THE ANTI-HYPOXIA-ADENOSINERGIC APPROACH TO
THE IMMUNOTHERAPY OF CANCER

A dissertation presented
by
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to
The Department of Biology
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the field of
Biology

Northeastern University
Boston, Massachusetts
December, 2011
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ABSTRACT OF DISSERTATION

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Abstract

The suppression of tumor-reactive T- and NK cells in the tumor microenvironment (TME) involves several functionally distinct mechanisms that include the modulation of immunological negative regulators\(^1,2\), suppressor cells\(^3,4\), and the secretion of soluble anti-inflammatory factors\(^5,6\) that allow tumors to escape immunosurveillance. In addition to these mainstream mechanisms of immunosuppression in the TME, Sitkovsky and co-authors have suggested that it is the tumor hypoxia-driven accumulation of extracellular adenosine and subsequent signaling by engagement of the cAMP-elevating A2 adenosine receptors on tumor-reactive effector T-\(^7\) or NK cells\(^8\) that is instrumental in aiding the process of tumor evasion. Here, we show that the reversal of hypoxia by breathing high oxygen (60%-containing gas mixtures breaks tumor-induced hypoxia-adenosinergic suppression and improves the regression of MCA205 fibrosarcoma and B16 melanoma pulmonary metastases by adoptively transferred tumor-reactive T cells and by endogenously developed anti-tumor T- and NK cells. We have demonstrated that breathing 60% oxygen i) increased oxygen levels in previously hypoxic tumor tissue; ii) decreased intratumoral extracellular adenosine levels; iii) prevented the inhibition of intratumoral infiltration and proliferation of tumor-reactive T cells and iv) enhanced immune-stimulating cytokines while decreasing negative regulators of the anti-tumor immunity. We propose that this novel mechanism is mediated by T- and NK cells and suggest hyperoxia is acting upstream and downstream of the same tumor-protecting pathway. These previously unappreciated immunoenhancing capabilities of supplemental oxygen clarify an important aspect of tumor biology and anti-tumor immunity and offer a conceptually novel medical use of supplemental oxygen.
In studies of A2A or A2B adenosine receptor gene-deficient mice, we found that A2A adenosine receptor (A2AR) deletion solitarily liberates endogenous CD8 T cell anti-tumor immunity against progressively growing weakly immunogenic MCA205 sarcomas. Analyses of the therapeutic efficacy of adoptively transferred A2AR^{-/-}, A2BR^{-/-} or A2AR^{-/-}/A2BR^{-/-} T cells from culture-activated tumor draining lymph nodes (TDLN) confirmed that immunosuppression in the TME was mediated by A2AR on CD8 tumor-reactive T cells. Of potential clinical significance, treatment with the synthetic, selective A2AR antagonist KW6002 mimicked A2AR gene-deletion in adoptive T cell immunotherapy against pulmonary metastases. This therapeutic benefit of A2AR antagonism was independent of the anatomical location of tumor growth since anti-tumor reactivity was also observed in the eradication of established intracranial tumors, resulting in mouse survival and the development of long-lasting tumor-specific memory. Importantly, antagonistic blockade of the A2AR on adoptively transferred T cells prevented the inhibition of anti-tumor effector functions, as reflected by the increased levels of IFN-γ secretion by tumor-infiltrating CD8 T cells.

Taken together, these data reveal the principle mechanisms of hypoxia-driven immunosuppression in the TME and offer a therapeutic solution in the selective antagonism of the A2AR or the use of supplemental oxygen to augment T cell-based immunotherapy. In addition, the data presented here survived rigorous critical review in preparation for clinical trials at the University of Miami Sylvester Cancer Center, where the treatment of lung cancer patients with cancer vaccines will be combined with breathing 40% to 60% oxygen and A2AR antagonism.
Acknowledgements

I would like to thank Dr. Michail Sitkovsky for his continuous support and commitment to both his lab and graduate students. As advisor and mentor, his passion for science has truly been inspirational. Additionally, the help of the senior investigators in the Sitkovsky Lab have been paramount to accomplishing this research. Many thanks go to Dr. Akio Ohta, Akiko Ohta and Dr. Dmitriy Lukashev for their training and patience. I would like to make special mention of Dr. Jorgen Kjaergaard for his guidance. His expertise in the field of cancer immunotherapy and his dedication to my development as a scientist has been essential to this process.

Many others contributed to this body of work and deserve thanks, particularly Robert Abbott, Taylor Schreiber, Kami Ko, Manasa Madasu, Radhika Kini, Meenakshi Subramanian and Margaret McCrann. I would also like to thank my committee members whose time, support and advice have been greatly appreciated. Thanks go to Dr. Wendy Smith, Dr. Erin Cram and Dr. Graham Jones. I would also like to thank Dr. Richard Marsh, Dr. Veronica Godoy-Carter, Dr. Adrian Gilbert, Dr. Aaron Roth, Frauke Argyros and Janeen Greene for their additional support.

Lastly, I would like to thank my family, whose encouragement and support have never gone unnoticed.
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<th>Full Form</th>
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<tbody>
<tr>
<td>A2AR</td>
<td>A2A adenosine receptor</td>
</tr>
<tr>
<td>A2BR</td>
<td>A2B adenosine receptor</td>
</tr>
<tr>
<td>A-NK</td>
<td>Activated natural killer</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP response element</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic lymphocyte antigen-4</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>Fio2</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box p3</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1 alpha</td>
</tr>
<tr>
<td>HP-1</td>
<td>Hypoxprobe-1</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>mAB</td>
<td>Monoclonal antibody</td>
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</table>
MCA – 3-methylcholanthrene

MFI – Mean fluorescent intensity

MHC – Major histocompatibility complex

NAC – N-acetylcysteine

NK – Natural killer

NSCLC – Non-small-cell lung carcinoma

OD – Optical density

PD-1 – Programmed death receptor-1

PECAM-1 – Platelet endothelial cell adhesion molecule-1 (CD31)

PEG – Polyethylene glycol

PKA – Protein kinase A

pO₂ – Partial pressure of oxygen

Rag-2 – Recombinase activation gene-2

ROS – Reactive oxygen species

RT – Reverse transcription

TDLN – Tumor draining lymph node

TGF-β – Transforming growth factor-beta

TIL – Tumor infiltrating lymphocytes

TME – Tumor microenvironment

TNF-α – Tumor necrosis factor-alpha

T reg – T regulatory cell

TRITC – Tetramethyl rhodamine isothiocyanate

VEGF – Vascular endothelial growth factor
Chapter 1

Introduction

The hypoxia-adenosinergic immunosuppressive mechanism that protects tissues of vital organs from excessive collateral damage

The hypoxia-adenosinergic immunosuppressive pathways have evolved to protect normal tissues from excessive collateral damage by overactive immune cells during the anti-pathogen immune response\(^9,10\). The inflammatory response to pathogens is often accompanied by significant damage to innocent bystander cells. It has been proposed that in addition to the danger signals that trigger the anti-pathogen immune response, there exists a second signal that indicates the need to down-regulate or to stop the immune response and minimize collateral damage to normal tissues\(^11\). This suggested the existence of molecular sensors that trigger immunosuppression, thereby enabling a fine balance between pro-inflammatory and anti-inflammatory processes that ensure the majority of humans survive infections without permanent loss of vital organ function\(^11\).

We hypothesize that it is the collateral damage to blood vessels that acts as the initial event triggering hypoxia-adenosinergic immunosuppression as a result of the diminished capacity to deliver oxygen in local inflamed tissue microenvironments. The ensuing low-oxygen tension (i.e., hypoxia) in local tissues leads to the accumulation of extracellular adenosine. In turn, extracellular adenosine stimulates adenosine receptors that elevate the immunosuppressive, intracellular second messenger cAMP, which inhibits overactive immune cells\(^10,12\).
**Four adenosine receptor subtypes (A1, A2A, A2B, A3)**

There are four subtypes of seven trans-membrane spanning adenosine receptors\(^{13}\) (Fig. 1.1). The A2 receptors are subdivided into the high-affinity A2A receptor (A2AR) and low-affinity A2B receptor (A2BR) and are G\(_s\) protein-coupled\(^{13-17}\). Signaling through A2AR or A2BR in T cells results in the intracellular accumulation of cAMP by adenylyl-cyclase and the subsequent inhibition of TCR-triggered activation\(^{10,17}\) as well as many effector functions including proliferation, cytotoxicity and the secretion of important pro-inflammatory cytokines such as IFN-\(\gamma\)^{9,10,18} and TNF-\(\alpha\)^{19,20}. Interestingly, the high-affinity A1 receptor (A1R) and low-affinity A3 receptor (A3R) are inhibitory G\(_i\) protein-coupled, acting in opposition of A2 receptors by inhibiting the intracellular accumulation of cAMP\(^{13,21}\). However, CD8 and CD4 T cells express A2AR and A2BR predominantly\(^{22}\), with very low levels of A1R or A3R\(^{16,23}\).

**Hypoxia and intracellular sources of extracellular adenosine**

We suspect this mechanism to be triggered by local tissue hypoxia caused by damage to endothelial cells and microcirculation, thereby interrupting normal blood and oxygen supply. It is well established that inflamed and cancerous tissues are hypoxic\(^{10,12}\). Hypoxia is associated with a decrease in intracellular ATP\(^{24}\); an increase in intracellular AMP\(^{24}\); the inhibition of critical enzymes in adenosine modulation, i.e. adenosine kinase\(^{25}\) and 5’ nucleotidases\(^{26,27}\); the accumulation of intracellular adenosine\(^{25}\); the transport or diffusion of intracellular adenosine and the subsequent accumulation of adenosine in the extracellular space. Moreover, recent studies of the transcription factor Hypoxia Inducible Factor-1\(\alpha\) (HIF-1\(\alpha\)) have indicated a role in the transcriptional repression of adenosine kinase, providing a direct link between hypoxia and adenosine accumulation\(^{25,28-30}\).
Figure 1.1. The high affinity A2AR and low affinity A2BR are the primary adenosine receptors on CD4 and CD8 T cells\textsuperscript{10}. A2AR and A2BR are G\textsubscript{s}-protein-coupled receptors that stimulate adenylyl-cyclase to produce cAMP. Subsequent intracellular signaling mediated by cAMP results in the suppression of T cell effector functions, proliferation and cytokine secretion. The high-affinity A1 receptor and low-affinity A3 receptor are G\textsubscript{i} protein-coupled and inhibit the intracellular accumulation of cAMP, but are expressed at very low levels on cells of the adaptive immunity.
**Hypoxia and HIF-1α**

HIF-1α is known as the master regulator of oxygen homeostasis\(^{31,32}\). Under normal oxygen tension, HIF-1α is rapidly degraded. Oxygen-sensing prolyl hydroxylase (PHDs) enzymes target HIF-1α for proteosomal degradation\(^{33}\) (Fig 1.2). During hypoxia, HIF-1α is stabilized and sequestered in the nucleus where it modulates the transcriptional changes necessary to allow cells to adapt under low oxygen conditions\(^{33}\). Recently, it was established that HIF-1α also has a role in the regulation of the immune response\(^{34,35}\). Studies in our lab have indicated HIF-1α may cooperate with A2AR and A2BR in the inhibition of activated T cells\(^{23,31,32,36-38}\). Specifically, it was shown that T cells lacking HIF-1α had increased TCR-triggered production of cytokines such as IFN-γ\(^ {34}\). The negative role of HIF-1α in the regulation of activated T cells was also supported by *in vivo* studies of T cells in inflamed, hypoxic areas. Mice with a T cell-specific deletion of HIF-1α exhibited significantly higher cytokine production, stronger antibacterial effects and improved survival in a model of sepsis\(^ {35}\). Furthermore, it was shown that HIF-1α levels in T cells increase not only after exposure to hypoxia but also through antigen receptor (TCR)-triggered activation of T cells\(^ {34}\). This suggests that activated T cells may be inhibited by the accumulation of HIF-1α even after exiting hypoxic tissue areas.
Figure 1.2. The oxygen-dependent regulation of HIF-1α. Under normal oxygen conditions, HIF-1α is targeted for destruction by oxygen-dependent hydroxylation and subsequent ubiquitin-mediated proteosomal degradation. Hypoxia stabilizes HIF-1α by preventing the activity of prolyl hydroxylase enzymes, allowing the transcriptional activity of HIF-1α.
**Hypoxia and extracellular sources of adenosine**

The ecto-enzymes CD39 (ATPase/ADPase) and CD73 (5′-nucleotidase) represent another important pathway in the generation of extracellular adenosine\(^{26,39-41}\). Nucleotides released into the extracellular space are phosphohydrolyzed by CD39 (converting ATP/ADP to AMP) in tandem with CD73 (converting AMP to adenosine). Studies using CD39 and CD73 null mice concluded that these enzymes serve as critical control points for endogenous adenosine generation\(^{42-44}\). Interestingly, CD73 expression is elevated in hypoxic conditions in a HIF-1α-dependent manner\(^{27}\). Along with a hypoxia response element (HRE), the promoter region contains a cAMP response element (CRE), suggesting CD73 expression is regulated by a feed forward mechanism orchestrated by hypoxia-adenosinergic signaling\(^{27}\).

