 cytokines/chemokines. The immune system detects molecular patterns found on various pathogens by TLRs. TLR9 on human immune cells is extensively studied on DCs and B cells which are shown to constitutively express TLR9. During this activation process, GAA is processed by DCs and tumor specific antigens are presented to immune cells such as CTLs. DCs are the most potent antigen-presenting cells, which reside in most organs and tissue as immature cells at the interface of potential pathogen entry sites. DCs continuously sample although they do not effectively present the antigens without danger signals, which can be activated with ligations of TLRs by pathogen associated molecular patterns (PAMPs), which are byproducts of various pathogens. Unmethylated CpG dinucleotides triggers danger signals to DCs. This process matures DCs and upregulates chemokine receptors that guide DCs to draining lymph nodes. DCs interact and induce priming of CD8+ T cells with upregulation of adhesion and co-stimulatory molecules.
such as CD80 (B7.1) and CD86 (B7.2) in addition to the secretion of type I interferon. These further initiate secondary effects in the immune system, including cytokines/chemokines secretion, NK activation thus linking the innate and adaptive immune response, and expansion of T cell population, especially Th1 cells and cytotoxic T lymphocytes.

Development of effective tumor immunotherapy needs optimized immune responses. Glioma has been shown to express several GAA including gp100, melanoma antigen, α2 chain of IL13R, tenacin, and EGFR isoform III. However, universally expressed GAA with the critical cell survival function is not yet identified, thus targeting individual GAA could lead to immune escape since there are some populations that do not express the targeted GAA. For this heterogeneity of the glioma population, using the whole tumor cell lysate is an attractive source of antigens. Regardless, the tumor lysate is poorly immunogenic, and glioma immunotherapy needs to break this immunologic tolerance. Advancements in our understanding of the immune system indicate that we should be able to improve the quality of our lysate production. To solve this problem we used whole tumor cell lysate as a preferred source of antigens with TLR9 agonist CpG and tumor lysate.

In this study, it was postulated that it is reasonable to use the hypoxic condition to culture the tumor lysate since the oxygen condition in the tumor and inside brain is hypoxic; thus it would mimic the tumor environment providing tumor associated antigens presented on the glioma in situ. Furthermore, stress factors induced by hypoxic conditions are reported to bind the TLRs to enhance the cross-presentation in which the exogenous antigen are presented to CD8+ T cells and thus improve the cytotoxicity of target cells. Stress inducible factors produced under the hypoxic conditions also bind to the TLRs and activate antigen-presenting cells. These remarks might be explained by the fact that PAMPs and damage associated molecular patterns share recognition receptors to initiate the inflammatory responses. Several studies have shown that hypoxia induces proteins such as HMGB1 and HSPs, which promote inflammatory cascade through
ligation of TLRs. For example, Tung et al. have shown that HMGB1 induced by liver ischemia can activate the inflammatory pathways via binding of TLR, a lack of TLR or impairment of TLR signaling resulting in attenuated inflammatory response in hepatic shock.

Our research characterized for the first time the immunogenicity of 5 % O\textsubscript{2} lysate compared to atmospheric 20 % O\textsubscript{2} tumor lysate against glioblastoma in a murine model. 20 % O\textsubscript{2} lysate showed significant increase in the total number of the tumor draining lymphocytes. Even greater increase was seen in the group treated with 5 % lysate, however, a significant difference was not seen in the spleen. This observation is supported by studies on the proliferation and priming of lymphocytes in the brain tumor model. Calzascia et al. measured expression patterns of adhesion receptors of tumor specific CD8\textsuperscript{+} T cells in lymphoid organs to find that priming and proliferation occurred mostly in cervical lymph nodes and very minimal responses in the spleen after the intracranial implantation of tumor, indicating that the site of antigen capture to CTLs is critical in determining the homing phenotype. A possible set back of this experiment is that lymphocytes were counted per each animal, rather than each lymph node. Thus, there could be a potential sample bias since the number of lymph nodes harvested might skew the results.