Recent studies have established CD73 as a critical negative regulator of the anti-tumor immunity by nature of its adenosine-generating capabilities\(^{45,46}\). Expression of CD73 has been reported in many types of cancers (bladder cancer, leukemia, glioma, glioblastoma, melanoma, ovarian cancer, thyroid cancer, esophageal cancer, prostate cancer and breast cancer) and is associated with invasiveness and poor prognosis\(^{46}\). It has been proposed that tumor cells upregulate expression of CD73 as a method of evading the anti-tumor response by increasing levels of extracellular adenosine in the tumor microenvironment (TME)\(^{46,47}\). Promisingly, the use of monoclonal antibodies to block CD73 activity in pre-clinical studies has demonstrated delayed progression of primary tumor growth as well as decreased metastasis\(^{46}\).
Figure 1.3. Hypoxia-adenosinergic immunosuppression inhibits T cell effector functions\textsuperscript{12}. Hypoxia alters tissue microenvironments causing physiological changes that result in the accumulation of extracellular adenosine. This, in turn, induces suppressive A2 adenosine receptor signaling. Simultaneously, hypoxia stabilizes the transcription factor HIF-1\textalpha, which may cooperate with A2 adenosine receptors to suppress T cell effector functions. (AK: adenosine kinase, NT5: 5'-nucleotidase, i.e. CD73, TCR: T cell receptor)
Cancer immunotherapy

Cancer immunotherapy by endogenous or adoptively transferred anti-tumor T cells is complementary to conventional treatments. For the past several decades, tumor-infiltrating lymphocytes (TIL) isolated from solid tumors have been used as a source of tumor-sensitized T cells.\textsuperscript{48-50} To obtain sufficient numbers for adoptive immunotherapy, TILs must be supplemented \textit{in vitro} with Interleukin-2 (IL-2), an immune-stimulating cytokine necessary for T cell proliferation\textsuperscript{48}. However, clinical studies have demonstrated that co-administration of exogenous IL-2 causes serious detrimental side effects \textit{in vivo}\textsuperscript{140} while TILs alone are largely ineffective\textsuperscript{50}. Thus, a major focus of immunotherapy has been the identification of factors that are capable of enhancing T cell potency without exhibiting harmful side effects or significant auto-immunity.

Pre-clinical studies have established the inguinal lymph nodes draining a progressively growing tumor as an abundant source of tumor-specific T cells\textsuperscript{51}. While having little anti-tumor capacity when transferred directly, these cells can be expanded into mature anti-tumor effector T cells \textit{in vitro} by culture-activation with anti-CD3 (T cell co-receptor) monoclonal antibody and IL-2\textsuperscript{52}. Using this as a model of adoptive immunotherapy, Kjaergaard et al. demonstrated that these tumor draining lymph node (TDLN) T cells were tumor antigen-specific since TDLN sensitized by the weakly immunogenic MCA205 fibrosarcoma demonstrated therapeutic efficacy only against transplantable tumors of MCA205 origin, and not of the antigenically distinct MCA207\textsuperscript{53}. It was shown that transferred tumor-reactive TDLN T cells began to infiltrate tumors as early as 2 hours after infusion, with maximal infiltration occurring between 16 and 24 hours\textsuperscript{53} (Fig. 1.4).
Figure 1.4. Adoptively transferred tumor-reactive T cells begin to infiltrate pulmonary metastases 2 hours after transfer with maximal infiltration occurring between 16-24 hours\textsuperscript{53}. Fluorescence micrographs of MCA205 pulmonary tumor-infiltrating cells (A) 2 hours and (B) 16 hours after adoptive transfer of $50 \times 10^6$ TRITC-labeled TDLN T cells. A hemotoxylin stain of the same tissue section is provided to the right. By 16 hours, nearly all of the tumor-reactive T cells remaining in the lung are found within tumor tissue and not in surrounding lung parenchyma.
Interestingly, tumor infiltration was not dependent on specific antigen recognition since TDLN T cells accumulated into both MCA205 and MCA207 indiscriminately (Fig. 1.5)\textsuperscript{53}. Tumor-reactive T cell proliferation, however, did require antigen specificity\textsuperscript{53,54}. These studies suggest the fate of adoptively transferred T cells recruited to the TME may depend on the quality of their antigen interaction and their ability to perform effector functions following tumor infiltration. However, even when tumors express antigens that elicit a massive influx of tumor antigen-specific T cells to the tumor site, the incidence of complete tumor destruction is very low\textsuperscript{2,55,56}. Thus, T cell-based treatments have had only limited success in large part because of the immunosuppressive TME that protects malignant cells from anti-tumor T cells, thereby negating advances in immunotherapy. Understanding the mechanisms that inhibit T cells in this poorly understood, hostile TME may help to explain the coexistence of tumors and anti-tumor T cells (Hellstrom Paradox)\textsuperscript{57}.

There are a myriad of mechanisms protecting tumors from the immune system including i) the shedding of the major histocompatibility complex (MHC) class I\textsuperscript{58,59}; ii) the masking of co-stimulatory molecules\textsuperscript{60,61}; ii) the secretion of immunosuppressive soluble factors such as TGF-\textbeta\textsuperscript{5} and IL-10\textsuperscript{6}; iii) the recruitment of host-derived suppressor cells, i.e. tumor-associated macrophages\textsuperscript{62}, myeloid-derived suppressor cells (MDSC)\textsuperscript{63,64} and CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} regulatory T cells (Tregs)\textsuperscript{3,4} and iv) the preponderance of negative regulators of the immune response\textsuperscript{1,2,10}. Thus a prevalent strategy of bolstering the therapeutic potency of anti-tumor immunity is targeting physiological negative regulators that sculpt the immunosuppressive TME.
Figure 1.5. The infiltration of tumor-reactive TDLN T cells is not tumor-antigen specific\textsuperscript{53}. The tumor infiltration of (A) naïve splenic T cells, (B) culture-activated splenic T cells, (C) culture-activated MCA207 TDLN and (D) culture-activated MCA205 TDLN was analyzed in a time course assay of 10-day established MCA205 pulmonary metastases.
Negative regulators and hypoxia-adenosinergic immunosuppression

It is well established that tumors are hypoxic due to disrupted oxygen delivery from highly irregular or damaged tumor microvasculature\(^6^5\). Tumor hypoxia is a poor prognosis factor in many types of cancers\(^6^5\) and is associated with the accumulation of extracellular adenosine, which may inhibit the anti-tumor response via adenosine signaling\(^1^0\). Interestingly, hypoxia also modulates the expression of many negative regulators of the immune response suggesting that oxygen deprivation may act as the driving force shaping the immunosuppressive TME. It has been proposed that negative regulators of the anti-tumor response may collaborate with the hypoxia-adenosinergic pathway to suppress effector cells in the TME\(^1^0,4^7\). Current studies of immunosuppressive T regs have provided direct evidence in support of this.

While inhibition by T regs has been a major focus of immunology for the past several decades, the mechanisms by which they cause this suppression remains poorly understood. Studies by Naganuma et al. have indicated that adenosine signaling is important for T reg function since T regs from A2AR\(^-/-\) mice demonstrated impaired suppression in a T cell-mediated colitis model\(^6^6\). These findings are in agreement with similar observations of the reduced functionality of T regs lacking the adenosine-generating enzymes CD73 or CD39\(^4^0\). T regs can also exert their suppressive effects in a more direct manner by shuttling cAMP into adjacent cells via gap junctions\(^6^7\). Other important suppressive mechanisms adopted by T regs involve the secretion of soluble factors such as IL-10 and TGF-β that bind to receptors on effector cells to prevent proliferation and induce inhibitory signaling\(^4^7,6^8\). The immunosuppressive cytokines IL-10 and TGF-β are upregulated by the T reg-associated transcription factor Foxp3, which has both hypoxia- and cAMP response elements, suggesting that the transcription of these soluble factors may be under control of cAMP-elevating adenosine signaling\(^4^7,6^8\). Taken together, these findings
indicate that T regs may control their own suppressive capacity by generating extracellular adenosine, thereby acting in an autocrine manner to promote their own suppressive functionality.

Additionally, T regs express high levels of the anti-inflammatory mediator Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)\(^69\). CTLA-4 on the surface of T regs or T effector cells can bind the B7 receptor with high affinity on antigen-presenting cells, blocking the ligation between B7 and the important T cell co-stimulatory receptor CD28\(^60,61\). The competitive binding of CTLA-4 not only prevents T cell activation but simultaneously transmits negative signaling that inhibits effector cells\(^61\). The interaction between CTLA-4 and B7 has been demonstrated as a vital mechanism for T reg-mediated suppression\(^70\). Studies of mice with a T reg-specific deletion of CTLA-4 showed impairment of T reg suppression both \textit{in vivo} and \textit{in vitro}\(^70\). Also relevant is the finding that CTLA-4 is under control of the T reg transcription factor Foxp3\(^70\). Possessing both an HRE and CRE\(^47,68\), CTLA-4 expression on T regs could be regulated by hypoxia and adenosine signaling. Pre-clinical studies have established that CTLA-4 blockade by monoclonal antibodies represents a promising strategy to overcome T cell tolerance during the anti-tumor response\(^61,71-73\).

**Reversing hypoxia-adenosinergic immunosuppression**

The tumor microenvironment has been described as a complex equilibrium in which the anti-tumor immunity is constantly challenged by the tumor-promoting mechanisms co-opted by the tumor itself\(^74\). We, and others, suggest that hypoxia coordinates the physiological changes that tip the balance in favor of advancing tumor growth. We propose that the reversal of tumor hypoxia may be the fundamental first step in re-establishing immune competence in the TME. By inspiring high oxygen-containing gas mixtures (60% oxygen), we aim to eliminate tumor
hypoxia and reduce levels of extracellular adenosine, thereby breaking the tumor-induced hypoxia-adenosinergic signaling and unleashing the otherwise inhibited anti-tumor effector cells. Supplemental oxygen (e.g. carbogen, 95% oxygen: 5% carbon dioxide) has been established as an approach to normalize tumor oxygenation in order to stabilize reactive oxygen species (ROS) generated during chemotherapy and radiotherapy.\textsuperscript{65,75,76} However, such treatment protocols only permit short-term hyperoxic breathing due to the acute pulmonary tissue damage associated with breathing extremely high concentrations of oxygen. Intermittent and infrequent supplemental oxygen breathing is likely to only reduce hypoxia transiently, and may not be sufficient to address the physiological mechanisms inhibiting the anti-tumor response.

Our approach of using hyperoxia to eliminate A2AR-mediated immunosuppression in the TME is in conceptual contrast to all other medical uses of oxygen. This novel strategy was motivated by our previous findings in an acute lung injury model, where treatment with supplemental oxygen exacerbated lung injury by heightening inflammation. Importantly, the powerful effects of supplemental oxygen were shown to be A2AR-mediated, since treatment with a selective A2AR agonist (CGS21680) prevented lung injury\textsuperscript{77}. These studies along with others\textsuperscript{78,79}, have demonstrated the feasibility of our approach by establishing that breathing 60% oxygen is safe for long-term use in both mice and humans.

**Targeting hypoxia-adenosinergic immunosuppression**

Subverting adenosine-mediated suppression may also be achievable by treatment with A2AR antagonists. In a solid immunogenic CL8-1 melanoma tumor model, genetic deletion of A2AR enabled the survival of 60% of mice with established tumors but not of the WT littermate controls (Fig. 1.6)\textsuperscript{7}. Moreover, while the use of non-specific A2AR antagonists (Caffeine,
ZM241385) alone were insufficient to promote tumor regression of CMS4 sarcoma pulmonary metastases, combinatorial treatment of antagonist plus adoptively transferred CMS4 CD8 T cells showed significant enhancement of T cell immunotherapy (Fig. 1.6)\textsuperscript{7}. The overall effectiveness of the antagonist treatment may be underappreciated due to an extremely brief half-life (~0.5-1.5 hr) in mice\textsuperscript{7}. It is noteworthy to mention that high-dose caffeine is also capable of inhibiting phosphodiesterases, which can result in the inadvertent accumulation of cAMP\textsuperscript{80}. To overcome these obstacles, it will be important to test the more efficient and longer-lasting A2AR-specific antagonist, KW6002.

Pharmacologically, KW6002 has been shown to be a clinically safe medical prescription in disorders such as Parkinson’s disease, where it is used to antagonize the A2AR to prevent dimerization with dopamine receptors\textsuperscript{81}. Studies have demonstrated a greater than 50-fold selectivity of KW6002 for A2A receptors compared to A1 receptors, the only other high affinity adenosine receptor\textsuperscript{81,82}. Additionally, KW6002 has a significantly longer half-life (up to 8 hours) compared to other A2AR antagonists, making it the superior option as an anti-adenosinergic drug\textsuperscript{83}. Since current use of KW6002 is restricted to testing in clinical trials for Parkinson’s disease, our collaborators in the Jones Laboratory at Northeastern University have developed a novel method of synthesizing KW6002\textsuperscript{83}. Here, we demonstrate the use of KW6002 in the antagonistic targeting of the hypoxia-adenosinergic pathway as a feasible strategy to enhance the adoptive immunotherapy of cancer.
Figure 1.6. Deficiency of the A2AR promotes survival in mice with immunogenic CL8-1 tumors while antagonism enhances tumor regression by adoptively transferred CD8 T cells. (A) A2AR<sup>-/-</sup> or A2AR<sup>+/+</sup> control mice were inoculated with intradermal CL8-1 tumors and followed out for 80 days. (B) The antagonist ZM241385 enhanced the efficacy of anti-tumor CD8 T cells against CMS4 lung metastasis.
Chapter 2

Hyperoxic reversal of hypoxia-adenosinergic immunosuppression in lung metastases

2.1 Introduction

Activities of T-lymphocytes and natural killer (NK) cells are inhibited in hypoxic (i.e. oxygen-poor) and extracellular adenosine-rich, inflamed and cancerous tissues (Fig. 2.1). This physiological immunosuppression is mediated by cAMP-elevating A2A adenosine receptors (A2AR) on the surface of T-lymphocytes and NK cells. Since activated T cells are also inhibited by activities of the Hypoxia Inducible Factor-1α (HIF-1α), hypoxia response elements (HRE) as well as cAMP response elements (CRE) are believed to be important in governing this hypoxia-adenosinergic immunosuppression in the tumor microenvironment (TME) and in inflamed tissues. Adding to this suppression, A2AR may also interfere in the trafficking of T cells due to the heterologous desensitization of chemokine receptors. It has been suggested that hypoxia-adenosinergic signaling and immunological negative regulators may collaborate to account for the inhibition of tumor-reactive T cells within the TME.

The paradoxical co-existence of tumors and anti-tumor T cells in cancer patients and mice can be at least partially accounted for by hypoxia-driven immunosuppression in the TME. We reasoned that clinical supplemental oxygen protocols may increase the oxygen tension in tumor tissue, thereby weakening hypoxia-driven and [adenosine]→A2AR-mediated immunosuppression. This, in turn, could unleash the otherwise inhibited anti-tumor immunity (Fig. 2.1).
Figure 2.1. Eliminating tumor hypoxia is hypothesized to promote anti-tumor immunity. Breathing 60% oxygen is proposed to prevent the tumor hypoxia-driven generation of extracellular adenosine ([Ado]_{high}), thereby abolishing signaling through immunosuppressive cAMP-elevating A2AR and de-inhibiting tumor-reactive T cells resulting in stronger anti-tumor effects.
Breathing \([\text{oxygen}]^{\text{High}}\) (e.g. 95% oxygen [carbogen]) represents a classic approach to normalize tumor oxygenation in order to generate reactive oxygen species (ROS) in non-surgical therapeutic approaches, including chemotherapy, radiotherapy and photodynamic therapy of cancer\(^ {65,75,76}\). However, our approach of using hyperoxia to eliminate the A2AR-mediated immunosuppression in the TME is in conceptual contrast to all other medical uses of oxygen.

We hypothesized that targeted elimination of the hypoxia-driven immunosuppression\(^ {7,8,34,47,85-87}\) in the TME may further improve promising cancer immunotherapy approaches\(^ {72,90,91}\) and enable better tumor rejection. Therefore, we asked whether weakening hypoxia by increasing tumor oxygenation may prevent the generation of immunosuppressive adenosine in the TME and lead to better tumor rejection by de-inhibited anti-tumor immunity. Here we show that hyperoxia, i.e. breathing 60% oxygen-containing gas mixtures, prevents the inhibition of anti-tumor T- and NK cells and thereby enables the regression of established MCA205 fibrosarcoma or B16 melanoma pulmonary metastases in mice.

The tumor-regressing effects of hyperoxia are lost in i) common gamma (\(\gamma_c\))/Rag-2 mice deficient in T- or NK cells, ii) wild type (WT) mice depleted of T- and/or NK cells and iii) A2AR gene-deficient mice. These controls validate the premise of our approach by demonstrating that the effects of hyperoxia are entirely T- and NK cell-dependent and that hyperoxia and A2AR gene deletion act upstream and downstream of the same tumor-protecting Hypoxia \(\rightarrow [\text{Adenosine}]^{\text{High}} \rightarrow A2AR\)-mediated pathway, respectively.

We also demonstrate that breathing 60% oxygen converts the hypoxia-adenosinergic immunosuppressive TME into an immunopermissive milieu by i) increasing the oxygen tension, ii) decreasing the levels of intratumoral extracellular adenosine, iii) increasing the infiltration of
endogenous and highly activated CD8 T cells, iv) increasing levels of pro-inflammatory cytokines and chemokines, iv) decreasing immunosuppressive cytokines (e.g. TGF-β) and downregulating the expression of immunosuppressive A2AR. In addition, hyperoxic breathing weakened suppression by T regulatory cells (T regs) as reflected by the decreased accumulation of CD4^+Foxp3^+ cells in the TME as well as their reduced expression of CTLA-4, which is considered important for T reg-mediated suppression\(^70\).

Additional mechanistic insights were obtained by findings that hyperoxia enhanced the therapeutic efficacy of adoptively transferred tumor-reactive T cells and enabled complete tumor regression by facilitating infiltration, preventing the inhibition of proliferation in pulmonary metastases and lymphoid organs, and increasing the production of IFN-γ by transferred T cells in the TME. Taken together, these studies clarify important issues of tumor biology and immunology, uncover the novel and potentially clinically important property of oxygen and provide proof-of-principle for the hyperoxic enabling of anti-tumor immunity.
2.2 Results

The anti-tumor effects of hyperoxia require the activities of endogenous T- and NK cells

To test our hypothesis, we took advantage of the weakly immunogenic MCA205 fibrosarcoma pulmonary metastasis model with a predictable time course and intensity of T cell response\(^{54}\). Our expectation was confirmed in Figure 2.2 A, demonstrating the improved regression of 11-day established pulmonary metastases in mice breathing 60% oxygen (identified as “60% O\(_2^*\)”) when compared to mice breathing ambient oxygen (identified as “21% O\(_2\)”). An even stronger regression was observed when mice began breathing 60% oxygen immediately following tumor inoculation (identified as “60% O\(_2\)”). Hyperoxia-induced tumor regression was also observed in the poorly immunogenic B16 melanoma metastasis model (Fig. 2.2 B).

We hypothesized that the anti-tumor effects of 60% oxygen could be explained by the increased activities of endogenously developed tumor-reactive T- and NK cells (Fig. 2.1). Alternatively, these effects could be the result of direct cytotoxicity and oxidative stress-mediated tumor damage caused by hyperoxia\(^ {75,76}\). To discriminate between these mechanisms, we tested whether the hyperoxia-induced regression would still be observed in common gamma (\(\gamma c\))/Rag-2\(^{-/-}\) mice, deficient in T- and NK cells\(^{92}\). Figure 2.3 shows that the improved regression seen in WT mice breathing 60% oxygen was completely abrogated in (\(\gamma c\))/Rag-2\(^{-/-}\) mice, demonstrating the critical necessity of T- and NK cells in the hyperoxia-induced anti-tumor response. Next, we examined the effects of hyperoxia on tumor-bearing mice depleted of CD4 and/or CD8 T cells, NK cells, or CD4/CD8 T cells and NK cells.
Figure 2.2. Breathing 60% oxygen promotes tumor regression. (A) Mice with 11-day established MCA205 pulmonary metastases were placed in small animal intensive care units infused with 60% oxygen (identified as “60% O₂*”) and lungs were harvested at day 21. An even stronger regression was observed when mice were placed in units immediately following tumor inoculation (identified as “60% O₂”). To the right is the enumeration of metastases for each set of lungs after counterstaining. Lungs with more than 250 metastatic nodules were assigned >250 as that is the maximum number that can be counted reliably (n = 5 mice per group; averages represented as horizontal bars) (B) Hyperoxia-enhanced tumor regression in mice with B16 melanoma pulmonary metastasis. Mice were placed in 60% oxygen immediately after tumor inoculation and lungs were harvested on day 21.
Figure 2.3. Tumor-regressing effects of hyperoxia are lost in common gamma (γc)/Rag-2−/− mice deficient in T- and NK cells. Wild type and (γc)/Rag-2−/− mice were inoculated with MCA205 tumor cells and placed in 60% oxygen units for 21 days. Depicted is an (A) image of tumor-bearing lungs after counterstaining along with (B) the enumeration of metastases from each group. As further confirmation, (C) tumor-bearing lung weights and (D) a histological analysis of the percent tumor saturation of each set of lungs is provided.
Figure 2.4. Hyperoxia-induced regression of pulmonary metastasis is mediated primarily by CD8 T cells and NK cells. Depletion of T cell subsets or NK cells using monoclonal antibodies significantly impaired or completely abrogated the anti-tumor effects of 60% oxygen. Mice were given intraperitoneal injections of respective antibodies 2 days prior to MCA205 tumor inoculation and placed in 60% oxygen units for 21 days. Depletion of cell subsets was maintained by weekly injections of monoclonal antibodies.
Figure 2.4 shows that the improved regression of pulmonary metastases by hyperoxia is mediated to a large extent by endogenous T cells, since mice depleted of CD4 and CD8 T cells demonstrated severely impaired tumor regression after 60% oxygen treatment. Particularly, CD8 T cells proved to be the more essential arm of the hyperoxia-induced anti-tumor T cell response.

**NK cells play a critical role in the anti-tumor effects of breathing 60% oxygen**

Interestingly, the depletion of NK cells alone virtually eliminated the anti-tumor effects of 60% oxygen, even though these mice still retained CD4 and CD8 T cells (Fig. 2.4). While it has been established that NK cells are important in anti-tumor immunity, our data suggest that their full potential may not be realized due to inhibition under normoxic conditions.

The in vivo observations that NK cells are very susceptible to hypoxia-adenosinergic inhibition in the TME are in agreement with previous in vitro demonstrations of A2AR-mediated inhibition of NK cells. These data offer the use of supplemental oxygen as a novel way to enhance the anti-tumor activities of NK cells by unleashing their powerful capacity to orchestrate CD4 and CD8 T cells during the anti-tumor response.

To confirm the hyperoxic tumor regression was indeed mediated by T- and NK cells and to exclude the possibility of non-specific tumor cell toxicity caused by oxygen free radicals, we used the potent free radical scavenger N-acetylcysteine (NAC). Our control assays demonstrated that the tumor-regressing effects of hyperoxia were still present in mice treated daily with NAC (Fig. 2.5). Taken together, these findings suggest that the hyperoxic tumor regression is indeed mediated by T- and NK cells and could not be accounted for by tumor destruction associated with reactive oxygen species.
Figure 2.5. The tumor-regressing effects of 60% oxygen are still present in mice treated with an oxygen free radical scavenger. Mice breathing 21% or 60% oxygen after MCA205 tumor inoculation were given daily intraperitoneal injections of the oxygen free radical scavenger N-acetylcysteine (NAC). After 21 days, lungs were harvested and the metastases were enumerated. No significant difference was observed in mice breathing 60% oxygen with or without NAC.
Hyperoxic breathing inhibits the hypoxia-adenosinergic pathway by reducing tumor hypoxia and preventing the accumulation of extracellular adenosine

To test our initial hypothesis, it was important to ask whether hyperoxic breathing was actually decreasing tumor hypoxia and adenosine levels in the TME (Fig. 2.1). This was confirmed in Figure 2.6 A, since breathing 60% oxygen increased the local tissue oxygen tension in the pulmonary TME, as reflected by the drastic reduction in immunostaining of the molecular *in vivo* hypoxia marker Hypoxyprobe-1. Hyperoxic breathing also reduced the exposure of lymphocytes to hypoxia in the TME as well as in lymphoid organs, since both CD4 and CD8 T cells harvested from the lung and spleen of tumor-bearing mice breathing 60% oxygen had significantly less hypoxic staining (Fig. 2.6 B). Using equilibrium microdialysis probes, we also show that the decrease in hypoxia was accompanied by a decrease in the levels of extracellular adenosine in the TME (Fig. 2.7). These observations validate the biochemical assumptions for the reversal of adenosine-mediated inhibition of anti-tumor immunity by hyperoxia.