When the populations of the subtypes of lymphocytes were analyzed in the 20 % O\textsubscript{2} lysate treated group, there was a significant increase in CD8\textsuperscript{+}CD69\textsuperscript{+}, and CD19\textsuperscript{+}CD45\textsuperscript{+} cell populations compared to the saline treated group. In 5 % O\textsubscript{2} lysate treated group, there was a significant increase in CD8\textsuperscript{+}CD69\textsuperscript{+}, CD4\textsuperscript{+}CD69\textsuperscript{+} and CD19\textsuperscript{+}CD45\textsuperscript{+} cell populations compared to the saline treated group. The CpG stimulation of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses were also seen in several studies. In addition, the same proliferation effect of CpG to stimulate B cells proliferation has been documented. Furthermore, when lymphocyte populations from 5 % O\textsubscript{2} lysate vaccinated animals were compared with animals in 20 % O\textsubscript{2} lysate treated animals, the 5 % O\textsubscript{2} lysate treated animals were significantly increased in CD8\textsuperscript{+}CD69\textsuperscript{+}, and CD45\textsuperscript{+}CD19\textsuperscript{+} levels. Since it is
possible that tumor lysate derived from hypoxic condition is more immunogenic for reasons mentioned previously, it is likely that although 20 % O\textsubscript{2} lysate is immunogenic, 5 % O\textsubscript{2} lysate is even more immunogenic than 20 % O\textsubscript{2} and proliferates several subsets of lymphocytes at the tumor draining lymph nodes.

Vaccination with CpG activated DCs which were pulsed with the tumor lysate induces a strong antitumor immune response with strong CTL and NK responses\textsuperscript{77, 78}. Generally, CTLs target the tumor cells upon the recognition of a MHC class I molecule and a GAA derived endogenously from the tumor cells. Although, the delivery of exogenous tumor lysate to the endogenous MHC class I pathway becomes a challenge in a cancer vaccine using exogenous tumor lysate in priming the CTL for antitumor effect. However, several studies have shown that signaling through TLR9 induces the cross presentation of exogenous antigen to MHC class I molecules\textsuperscript{79}. In addition, activation of the cross-presentation and the stimulation of CD8\textsuperscript{+} T cell subsets were observed when CpG plus peptides were used to stimulate a DC subset\textsuperscript{80}. Furthermore, it is possible that the cross presentation is further enhanced by using the tumor lysate grown in the 5 % oxygen condition due to increased stress factors acting as the TLR ligand. When lymphocyte isolated from cervical draining lymph nodes were used as effector cells in cytotoxicity assay, lymph nodes from 65 µg of 5 % O\textsubscript{2} lysate treated animals had a higher cytotoxicity compared to 65 µg of 20 % O\textsubscript{2} lysate treated animals. However, 5 % O\textsubscript{2} lysate was found to be suppressive when the lysate amount was increased to 650 µg, which is possibly due to the amount of lysate overwhelming the immune response.

CD8\textsuperscript{+} T cells are not the only critical player in tumor cytotoxicity because CD8\textsuperscript{+} T cell depletion only partially decreased the cytotoxicity (Figure 14), which indicates that there are other lymphocytes playing a role such as NK cells. This finding correlates with previous studies showing that NK cells can be activated by CpG administration, and activated NK cells are cytotoxic to the tumor cells directly\textsuperscript{81}. Several studies were done to elucidate the cells playing a role in tumor immunotherapy with CpG. Activated DCs and NK cells induces Th1 cytokines including IL-12 and IFN-\textgamma, and tumor cytotoxicity is
further enhanced\textsuperscript{82,83}. In a study of a murine glioma immunotherapy model using CpG and lysate, Wu et al. showed that CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells play a critical role by depleting those cells with mAb\textsuperscript{60} while depletion of CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells worsened the animal survival. Particularly, when animals were depleted with CD4\textsuperscript{+} T cells, the animals had a worse outcome than CD8\textsuperscript{+} T cells or NK depletion. Sandler et al. also reported that CD4\textsuperscript{+} T cells play a critical role in secreting cytokines that enhances the cytotoxic activity due to CD8\textsuperscript{+} T cells or NK cells\textsuperscript{84}. Therefore, a possible mechanism is that the effector cells in antitumor immunity are CD8\textsuperscript{+} T cells and NK cells, although their functions are regulated by CD4\textsuperscript{+} T cell functions.