Next, we used A2AR−/− mice to determine whether hyperoxia and A2AR gene deletion were acting upstream and downstream of the same immunosuppressive tumor-protecting pathway, respectively. Figure 2.8 shows the improved regression of pulmonary metastases in A2AR−/− mice compared to WT controls (identified as “21% WT” vs. “21% A2AR−/−”). This observation is parallel to previous findings that have demonstrated the enhanced rejection of solid tumors in A2AR−/− mice (Fig. 1.6 A). However, breathing 60% oxygen did not further improve tumor regression in A2AR−/− mice (Fig. 2.8; right side of histogram). Collectively, these results indicate that hyperoxia is acting upstream of the Hypoxia→[Adenosine]$^{\text{High}}$→A2AR→[cAMP]$^{\text{High}}$ immunosuppressive pathway by eliminating hypoxia and reducing levels of extracellular adenosine.
Figure 2.6. Breathing 60% oxygen reduces hypoxia. (A) The hypoxia marker Hypoxyprobe-1 was used in the immunohistochemical staining of pulmonary metastases in mice breathing 60% oxygen for 3 hours. The average OD/mm² per group is shown (mean ± s.e.m, n = 3 mice). (B) Hypoxic exposure of CD4 and CD8 T cells in the lung and spleen was also reduced in tumor-bearing mice breathing 60% oxygen for 48 hours as measured by the fluorescent intensity of Hypoxyprobe-1 using flow cytometric analysis.
Figure 2.7. Hyperoxia reduces intratumoral levels of extracellular adenosine. Mice were inoculated subcutaneously with $1 \times 10^5$ MCA205 tumor cells to initiate solid tumor formation. Since pulmonary metastases were too small for microdialysis, 11-day established intradermal tumors were used to analyze the levels of extracellular adenosine. Microdialysis probes were used to collect adenosine from MCA205 solid tumors in mice breathing 21% or 60% oxygen for 3 hours. Normal tissue next to the tumor was also probed as a control. Adenosine was quantified by liquid chromatography-tandem mass spectrometry.
Figure 2.8. Hyperoxia acts upstream of the A2AR in the hypoxia-adenosinergic pathway. WT and A2AR−/− mice were inoculated with MCA205 tumor cells and placed in 21% or 60% oxygen for 21 days. Therapeutic benefit of hyperoxic breathing shown in WT mice is absent in A2AR−/− mice. Also shown is the improved tumor regression seen in A2AR−/− mice compared to WT mice breathing 21% oxygen.
Hyperoxic breathing enables more robust anti-tumor immunity by reversing immunosuppression in the TME

To demonstrate mechanistically that the reversal of immunosuppression in the TME enables a more robust anti-tumor response, we analyzed the effect of hyperoxia on mice with 11-day established MCA205 pulmonary metastases. Figure 2.9 demonstrates histologically the increased intratumoral infiltration of CD8 T cells in mice breathing 60% oxygen. Interestingly this was a CD8 T cell-specific phenomenon since no significant improvement of intratumoral infiltration was observed in CD4 T cells. This was confirmed and extended in flow cytometric time course assays showing the hyperoxia-enhanced accumulation of highly activated CD8 T cells in the pulmonary TME (Fig. 2.10 A). No significant accumulation of CD4 T cells was observed in the lung TME of mice breathing 60% oxygen (Fig. 2.10 B).

In Figure 2.11, custom-made RT-PCR arrays were used to scan the pulmonary TME for hyperoxia-induced changes in the repertoire of 94 relevant immune response gene products that included 4 chemokine receptors, 20 chemokine ligands and 27 different cytokines, among others\(^4\). Remarkably, the hyperoxia-associated increase in the levels of pro-inflammatory cytokines (e.g. IL-2, IL-12\(\alpha\)) and chemokines (e.g. CXCL9, CXCL10, CXCL11) was accompanied by the complementary and profound decrease in immunosuppressive cytokines (e.g. TGF-\(\beta\)) and by the down-regulation of immunosuppressive A2AR, A2BR, and even of key enzymes of other anti-inflammatory pathways, e.g. prostaglandin E synthase-2 (COX-2). Interestingly, COX-2 has been shown to be regulated by hypoxia\(^5\) and A2AR-mediated cAMP induction\(^6\). Moreover, cAMP-dependent PKA signaling as well as COX-2 expression have been pharmacologically implicated in T reg-mediated suppression of the anti-tumor response\(^7\).
**Figure 2.9.** Hyperoxia enhances the infiltration of endogenous CD8 T cells. Mice bearing 11-day established MCA205 pulmonary metastases were placed in 60% oxygen for 4 days. The infiltration of endogenous CD4 and CD8 T cells was analyzed by immunohistochemical staining. Significantly more CD8 T cells, but not CD4 T cells, were observed in the tumors of mice breathing 60% oxygen compared to 21% oxygen (mean ± s.e.m, n = 3 mice).
Figure 2.10. **Hyperoxic breathing promotes the accumulation of highly activated endogenous CD8 T cells, but not CD4 T cells.** Mice with 11-day established MCA205 pulmonary tumors were placed in 60% oxygen units for up to 8 days and the time course of the accumulation of highly activated (A) CD8 and (B) CD4 T cells was analyzed by flow cytometry. Shown in both experiments is the mean ± s.e.m (n = 3 mice per time point).
Figure 2.11. Breathing 60% oxygen promotes the production of pro-inflammatory cytokines and chemokines while reducing negative regulators of the immune response. Custom-made RT-PCR arrays were used to screen for changes in 94 different chemokines, cytokines and immune response genes. Breathing 60% for 3 days (A) increases the levels of immune-stimulating cytokines and chemokines while (B) decreasing the levels of the negative regulators TGF-β, A2AR, A2BR, and COX-2 expression in the lung tissue of mice bearing 11-day established metastases. Shown in both experiments is the mean ± s.e.m (n = 3 mice).
Hyperoxic breathing enables more robust anti-tumor immunity by weakening immunosuppression by T regs

Fortuitously, the increased accumulation of TME-infiltrating CD8 T cells was not negated by the simultaneous increase in immunosuppressive T regs, which is a well-appreciated problem with many immunotherapy protocols\(^\text{22}\). Because of the proposed role of HRE- and CRE-mediated transcription in the development and function of T regs\(^\text{47}\), we hypothesized that hyperoxia may affect T regs in the TME. Therefore, we analyzed the effect of breathing 60% oxygen on the time course of CD4\(^+\)Foxp3\(^+\) T reg infiltration and CTLA-4 expression, which has been implicated in T reg-mediated suppression\(^\text{70}\).

Data from Figures 2.12-2.14 suggest that hyperoxia weakens T reg-mediated suppression by at least three separate mechanisms that may synergize to allow T- and NK cells to overcome hypoxia-adenosinergic inhibition. We show that hyperoxic breathing i) inhibited the time-dependent accumulation of T regs in the TME (Fig. 2.12 A), ii) reduced the exposure of T regs to hypoxia (Fig. 2.12 B) and iii) inhibited the time-dependent tumor-growth associated increase in CTLA-4 expression on T regs in the TME (Fig. 2.13, 2.14). Mice breathing 60% oxygen had a lower proportion of CTLA-4\(^+\) T regs (Fig. 2.13) as well as reduced CTLA-4 expression on those T regs as measured by mean fluorescent intensity (Fig. 2.14). These changes in immunosuppressive T regs are complementary to parallel observations of the strong decrease in TGF-\(\beta\) (Fig. 2.11 B), which is believed to be an important inhibitor of anti-tumor immunity\(^\text{68}\). These findings led us to test whether there was a relationship between TME hypoxia and CTLA-4 expression in T regs. Using the \emph{in vivo} tissue hypoxia marker Hypoxyprobe-1, we show that CTLA-4\(^{\text{High}}\) T regs from the lung TME and spleen of tumor-bearing mice were exposed to a much lower oxygen tension when compared to CTLA-4\(^{\text{Low}}\) T regs (Fig. 2.15).
Figure 2.12. Hyperoxia disrupts T reg-mediated suppression in the lung TME. (A) Hyperoxic breathing decreased the accumulation of CD4⁺FoxP3⁺ T regs in the lung TME. Mice with 11-day established MCA205 metastases were placed in 21% or 60% oxygen and the time course of T reg accumulation in the lung was determined by flow cytometry (mean ± s.e.m, n = 3-4 mice per time point). (B) Breathing 60% oxygen for 48 hours reduces the exposure of T regs to hypoxia in both the lung and spleen of mice bearing 11-day established MCA205 pulmonary metastases as measured by the mean fluorescent intensity of Hypoxyprobe-1 staining.
Figure 2.13. Hyperoxia reduces the proportion of T reg cells expressing CTLA-4.  
(A) Breathing 60% oxygen results in a lower percentage of T regs expressing CTLA-4 measured by flow cytometry. Percentages of CTLA-4⁻ and CTLA-4⁺ T reg cells are shown.  
(B) In a time course assay, hyperoxia inhibited the time-dependent tumor-growth associated increase in the percentage of T regs expressing CTLA-4 in the TME of mice with 11-day established MCA205 pulmonary metastases. The average increase in the percent of CTLA-4⁺ T regs from the lungs of tumor-bearing mice is compared to the baseline percentage of CTLA-4⁺ T regs from the lungs of non-tumor-bearing mice. Mice were breathing either 21% or 60% oxygen for up to 4 days (mean ± s.e.m, n = 3-4 mice per time point).
Figure 2.14. Hyperoxia reduces the expression of CTLA-4 on T reg cells. (A) Breathing 60% oxygen reduces the expression of CTLA-4 on T regs from the TME of mice bearing MCA205 pulmonary metastases as measured by the mean fluorescent intensity. (B) In a time course assay, hyperoxia inhibited the time-dependent tumor-growth associated increase in CTLA-4 expression on T regs in the TME of mice with 11-day established MCA205 pulmonary metastases. The average increase in the mean fluorescent intensity of CTLA-4 on T regs from the lungs of tumor-bearing mice is compared to the baseline CTLA-4 intensity on T regs from the lungs of non-tumor-bearing mice. Mice were breathing either 21% or 60% oxygen for up to 4 days (mean ± s.e.m, n = 3-4 mice per time point).
Figure 2.15. The expression of CTLA-4 on T regs is associated with hypoxia. CTLA-4<sup>High</sup> T regs in the (A) lung TME and (B) spleen are predominantly Hypoxyprobe<sup>High</sup>, reflecting their exposure in vivo to deeper levels of hypoxia. CTLA-4<sup>Low</sup> T regs had significantly lower levels of Hypoxyprobe-1 staining. Hyperoxic breathing is also shown to decrease the overall numbers of CTLA-4<sup>High</sup> T regs compared to mice breathing 21% oxygen (n = 4 mice).
While both CTLA-4$^{\text{High}}$ and CTLA-4$^{\text{Low}}$ T regs were present in mice breathing 21% and 60% oxygen, the hypoxia staining from the CTLA-4$^{\text{High}}$ T reg population was significantly lower in hyperoxia-treated mice. This, in turn, may lead to inhibited HRE- and CRE-mediated immunosuppressive transcription, supporting our findings of the dramatic down-regulation of TGF-β (Fig. 2.11 B).

**Hyperoxic breathing enables tumor regression by adoptively transferred tumor-reactive T cells by promoting enhanced intratumoral infiltration, proliferation and IFN-γ production**

To gain additional mechanistic insight into the hyperoxia-mediated enhancement of tumor-reactive T cells, we studied the fate of adoptively transferred T cells in mice breathing 60% oxygen$^{53,90,98}$. Mice with 11-day established pulmonary metastases were infused intravenously with in vitro culture-activated antigen-specific T cells derived from tumor draining lymph nodes (TDLN). The TDLN cells consisted of ~76% CD8 and ~12% CD4 T cells after culture-activation (Figure 2.16). As shown in Figure 2.17, commencing hyperoxic breathing on the same day as adoptive T cell immunotherapy enabled significantly better tumor regression when compared to mice treated with either oxygen or T cells alone (Fig. 2.17; group #5). However, an even stronger therapeutic effect, i.e. the complete regression of metastases by adoptively transferred tumor-reactive T cells, was achieved if mice were breathing 60% oxygen from the time of tumor inoculation until the assay completion (Fig. 2.17; group #6). This is best explained by the superimposed anti-tumor activities of hyperoxia-enhanced adoptively transferred and endogenously developed tumor-reactive T cells.
Figure 2.16. Culture-activated TDLN cells consist primarily of CD8 T cells. To generate TDLN, mice were inoculated subcutaneously with 1x10^6 MCA205 tumor cells in both flanks. Twelve days later, tumor draining LN cells (TDLN) were harvested and activated with anti-CD3 monoclonal antibody immobilized on 24-well tissue culture plates for 2 days followed by expansion with IL-2. Freshly harvested TDLN cells (prior to activation) typically consist of equal proportions of CD4 and CD8 T cells. Following 6-day culture-activation (immediately prior to infusion into tumor-bearing recipient mice), TDLN cells consist of mostly CD8 T cells with some CD4 T cells. The percentage of each cell subtype is shown in each panel.
Figure 2.17. Adoptive immunotherapy in combination with hyperoxic breathing enabled the complete regression of 11-day established MCA205 pulmonary metastases. Mice from group 5 (identified as “60%*”) were placed in the 60% oxygen units on the 11th day of tumor growth, the same day as adoptive T cell immunotherapy. Mice from group 6 (identified as “60%”) were placed in units for the duration of the assay (21 days), prior to T cell transfer. Breathing 60% oxygen for the duration of the assay combined with infusion of 5x10^6 culture-activated TDLN T cells on the 11th day of tumor growth results in virtually complete tumor regression.
Since limited tumor infiltration of anti-tumor T cells has been shown to diminish the effects of immunotherapy\textsuperscript{55}, we examined the effects of hyperoxia on the trafficking of adoptively transferred tumor-reactive T cells. Figure 2.18 shows the hyperoxic facilitation of intratumoral infiltration and the increased number of CFSE-labeled adoptively transferred T cells in pulmonary metastases. These results confirm the observation of increased intratumoral infiltration of endogenously developed CD8 T cells during hyperoxic breathing (Fig. 2.9, 2.10).

Hyperoxia also enhanced the proliferation of adoptively transferred tumor-reactive T cells in the TME and in lymphoid organs as measured by the CFSE dilution assay in Figure 2.19. Four days after adoptive transfer into Thy1.2\textsuperscript{+} tumor-bearing recipient mice, only 27% of the congeneric Thy1.1\textsuperscript{+}, CFSE-labeled tumor-infiltrating T cells divided (>4 rounds of division) in mice breathing 21% oxygen. However, 54% divided if mice were breathing 60% oxygen during the four days following adoptive transfer. These effects of hyperoxic breathing are also systemic, since better proliferation of tumor antigen-specific T cells was observed not only in metastatic tissue, but also in lymphoid organs, e.g. spleen, which have many hypoxic areas\textsuperscript{99}.

We also found that hyperoxia increased the production of IFN-\(\gamma\) by adoptively transferred Thy1.1\textsuperscript{+} lung tumor-infiltrating T cells (Fig. 2.20). Of the Thy1.1\textsuperscript{+}CD8\textsuperscript{+} cells recovered from the lung TME of Thy1.2\textsuperscript{+} mice breathing 60% oxygen, 72\% were expressing IFN-\(\gamma\) compared to 57\% in control mice breathing 21\% oxygen. This was also true of CD4\textsuperscript{+} cells, which constitute a much lower proportion of the adoptively transferred cells (Fig. 2.20 and 2.16).
Figure 2.18. Hyperoxia facilitates the infiltration of adoptively transferred T cells into 11-day established pulmonary metastases. To the left are fluorescent micrographs of pulmonary tumor infiltrating CFSE-labeled adoptively transferred T cells (green) in mice breathing (A) 21% or (B) 60% oxygen 48 hours after adoptive transfer. A hematoxylin stain of the same tissue section is also shown. (C) The graph to the right demonstrates the number of tumor-infiltrating transferred T cells per unit area of normal or metastatic tissue (mean ± s.e.m).
Figure 2.19. Hyperoxia enhances the \textit{in vivo} proliferation of adoptively transferred tumor-infiltrating T cells in pulmonary metastases and lymphoid organs. Culture-activated T cells derived from B6/Thy1.1 congenic mice were labeled with CFSE and infused intravenously into B6/Thy1.2 tumor-bearing recipients. Four days after transfer, lungs and spleens were harvested from the Thy1.2\(^+\) tumor-bearing recipient mice. The proliferation of Thy1.1\(^+\) T cells was analyzed by flow cytometry. Numbers in each panel represent the percentage of highly (>4 rounds of division) proliferating T cells.
Figure 2.20. Hyperoxia increases IFN-γ production by adoptively transferred tumor-infiltrating T cells in pulmonary metastases. Tumor-reactive T cells were prepared from Thy1.1⁺ mice and infused into tumor-bearing Thy1.2⁺ recipient mice and placed in 21% or 60% oxygen. After 4 days, tumor-infiltrating donor Thy1.1⁺ CD8 and CD4 T cells were harvested from the lung and evaluated for the production of IFN-γ by flow cytometry.
2.3 Discussion

We have shown that breathing 60% oxygen weakens the hypoxia-adenosinergic immunosuppressive pathway and thereby liberates the otherwise inhibited anti-tumor activities of T- and NK cells in the TME, enabling the regression of lung metastases. These findings i) further confirm the tumor-protecting function of hypoxia-adenosinergic immunosuppression in the TME, ii) establish the previously unappreciated property of oxygen to enhance the anti-tumor T- and NK cell immune response in vivo and iii) offer novel direction in attempts to improve the immunotherapy of cancer.

We propose that this novel property of oxygen will be most fully capitalized upon when combined with other established protocols of cancer immunotherapy. While hyperoxic breathing is shown here to reduce hypoxia-adenosinergic immunosuppression, the full therapeutic benefit may not be realized without the induction of potent anti-tumor immunity. It will be interesting to test whether breathing 60% oxygen will synergize with other methods of cancer immunotherapy that seek to ignite an anti-tumor response, i.e. cancer vaccines. It will also be important to examine whether the anti-tumor effects of hyperoxia, which decrease but do not completely eliminate the levels of extracellular adenosine in the TME, will have even stronger anti-tumor effects if combined with competitive A2AR antagonism7.

It remains to be sufficiently tested whether breathing 60% oxygen will be capable of eliminating hypoxia and promoting tumor regression in tumors of different anatomical locations. Our studies have focused on the use of pulmonary tumors, hypothesizing that the oxygen tension in the lung would be the most dramatically affected by inspiring high oxygen. Promisingly, data from Figure 2.6 showed a reduction of adenosine levels in intradermal tumors after 60% oxygen breathing. Interestingly, preliminary experiments using Hypoxyprobe-1 in solid tumors have
also indicated a reduction in the levels of hypoxia in mice breathing 60% oxygen (Figure 2.21). However, hypoxia seems to be much more heterogeneous in solid tumors compared to lung metastases. This could explain recent preliminary findings demonstrating only modest effects of 60% oxygen breathing on subcutaneous tumor regression (Figure 2.22). It will be important to determine whether hyperoxic breathing will affect oxygen gradients, and to what magnitude, in more distal sites. Some studies have indicated that a 5-fold increase in blood gas pO$_2$ after hyperoxic breathing only translates to a 2-fold increase in tumor arteriolar oxygen in subcutaneous tumor models$^{100-102}$. Clearly, irregular and porous vasculature will hinder oxygen transport to solid tumors. Thus, it may be beneficial to take advantage of hyperbaric oxygenation when treating more distant, subcutaneous tumors. Hyperbaric oxygen, which makes use of pressurized chambers and high oxygen therapy, is a more powerful method of oxygen delivery since it greatly enhances the concentration of dissolved oxygen. In tumor oxygenation studies, hyperbaric oxygen (100% oxygen at 3.0 atm) has been shown to elevate pO$_2$ 6-8 fold$^{101}$. We have shown in the lung metastasis model that hyperbaric oxygen, like normobaric 60% oxygen, is capable of enhancing adoptively transferred T cells (Figure 2.23). It will be interesting to determine whether hyperbaric oxygen will be more effective than 60% oxygen in promoting the regression of solid tumors. While hyperbaric oxygen is already clinically available for other indications, it has an important caveat in that it is unsafe for continuous or long-term treatment due to the effects of oxygen toxicity and the production of high levels of oxygen free radicals.

Unlike hyperbaric oxygen, 60% oxygen is not associated with oxygen toxicity or excessive free radical damage. Breathing ≤ 60% oxygen has been shown to be clinically safe and well tolerated in long-term treatments in both mice and humans$^{78,79}$.
Figure 2.21. Preliminary findings suggest breathing 60% oxygen alleviates hypoxia in solid tumors. Representative fluorescence micrographs of established MCA205 intradermal tumors from mice breathing (A) 21% or (B) 60% oxygen for 24 hours are shown above. Tumors were excised and sectioned for immunohistochemical staining with the hypoxia marker Hypoxyprobe-1. (C) A tumor section from control mice without Hypoxyprobe-1 is also shown.
Figure 2.22. Breathing 60% oxygen delays the progression of solid tumor growth. Mice were inoculated with $1 \times 10^5$ MCA205 tumor cells to generate intradermal tumors and placed in either 21% or 60% oxygen for 36 days. Tumor sizes were estimated twice weekly by measuring perpendicular diameters. The results are expressed as the mean diameter of tumors per group.
Figure 2.23. Hyperbaric oxygen enhances the efficacy of adoptively transferred tumor-reactive T cells. Mice bearing 11-day established MCA205 pulmonary metastases were infused with $10 \times 10^6$ culture-activated tumor-reactive T cells and treated with hyperbaric oxygen (100% oxygen at 2.5 ATM) for 1.5 hours twice a day. Treatment commenced on the day of adoptive T cell transfer and continued for 6 days. After 21 days, lungs were harvested and metastases were enumerated.
An appealing attribute of our proposed treatment is the availability of oxygen and the potential for immediate translation into clinical research, since protocols using high oxygen-containing gas mixtures are already established for radiotherapy\textsuperscript{68}. In addition, an oxygen mask that can achieve a F\textsubscript{O}2 (fraction of inspired oxygen) of \( \geq 60\% \) has recently been made available (Pulmanex Hi-Ox). However, implementation may be complicated by the impracticality of patients wearing uncomfortable masks at all times. Simple masks are much more convenient and patient-friendly but only deliver up to \( \sim 40\% \) oxygen. Thus, it was imperative to determine whether a lower concentration of oxygen (40\%) was capable of evoking an anti-tumor response. Figure 2.24 shows the linear tumor-regressing effects of 21\%, 40\% and 60\% oxygen. While breathing 60\% oxygen resulted in the most dramatic elimination of lung metastases, breathing as low as 40\% oxygen was capable of inducing significant tumor regression.

If hyperoxia is to be considered for clinical use, it will be necessary to maximize the therapeutic effects while minimizing patient discomfort and inconvenience. Since standard nasal cannula and simple masks can deliver a F\textsubscript{O}2 between 30\%-40\% oxygen, we can propose an alternative solution to patients who want to limit the burden of an elaborate oxygen mask. While continuous breathing of 60\% oxygen will likely have the best chance of inducing tumor regression, it may be a more feasible option for patients to use simple masks and nasal cannula (30\%-40\% O\textsubscript{2}) during the day when patients are more active and the higher F\textsubscript{O}2 mask (60\% O\textsubscript{2}) during the night and sleeping hours. To mimic this treatment protocol, mice with MCA205 pulmonary tumors were alternated between 60\%-40\% oxygen and 60\%-21\% oxygen every 12 hours for the duration of the experiment. As expected, Figure 2.25 shows mice breathing 60\% oxygen for the duration of the experiment had the most drastic tumor regression. However, mice alternating between 60\%-40\% oxygen also demonstrated significantly improved tumor
regression, suggesting alternating hypoxic exposure may be a reasonable clinical solution. Interestingly, breathing 60% oxygen for only 12 hours a day was enough to induce tumor regression.