Furthermore, in this study, a decreased number of NK 1.1\textsuperscript{+} cells in both draining lymph nodes and spleen were observed when animals were vaccinated with 20 % O\textsubscript{2} lysate compared to saline, or 5 % O\textsubscript{2} lysate treated groups, despite the fact that lymphocyte from 20 % O\textsubscript{2} lysate vaccinated animals had a higher cytotoxicity than the saline treated group (Figure 9 and Figure 12). A possible explanation is that the vaccination of 20 % O\textsubscript{2} lysate only suppresses the NK cell marker, NK 1.1 on the NK cell surface without suppressing the NK cell population and its functions and as a result, the number of NK cells is unchanged and pathways independent of NK 1.1 regulate its cytotoxicity. Another possibility is that NK cells and 20 % O\textsubscript{2} lysate share the same antigen, thus the NK cell population is suppressed by autoimmune response when mice were treated with 20 % O\textsubscript{2} lysate, and cells other than NK cells such as CD4\textsuperscript{+} T cells or macrophages mediate the cytotoxicity by death receptor mediated pathways including FAS pathways and phagocytosis.

Many studies have shown that regulatory T cells migrate to the site where regulation is required, such as the site of inflammation\textsuperscript{85}. This supports our study that the 5 % O\textsubscript{2} lysate vaccination is more immunogenic and induces a stronger inflammation as there was a significant increase in CD4\textsuperscript{+}Foxp3\textsuperscript{+} population in draining lymph nodes when animals were treated with 5 % O\textsubscript{2} lysate compared to animals treated with 20 % O\textsubscript{2} lysate or control group.
Our study showed that lymphocytes from 5% O\textsubscript{2} lysate vaccinated animals had higher cytotoxicity compared to the animals vaccinated with 20% O\textsubscript{2} lysate. However, CD4\textsuperscript{+}Foxp3\textsuperscript{+} regulatory T cell population that has suppressive effects to inflammatory response was also increased in the draining lymph nodes when animals were vaccinated with 5% O\textsubscript{2} lysate. This was supported by a study in which the treatment of mice with CpG ODN increased the generation of regulatory T cells\textsuperscript{86}. A possible explanation is that 5% O\textsubscript{2} lysate are immunogenic to activate TLR ligation pathways, and activation of this pro-inflammatory response is so strong that it can override the suppressive effects of T regulatory cell population.

Antibody production in tumor immunotherapy is important since it can target the tumor cells by several mechanisms such as ADCC. It has been shown that vaccination with CpG can induce the proliferation, production of antibodies and cytokines. Moreover, recent studies have observed that activating TLRs on B cells is required for autoantibody productions\textsuperscript{76}. Our study also showed that a higher amount of tumor lysate increases the antibody response as it was shown that 650 µg lysate could induce higher antibody response than 65 µg lysate. Significant difference between 5% O\textsubscript{2} and 20% O\textsubscript{2} in induction of the antibody response was not seen. This result might indicate that even though CpG and lysate can stimulate antibody response, activation of TLR pathway via the putative stress ligands produced during the 5% O\textsubscript{2} condition is not a requirement of the induction of antibody response.

During the activation of DCs via TLR ligands, several cytokines are produced to program the immune response. If there is any difference in cytokine levels, it further describes the specific types of immune response such as Th1 or Th2 skewed, or anti-inflammatory response. Among the cytokines we measured, IL-2 and IL-12 were Th1 cytokines, and IL-4 was Th2 cytokine. However, any significant difference was not seen among the treatment groups, and the effect in the cytotoxicity, and proliferations seen in 5% O\textsubscript{2} or 20% O\textsubscript{2} was not due to the effect of cytokines. Likewise, the levels of IL-10 anti-
inflammatory cytokine, TNF-alpha pro inflammatory cytokine, and VEGF angiogenic cytokine did not change. A possible explanation is that differences in the cytokine level on day 13 are not significantly large enough to detect. Also, the increased cytotoxicity in the lymphocyte from animals treated with 5 % O₂ lysate compared to the 20 % O₂ lysate treated group could be mainly due to the cross presentation which was enhanced by the lower oxygen culturing condition of lysate, which could induce up regulations of ligands of the TLRs. Thus enhancing the CTL activity and cytokines do not play a role in the increase in cytotoxicity.

We have shown for the first time that 5 % O₂ lysate can increase the number of lymphocytes, and the total cell numbers of several lymphocytes subtypes were increased in draining lymph nodes. We have also shown the amount of lysate correlates with the tumor reactive antibody and 5 % O₂ lysate profoundly increases cytotoxicity of lymphocytes. The implication of these data could have a great impact on the current clinical application of vaccinations derived from tumor lysate and CpG.
Chapter 7 Bibliography