We have also considered that hyperoxia may be exerting some of its anti-tumor effects through other physiological events such as angiogenesis (VEGF, PECAM-1), MHC expression or the regulation of adenosine-generating enzymes such as CD73 and CD39. Interestingly, these enzymes along with other negative regulators of the immune response have both hypoxia- and cAMP response elements and have been shown to be regulated by hypoxia\textsuperscript{27,34,87}. Of particular interest to us and in support of our approach, the generation of adenosine by CD73 has recently been implicated as an important mechanism taken advantage of by tumors to avoid immunosurveillance\textsuperscript{46,47}. Its presence on both immune cells and tumor cells makes it a prime target for cancer immunotherapy. Future studies will focus on how hyperoxia effects the regulation of these enzymes and characterizing their contribution to hypoxia-adenosinergic immunosuppression.
Figure 2.24. Breathing as low as 40% oxygen significantly improves pulmonary tumor regression. Mice were inoculated with MCA205 tumor cells and placed in 60%, 40% or 21% oxygen. After 21 days, lungs were harvested and metastases were enumerated by counterstaining.
Figure 2.25. Alternating between 60% and 40% oxygen offers a reasonable solution to clinical obstacles of hyperoxic breathing. Mice were inoculated with MCA205 tumor cells and control mice were placed in 21% or 60% oxygen for the duration of the assay. Experimental mice were alternated between 60% and 40% oxygen or 60% and 21% oxygen every 12 hours.
2.4 Materials and Methods

Animals

Female C57BL/6N (B6) mice, 8-12 weeks old, were purchased from Charles River Laboratories; B6/Thy1.1 mice were purchased from The Jackson Laboratory; γc/Rag-2-/- mice were purchased from Taconic. A2AR-/- knockout mice on a mixed genetic C57BL/6 background along with A2AR+/- (WT) controls were routinely maintained as breeding colonies at Northeastern University. These animals were housed in a specific pathogen-free environment according to the National Institute of Health guidelines.

Tumors

MCA205 fibrosarcoma is a 3-methylcholanthrene-induced tumor of B6 origin and B16-F10.P1 is a poorly immunogenic subclone of the spontaneously-arising B16/BL6 melanoma. These tumor cells were maintained in culture in complete medium (CM). CM consisted of RPMI-1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 µM sodium pyruvate, 2 mM fresh L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 50 µg/ml gentamicin, and 0.5 µg/ml fungizone (Lonza Biologics), and 5x10^-5 M 2-mercaptoethanol (Sigma). Cultured tumor cells were harvested after a short incubation at 37°C with a solution containing 0.25% trypsin and 0.02% EDTA (Lonza Biologics). For establishment of pulmonary metastases, B6 mice were injected intravenously (i.v) with either 3x10^5 MCA205 or B16-F10.P1 tumor cells suspended in 200 µl of HBSS. On day 21, lungs were counterstained with India ink (15%) and MCA205 metastases were enumerated. Lungs with more than 250 nodules were assigned >250; this is the maximum number of metastases that can be counted reliably.
Hyperoxic breathing

Mice were placed in small animal intensive care units with well-controlled gas composition to mimic protocols of supplemental oxygen delivery to humans\textsuperscript{77}. Self-contained oxygen generators (Airsep) were used to ensure desired levels of oxygen were maintained inside each unit. Hypercapnic acidosis was avoided by replacing traditional mouse cage tops with aerated wire lids and by using Sodasorb (Grace&Co)\textsuperscript{77,104,105}. CO\textsubscript{2} levels inside the chamber never exceeded 0.4\%, while hypercapnia typically occurs at levels higher than 2\%. For hyperbaric oxygen experiments, mice were treated with 100\% oxygen at 2.5 atm in a Seachrist animal research chamber for 1.5 hours twice a day. Treatment commenced on the day of adoptive T cell transfer and continued for 6 days.

Depletion of subsets of T- and NK cells

Two days prior to i.v. injection of MCA205 fibrosarcoma cells, mice were depleted of CD4 and CD8 T cells and/or NK cells with intraperitoneal injections of 500 mg/mouse of GK1.5, YTS 169, and PK-136 (BioXcell). Control mice were injected with 500 mg of rat IgG. Mice were given maintenance doses of 250 mg of antibody each week until assay completion at day 21.

Free radical scavenger

Mice were inoculated with 0.3 x 10\textsuperscript{6} MCA205 tumor cells and placed in either 21\% or 60\% oxygen. Mice were given daily intraperitoneal injections of 150 mg/kg of N-acetylcysteine (NAC). This dose was selected since it is suggested to be the highest dose of NAC that will maximize the antioxidant effects without causing the immunosuppression that occurs at higher concentrations.
Evaluation of hypoxia in tumor tissue

Hypoxyprobe™-1 Plus Kit (Millipore) was used to detect hypoxia in pulmonary metastases and solid tumors. Mice with 11-day established MCA205 pulmonary metastases were placed in 21% or 60% oxygen for 3 hours then injected with 80 mg/kg of Hypoxyprobe-1™. For solid tumors, mice were placed in 60% oxygen units for 24 hours. After 1.5 hours of Hypoxyprobe-1 labeling, lungs were snap frozen in n-hexane at -70°C, 4-mm cryosections were prepared from 10-20 different cutting surfaces and immunohistochemistry was performed. For flow cytometric analysis with Hypoxyprobe-1, mice with 11-day established MCA205 metastases were placed in 21% or 60% oxygen for 48 hours and the mean fluorescent intensity of Hypoxyprobe-1 on T cells from the lung and spleen was analyzed.

**In vivo extracellular adenosine measurements**

Mice were inoculated subcutaneously with $1 \times 10^5$ MCA205 tumor cells to initiate solid tumor formation. Intradermal tumors established for 11 days were used to analyze the levels of extracellular adenosine since pulmonary metastases were too small for microdialysis. This may underestimate the effects of hyperoxia, since it seems likely that pulmonary metastases would be more susceptible to oxygenation than solid tumors. Microdialysis probes (Bioanalytical Systems) were placed in the center, edge and in normal tissue next to tumors. Microdialysate (isotonic saline, 100 U of heparin, and 20 mm EHNA adenosine deaminase inhibitor) was perfused at 2 ml/min for 2.5 hours using an infusion pump (Braintree Scientific). Twelve hours after first probe collection, mice were placed in 21% or 60% oxygen for 3 hours. Mice were re-probed for levels of adenosine in the same tumor area. Adenosine levels were measured by
reversed-phase liquid chromatography-tandem mass spectrometry using a triple quadrupole mass spectrometer with $^{13}$C$_{10}$-adenosine as an internal standard$^{106-108}$.

**Immunohistochemistry and analysis of intratumoral T cells**

Mice with 11-day established MCA205 pulmonary metastases were treated with 60% oxygen or maintained at 21% oxygen. After 4 days, the infiltration of CD4 and CD8 T cells into metastatic nodules was analyzed in tissue sections. To characterize tumor-infiltrating immune cells, immunohistochemistry was performed using 4-mm thick acetone-fixed, O.C.T-embedded tissue sections as described previously$^{109,110}$. The slides were soaked in -20°C methanol-acetic acid for 2 min then air-dried for 20 min at room temperature. Slides were pre-treated with Peroxidase Block (DAKO). Primary rabbit anti-CD8 or anti-CD4 antibody (BD Pharmingen) was applied at a concentration of 1:100 at room temperature for 1 hour. Rabbit anti-rat immunoglobulin antibody was applied at a concentration of 1:750 in DAKO diluent for 1 hour. Slides were detected with anti-rabbit Envision$^+$ kit (DAKO). Immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin. Cell number per unit area was calculated after tumors were annotated using Spectrum$^\text{TM}$ Plus and Aperio's ScanScope® slide scanners.

**RT-PCR arrays**

Mice with 11-day established MCA205 pulmonary metastases were placed in either 21% or 60% oxygen for 72 hours. Lungs were perfused with cold PBS and RT-PCR was performed using RT2 SYBR green PCR master mix (Superarray) on an Applied Biosciences 7300 PCR platform.
Custom-made 96-well RT-PCR arrays were used to analyze the fold-difference in the expression of 94 different immune-related genes.

**Lymphocyte isolation and flow cytometry**

Whole tumor-bearing lungs were homogenized manually and passed through 70 µm strainers. Red blood cells were lysed using ACK lysing buffer and lymphocytes were isolated using 40% Percoll (GE Healthcare) in cold culture media. Lymphocytes were incubated with fluorescent antibodies (BD Pharmingen and eBioscience) at concentrations between 1:50 and 1:200 for flow cytometry.

**Analysis of CTLA-4 expression on T regs**

Mice with 11-day established MCA205 pulmonary metastases were placed in either 21% or 60% oxygen for up to 4 days. Lymphocytes were incubated with fluorescent antibodies (BD Pharmingen and eBioscience) at concentrations of 1:50 and 1:200 for flow cytometry. The increase in the percentage of T regs expressing CTLA-4 or the mean fluorescent intensity of CTLA-4 on T regs from the lungs of tumor-bearing mice was compared to a baseline expression of CTLA-4 on T regs from the lungs of non-tumor-bearing mice.

**Preparation of TDLN T cells for adoptive immunotherapy**

B6 mice were inoculated subcutaneously with 1x10^6 MCA205 tumor cells in both flanks. Twelve days later, tumor draining LN (TDLN) cells were activated with anti-CD3 mAb (145-2C11) immobilized on 24-well tissue culture plates at 4x10^6 cells/2 ml of CM for 2 days. After anti-CD3 activation, cells were harvested, washed, and further cultured in gas-permeable culture
bags (Baxter Healthcare, Deerfield, IL) at 3x10^5 cells/ml of CM supplemented with 10 U/ml IL-2. Four days later, culture-activated TDLN T cells were harvested, washed and resuspended in HBSS for adoptive immunotherapy. Therapeutic efficacy of transferred T effector cells was assessed in the treatment of 11-day established MCA205 pulmonary metastases by i.v. injection of 5x10^6 culture-activated T cells into each mouse. All tumor-bearing mice were pretreated with 100 mg/kg cyclophosphamide one day before infusion of T cells.

**Assessment of in vivo trafficking, proliferation and cytokine production of tumor-reactive T cells**

For fluorochrome labeling, cells were resuspended at 1x10^7/ml in HBSS containing 5 µM CFSE (Molecular Probes) as previously described. Following incubation at 37°C for 10 min, the labeling was stopped by the addition of ice-cold HBSS, and washed twice with HBSS containing 5% FCS before being resuspended in HBSS prior to adoptive transfer. Forty-eight hours after transfer of 5x10^6 CFSE-labeled culture-activated TDLN T cells into tumor-bearing mice, lung samples were harvested and fixed in 4% formalin for 24h then placed in 30% sucrose. Tissues were snap frozen in n-hexane at -70°C and 8-mm cryosections were prepared from 10–20 different cutting surfaces. The sections were air-dried and examined under a fluorescent microscope (Olympus, New Hyde Park, NY) equipped with a filter combination of band-pass 490 for CFSE detection. The number of CFSE-labeled cells in 15-20 metastases from each lung was averaged and presented as the number of cells per mm^2 tumor tissue using 40x objective and an eyepiece reticle containing 100 squares. Afterward, the sections were counterstained with Meyer’s hematoxylin to confirm the presence of metastases by light microscopy. For proliferation studies, culture-activated T cells derived from B6/Thy1.1 congeneric mice were labeled with CFSE and infused i.v. into B6/Thy1.2 tumor-bearing recipients. Four days after
transfer, lungs and spleens were harvested, and single cell suspensions were prepared by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 units/ml hyaluronidase (Sigma) for 4 hours at room temperature. Thy1.1+ donor cells (2000–4000 events) were collected and the decay in intensity of CFSE was recorded as separated peaks on FACS histograms. Culture-activated Thy1.1+ TDLN T cells were also used for flow cytometric analysis of IFN-γ production 4 days after infusion into Thy1.2+ tumor-bearing recipients.

Statistics

The significance of differences in the numbers of pulmonary metastases between groups was analyzed by the Wilcoxon rank-sum test. Difference in hypoxic staining, adenosine levels, RNA levels, and tumor-infiltrating cells was analyzed by the Student’s t test. Two-tailed p values are listed.
Chapter 3

A2A adenosine receptor gene-deletion or selective antagonism liberates anti-tumor CD8 T cells from immunosuppression

3.1 Introduction

Cancer immunotherapy achieved by triggering endogenously developed antigen sensitized anti-tumor immunity or adoptively transferred tumor-reactive T cells, is complementary to conventional cancer treatments. Many immunotherapy protocols have focused on developing strategies to break tumor-induced tolerance. A common approach has been vaccination to boost natural immunity sufficient to eradicate disease without the induction of harmful auto-immunity. Immunotherapy by the transfer of tumor-reactive lymphocytes offers an appealing method to enhance the immune response beyond what has been accomplished by vaccination alone. While studies have demonstrated the value of a number of effector cell populations participating in the anti-tumor response, the critical necessity of the T cell contribution has been very well documented. However in clinical studies, T cell-based treatments have demonstrated only limited success, in large part due to the immunosuppressive tumor microenvironment (TME) that protects malignant tissue from tumor-reactive T cells thereby negating advances in immunotherapy. Even when tumors express antigens that can be recognized by T cells (e.g. melanoma), the incidence of complete tumor eradication is very low despite the massive influx of tumor antigen-specific T cells to the tumor site. The molecular mechanisms of the inhibition of tumor-reactive T cells in vivo have been a research focus for decades, since a rational treatment that prevents the
inhibition of anti-tumor T cells represents a promising therapeutic strategy.

Our focus is on the hypoxia-adenosinergic pathway of immunosuppression in inflamed and cancerous tissues. While the hypoxia-adenosinergic system has been widely studied in models of inflammation and autoimmune disease, it has received relatively little attention for its exploitability in anti-tumor immunity. Adenosine mediates a variety of physiological effects that become more pronounced under hypoxic conditions, when levels of extracellular adenosine are elevated. Signaling through cAMP-elevating Gs-coupled A2 receptors on tumor-reactive T cells may account for suppression in the TME\textsuperscript{16,23,37}. Our recent findings have indicated that the A2A receptor (A2AR)-adenosinergic pathway has been adopted by cancerous tissue as a mechanism to evade anti-tumor immunosurveillance\textsuperscript{7}. The demonstration of the enhanced rejection of solid tumors in A2AR-deficient mice provided the necessary in vivo genetic evidence to firmly implicate adenosine signaling as an important tumor-protecting mechanism. Indeed, tumors are hypoxic, which contributes to the accumulation of immunosuppressive extracellular adenosine in the TME\textsuperscript{87}.

Accumulating pre-clinical studies have established the promise of pharmacological antagonistic targeting of A2AR and/or A2BR to accomplish anti-tumor T cell liberation of immunosuppression in the TME\textsuperscript{9,10,120,121}. Our studies have shown that although commercially available A2 receptor antagonists, e.g. the non-specific antagonists caffeine and ZM241385 (ZM), were able to delay tumor progression, they were insufficient to induce complete regression or improve survival\textsuperscript{7}. Therefore, in the current study, we have used a novel method\textsuperscript{83} to synthesize the selective A2AR antagonist KW6002 and show that this compound demonstrates remarkable therapeutic efficacy in the i) enhancement of pulmonary and intracranial tumor rejection, ii) improvement of survival and iii) development of specific immunological memory if
used in combination with adoptive T cell-mediated immunotherapy. The *in vivo* genetic and pharmacological evidence presented here provide proof-of-principle for the therapeutic feasibility of targeting the downstream molecules of the hypoxia-adenosinergic pathway by using a novel and selective A2AR antagonist as an anti-tumor immune response-stimulating agent. We also show that it is genetic deletion or direct antagonism of the high affinity A2AR, and not of the low affinity A2BR that liberates T cells from tumor-induced suppression.
3.2 Results

**Gene-deletion of A2AR, but not of A2BR, on CD8 T cells augments the development of functional endogenous anti-tumor immunity**

Tumor-induced immunosuppressive adenosine signaling through A2AR and/or A2BR on the surface of anti-tumor T cells has been considered to be a critical factor protecting cancerous tissue\(^7,8^7\). To test this, gene-modified A2AR\(^{-/-}\) or A2BR\(^{-/-}\) single knockout mice and A2AR\(^{-/-}\)/A2BR\(^{-/-}\) double knockout mice were initially challenged intradermally (i.d.) with weakly immunogenic MCA205 fibrosarcoma cells. In mice with genetic deletion of the A2BR, tumor inoculation led to progressive growth not significantly different from wild type control mice (Fig. 3.1 A). Both wild type and A2BR\(^{-/-}\) knockout mice succumbed to progressively growing tumors by day 33-37. In contrast, mice with gene deletion of the A2AR or A2AR/A2BR demonstrated significant retardation of tumor growth between days 9 and 28, indicating a liberation of functional endogenous anti-tumor immunity from tumor-induced immunosuppression. Although the A2AR\(^{-/-}\) and A2AR\(^{-/-}\)/A2BR\(^{-/-}\) knockout mice all eventually succumbed to the progressive tumors by day 50 to 55, their survival time was significantly prolonged when compared to wild type or A2BR\(^{-/-}\) knockout mice challenged with the same tumor burden.

After demonstrating A2AR signaling as a crucial mechanism inhibiting anti-tumor immunity, we focused specifically on the T cell arm of the immune response. A2AR\(^{-/-}\) knockout mice were depleted of CD8 or CD4 T cell subsets using monoclonal antibodies before being challenged with 1x10\(^5\) MCA205 tumor cells.
Figure 3.1. Deletion of A2AR liberates functional anti-tumor immunity from immunosuppression by preventing signaling on CD8 tumor-reactive T cells. (A) A2AR+/−, A2BR−/− and A2AR+/−/A2BR−/− knockout mice in groups of five were challenged i.d. with 1x10⁵ MCA205 tumor cells and compared to wild type B6 mice with functional A2AR. Tumor sizes were estimated twice weekly by measuring perpendicular diameters. The results are expressed as the mean diameter of tumors per group. (B) A2AR−/− knockout mice were depleted of CD4 or CD8 T cells by i.p. injections of 300 mg GK1.5 or YTS 169 mAb, respectively, one day before MCA205 tumor inoculation. Depletion with mAb was repeated on a weekly interval.
The control group of A2AR$^{-/-}$ knockout mice received anti-rat IgG. As depicted in Figure 3.1 B, depleting CD8 T cells in A2AR$^{-/-}$ knockout mice resulted in the re-establishment of tumor-induced immunosuppression of endogenously developed anti-tumor immunity. In contrast, A2AR$^{-/-}$ gene-deficient mice depleted of the CD4 T cell population demonstrated tumor progression not significantly different from the non-T cell depleted A2AR$^{-/-}$ knockout mice. These results indicate a major role of the A2AR in tumor-induced immunosuppression of endogenous CD8 T cell immunity.

**Immunosuppression of culture-activated tumor-reactive T cells by A2AR in vitro**

The expression of A2AR and/or A2BR on adoptively transferred tumor-reactive T cells could be responsible for the inhibition of a therapeutic response in the local TME due to hypoxia-driven accumulation of extracellular adenosine$^{87}$. A reliable source of tumor-reactive T cells has been identified in the lymph nodes (LNs) draining a progressively growing tumor$^{53}$. Although lacking anti-tumor reactivity when transferred directly, these T cells can be culture-activated with immobilized anti-CD3 for 2 days followed by low-dose IL-2 expansion for 4 days to mature into functional tumor-reactive T cells (Fig. 3.2 A). Moreover, culture-activated tumor-reactive T cells display differential expression of cell adhesion molecules as well as T cell activation markers. Both CD4 and CD8 T cells down-regulate the lymph node homing molecule L-selectin (CD62L) and up-regulate the migratory adhesion molecule CD44 (Fig. 3.2 B). Furthermore, the early activation marker CD69 as well as CD25 are upregulated during the antigen sensitization and culture-activation procedure.
Figure 3.2. Culture-activated TDLN cells for adoptive immunotherapy are primarily CD8 with some CD4 T cells.  (A) Freshly harvested inguinal LN cells draining MCA205 fibrosarcomas contained ~40% CD3 T cells with equal proportion of CD4 and CD8 T cells. During anti-CD3/IL-2 culture-activation, these expanded TDLN T cells were virtually all CD3 cells (>90%) with predominantly CD8 (~70%) and some CD4 (~20%) T cells.  (B) Characterization of activation and adhesion molecules on the surface of freshly harvested and culture-activated CD4 and CD8 TDLN T cells. Percentages of cells in each quadrant are shown.
Because both CD4 and CD8 culture-activated TDLN T cells have anti-tumor effector T cell function\textsuperscript{111}, these cell populations were separated and analyzed for functional A2AR and A2BR expression by selective agonistic induction of cAMP. Individually, both subtypes demonstrate high expression of the A2AR as demonstrated by the production of cAMP after treatment with the A2AR-specific agonist, CGS-21680 (CGS) (Fig. 3.3 A). Interestingly, culture-activated CD4 and CD8 TDLN T cells seem to have very minimal A2BR expression since no difference in cAMP production was observed between the A2AR-specific agonist (CGS) and the non-specific A2AR/A2BR agonist (NECA). Importantly, the CGS- and NECA-induced production of cAMP in both CD4 and CD8 culture-activated TDLN T cells could be suppressed significantly by treatment with the antagonist ZM. In light of these initial findings, we wanted to determine whether A2AR and/or A2BR signaling on CD4 and CD8 culture-activated T cells during antigen encounter would affect the secretion of IFN-$\gamma$ \textit{in vitro}. Each subset was stimulated with MCA205 tumor cells in the presence or absence of NECA or CGS. In the absence of NECA or CGS, CD8 TDLN T cells demonstrated high IFN-$\gamma$ secretion during the first 24 hours of antigen encounter. When exposed to NECA and CGS, CD8 effector T cells produced approximately one-third of the normal levels of IFN-$\gamma$ (Fig. 3.3 B). Surprisingly we could not detect any IFN-$\gamma$ production in the CD4 T cell population whether they were exposed to NECA/CGS or not (data not shown). These data are consistent with the hypothesis that the induction of cAMP by extracellular adenosine in the TME promotes tolerance and inhibition of culture-activated TDLN T cells due to signaling via A2AR on tumor-reactive CD8 T cells.
Figure 3.3. A2AR is expressed on both CD4 and CD8 subsets of culture-activated TDLN T cells and inhibits IFN-γ production of CD8 tumor-reactive T cells via agonistic induction of cAMP. (A) Expression of functional A2AR on magnetic T cell sorted culture-activated CD4 and CD8 TDLN T cells. The levels of cAMP induced by the A2AR-selective agonist CGS reflect the expression of A2AR. The production of cAMP by CGS or the non-specific agonist NECA could be suppressed by ZM. (B) IFN-γ secretion by culture-activated CD8 TDLN T cells exposed to MCA205 tumor cells is inhibited by CGS- or NECA-induced cAMP production.
Gene-deletion of A2AR, but not A2BR, on TDLN T cells augments anti-tumor immunity by adoptively transferred T cells that are liberated from tumor-induced immunosuppression

To assay whether therapeutic tumor regression of adoptively transferred T cells was affected by hypoxia-adenosinergic inhibition as a consequence of their A2 receptor expression, culture-activated T cells were prepared from either A2AR\(^{-/-}\), A2BR\(^{-/-}\), A2AR\(^{+/+}\)/A2BR\(^{-/-}\) gene-modified or control wild type donor mice. Of note, the independently culture-activated T cells demonstrated no significant differences in either CD8 or CD4 T cell content as well as their cell-surface expression of adhesion and activation molecules.

Initially, in a 4-hour \(^{51}\)Cr release assay against a dose gradient of antigen-sensitizing MCA205 tumor targets, wild type control and A2BR\(^{-/-}\) culture-activated TDLN T cells demonstrated low or moderate capacity for \textit{in vitro} cytotoxicity (Fig. 3.4). In contrast, much higher escalating cytotoxicity was observed when testing A2AR\(^{-/-}\) or A2AR\(^{+/+}\)/A2BR\(^{-/-}\) T cells. It seems likely that the adenosine concentration in the culture media may increase due to the destruction of tumor cells, thus decreasing the effects of wild type and A2BR\(^{-/-}\) tumor-reactive T cells, while A2AR\(^{-/-}\) and A2AR\(^{+/+}\)/A2BR\(^{-/-}\) T cells remain unaffected. None of the culture-activated TDLN T cells demonstrated cytotoxicity against the antigenically distinct MCA207 tumor targets, indicating maintenance of antigen-specific anti-tumor reactivity (Fig. 3.4).

The ability of TDLN T cells with A2 receptor gene deletion to secrete cytokines during 24 hours of tumor-antigen encounter was also analyzed with or without NECA or CGS by quantization of the levels of IFN-\(\gamma\) by ELISA (Fig. 3.5). A2AR\(^{-/-}\) and A2AR\(^{+/+}\)/A2BR\(^{-/-}\) culture-activated TDLN T cells secreted considerable amounts of IFN-\(\gamma\) when stimulated by MCA205 tumor cells and the secretion was not reduced significantly by the A2AR-specific agonist CGS or the non-specific A2 receptor agonist NECA.
Figure 3.4. A2 receptor gene-deletion enhances cytotoxicity of tumor-reactive TDLN cells. In vitro cytotoxicity assay was conducted using culture-activated MCA205 TDLN T cells isolated from control wild type mice or A2AR^{−/−}, A2BR^{−/−} and A2AR^{−/−}/A2BR^{−/−} mice against ^{51}Cr-labeled MCA205 or the antigenically distinct MCA207 tumor cells. After 4 hours, ^{51}Cr release in the supernatant from target cells was measured using γ-radiation counter.
Figure 3.5. A2AR<sup>−/−</sup> and A2AR<sup>−/−</sup>/A2BR<sup>−/−</sup> culture-activated T cells are resistant to the CGS- or NECA-induced suppression of cytokine production. Culture-activated T cells from WT, A2BR<sup>−/−</sup>, A2AR<sup>−/−</sup>, and A2AR<sup>−/−</sup>/A2BR<sup>−/−</sup> mice were incubated with MCA205 tumor cells in vitro for 24 hours with or without 10 µM (A) CGS or (B) NECA agonists. The concentration of IFN-γ in the supernatant was measured by ELISA.
By contrast, wild type and A2BR−/− TDLN T cells secreted substantially less IFN-γ and this production was decreased significantly by the cAMP-inducing CGS or NECA treatment. This indicates that selective engagement of the A2AR during tumor antigen encounter induces immunosuppression that prevents the down-regulation of immunostimulating cytokine secretion essential for anti-tumor immunity.

In previous studies, culture-activated TDLN T cells have demonstrated therapeutic efficacy when adoptively transferred in high doses to recipient mice with advanced tumors. To examine whether gene-deletion of A2 receptors on transferred T cells augments therapeutic outcome, A2AR−/−, A2BR−/−, A2AR−/−/A2BR−/− or control wild type tumor-reactive T cells (10×10^6) were injected into recipient wild type tumor-bearing mice with 11-day established MCA205 pulmonary metastases. To mimic clinical protocols, non-myeloablative depletion was accomplished by cyclophosphamide administration prior to T cell transfer. As depicted in Figure 3.6, A2BR−/− tumor-reactive T cells demonstrate minor therapeutic tumor regression not significantly different from recipient mice receiving control wild type T cells. In contrast, complete tumor regression was achieved by injection of the same number of A2AR−/− or A2AR−/−/A2BR−/− tumor-reactive T cells. Thus, A2AR-mediated immunosuppression seems to limit the therapeutic benefit of adoptively transferred T cells, preventing tumor regression.

Selective A2AR antagonism by KW6002 treatment mimics the tumor-regressing effects of A2AR−/− T cells in adoptive T cell immunotherapy

Immunostimulating adjuvants are often used in adoptive T cell immunotherapy against cancer to enhance therapeutic outcome. However, this treatment is often associated with considerable side effects. Therefore, we investigated the potential use of the selective A2AR
Figure 3.6. A2AR-gene deletion in culture-activated TDLN T cell immunotherapy prevents immunosuppression and induces complete regression of 11-day established MCA205 pulmonary metastases. Culture-activated TDLN T cells (10x10^6) isolated from control mice or A2AR-，A2BR- and A2AR/A2BR gene-deleted mice were adoptively transferred to B6 mice with 11-day established MCA205 pulmonary metastases. Tumor-bearing mice were pretreated with 100 mg/kg cyclophosphamide before i.v. infusion of culture-activated T cells. Lungs were harvested on day 21 and metastatic tumor nodules on the surface of the lung were enumerated after counterstaining with India ink. Each experimental group consisted of five mice.
antagonist KW6002 to augment anti-tumor reactivity by preventing tumor-induced hypoxia-adenosinergic inhibition in adoptive T cell immunotherapy. Studies have demonstrated greater than 50-fold selectivity of KW6002 for A2AR versus A1R\(^8\). Our preliminary assays also indicated that KW6002 was capable of preventing CGS- and NECA-induced accumulation of cAMP \textit{in vitro} (Fig. 3.7). To test the therapeutic efficacy of KW6002 \textit{in vivo}, we combined KW6002 treatment with increasing doses of adoptively transferred culture-activated TDLN T cells. As shown in Figure 3.8, wild type tumor-reactive T cells demonstrated a therapeutic response against 11-day established MCA205 pulmonary metastases in a dose-related manner. However, to achieve complete regression 50x10\(^6\) T cells had to be administered. In contrast, if blockade of the A2AR was achieved by daily antagonistic KW6002 treatment (20 mg/kg) commencing on the day of cell transfer, only 12.5x10\(^6\) tumor-reactive T cells were sufficient to promote complete tumor regression. It is noteworthy to mention that KW6002 by itself had only minimal therapeutic effects. Adoptively transferred tumor-reactive T cells given together with KW6002 adjuvant treatment were 3 to 4 times more efficient when compared to transferred T cells alone.

Our previous studies also demonstrated that A2R antagonists (caffeine and ZM) were capable of enhancing anti-tumor efficacy of transferred T cells, although complete tumor regression was never achieved\(^7\). To determine whether KW6002 was more effective than other A2AR antagonists in preventing hypoxia-adenosinergic immunouppression in the TME, mice with 11-day established pulmonary metastases were treated with adoptively transferred tumor-reactive T cells in conjunction with caffeine, ZM or KW6002. Figure 3.9 demonstrates the superiority of KW6002 over caffeine and ZM in the enhanced efficacy of adoptively transferred T cells since only treatment with KW6002 was capable of inducing complete tumor regression.
Figure 3.7. KW6002 is capable of preventing CGS- or NECA-induced cAMP production *in vitro*. Splenocytes from naïve mice were incubated with CGS or NECA (10 µm) with or without KW6002 (1 µM) and the amount of cAMP was quantified by ELISA.
Figure 3.8. Treatment with the A2AR-specific antagonist KW6002 enhances adoptive T cell immunotherapy of advanced-established MCA205 pulmonary metastases. Recipient mice bearing 11-day established MCA205 pulmonary metastases pre-treated with 100 mg/kg cyclophosphamide were infused with culture-activated TDLN T cells with or without adjuvant (20 mg/kg daily i.p. injection of KW6002). Lungs were harvested on day 21 and metastatic tumor nodules on the surface of the lung were enumerated after counterstaining with India ink. Each experimental group consisted of five mice.
Figure 3.9. **KW6002 is the most effective A2AR antagonist in the therapeutic enhancement of adoptively transferred TDLN T cells.** Mice with 11-day established MCA205 pulmonary metastases were infused with $10 \times 10^6$ TDLN T cells with or without daily intraperitoneal injections of the A2AR antagonists caffeine, ZM or KW (20mg/kg). After 21 days, lungs were harvested and metastases were enumerated by counterstaining with India ink.
The enhanced anti-tumor effects of tumor-reactive T cells by A2AR antagonism is only observed after non-myeloablative depletion of recipient tumor-bearing mice

Adoptive T cell immunotherapy of advanced tumors has repeatedly been reported to be dependent on non-myeloablative pre-treatment of recipient mice with either total body irradiation or cyclophosphamide treatment\textsuperscript{54,126}. This pre-treatment may be associated with i) creating space within the tumor for incoming immune cells, ii) depleting suppressor cells in the TME or iii) promoting a cytokine storm to enhance T cell immunotherapy\textsuperscript{127-131}. To assay whether selective A2AR antagonistic treatment combined with adoptive T cell therapy can substitute for non-myeloablative depletion, groups of mice were left untreated with cyclophosphamide before T cell immunotherapy and given daily administration of KW6002 or vehicle control. As demonstrated in Figure 3.10 A, the effects of A2AR blockade by KW6002 on the regression of 11-day established tumors require cyclophosphamide treatment. We then asked whether pre-treatment with cyclophosphamide was necessary in the adoptive immunotherapy of early (3-day) pulmonary metastases with KW6002 administration. In this stage of metastatic outgrowth, intratumoral vascular structure has not been fully developed. As shown in Figure 3.10 B, pre-treatment with cyclophosphamide was not necessary to achieve therapeutic benefit by A2AR antagonistic blockade in this experimental setup.
Figure 3.10. Pre-treatment with cyclophosphamide is essential to achieve the full therapeutic benefit of KW6002 in adoptive immunotherapy of advanced, but not early metastatic outgrowth. (A) Recipient mice bearing 11-day established MCA205 pulmonary metastases were pre-treated with 100 mg/kg cyclophosphamide or vehicle control and infused with culture-activated TDNL T cells with or without KW6002 (20 mg/kg daily). (B) In mice with early (3-day) pulmonary MCA205 metastases, KW6002 improves therapeutic outcome without cyclophosphamide pretreatment in dose-related TDNL T cell-mediated tumor regression.
KW6002 does not affect the intratumoral accumulation and proliferation of tumor-reactive CD8 T cells, but prevents the inhibition of their IFN-γ secretion during in vivo recognition of specific tumor-associated antigens

The enhanced therapeutic efficacy achieved by A2AR antagonism during adoptive T cell immunotherapy may be explained by the i) increased accumulation within the tumor microenvironment, ii) liberation of the intratumoral proliferative capacity and/or iii) heightening of anti-inflammatory cytokine secretion by tumor-reactive T cells. The essential first step after infusion of the cultured-activated TDLN T cells is the trafficking and accumulation within metastatic lesions. TDLN T cells labeled with CFSE were used to study the in vivo distribution of transferred T cells within pulmonary tumors from mice treated with or without selective A2AR antagonistic treatment.

As depicted in Figure 3.11, adoptive transfer of 10×10⁶ TDLN T cells together with antagonistic treatment resulted in significantly higher accumulation of fluorescent T cells in MCA205 metastatic nodules compared to surrounding normal lung parenchyma (P < 0.01). However, this selective tumor accumulation was not associated with antagonism by KW6002 since similar tumor infiltration was found if antagonistic treatment was excluded from the treatment protocol. Moreover, in a CFSE dilution assay, the proliferative capacity of tumor-reactive T cells during in vivo antigen-specific recognition did not demonstrate significant change by antagonistic treatment (Fig. 3.12) during the four days following T cell infusion. The most likely explanation for the inability of KW6002 to affect T cell proliferation is exposure to the hypoxic TME and subsequent suppression by HIF-1α, which has been demonstrated as a strong negative regulator of T cell proliferation³⁴,³⁵.
Figure 3.11. KW6002 does not affect the ability of tumor-reactive T cells to infiltrate pulmonary metastatic tissue. (A) Fluorescent micrograph of pulmonary-tumor infiltrating CFSE-labeled adoptively transferred T cells (green) in mice treated with KW6002 (20 mg/kg/day) 48 hours after adoptive transfer. (B) The numbers of fluorescent cells per mm² tumor tissue or surrounding normal lung parenchyma is shown. Twenty random sections of tumor from three mice were used to calculate average numbers ± SD of fluorescent cells per mm² metastatic tissue.
Figure 3.12. The *in vivo* proliferation of tumor-reactive CD4 and CD8 T cells is not affected by KW6002 treatment. Culture-activated MCA205 TDLN T cells from congenic B6/Thy1.1 donor mice were labeled with CFSE and transferred into Thy1.2 B6 mice bearing 11-day established pulmonary MCA205 metastases with or without conjunctival daily i.p. injection of KW6002 (20 mg/kg). By day 4, Thy1.1$^{+}$ CD4 and CD8 T cells were harvested from the lung and spleen and analyzed by flow cytometry and displayed as FACS histograms. Numbers indicate the percentage of dividing cells. Depicted images are representative of three independent experiments.
The *in vivo* capacity of transferred CD8 and CD4 TDLN T cells to secrete IFN-γ during *in vivo* recognition of tumor antigen was also analyzed. In studies of adoptive immunotherapy of established tumors (Fig. 3.13), transferred CD8 T cells isolated from the TME of mice treated with KW6002 secreted significantly higher amounts of IFN-γ (~46%) compared to mice receiving T cell therapy alone (~21%). Transferred tumor-localized CD4 T cells also demonstrated a detectable but insignificant increase in the secretion of IFN-γ during antigen encounter (Fig. 3.13). Interestingly, these differences were not observed in the spleen, indicating antigen recognition-dependent secretion of pro-inflammatory cytokines by T cells (data not shown). These results suggest that the observed enhancement in adoptive T cell therapeutic response using the A2AR-specific antagonist KW6002 is not associated with increased intratumoral recruitment or proliferation of transferred T cells, but with the prevention of the inhibition of antigen-triggered IFN-γ production.

**Adoptive T cell immunotherapy of advanced intracranial tumors is enhanced by selective A2AR antagonistic treatment and subsequently accompanied by the development of long-lasting tumor specific memory**

Previous studies have demonstrated the feasibility of treating intracranial tumors by the systematic transfer of culture-activated TDLN T cells. It was shown that the blood-brain barrier (BBB) did not inhibit the ability of transferred T cells to infiltrate intracranial tumors and prevent an anti-tumor immune response. A2AR-specific antagonist KW6002 was initially developed for the treatment of Parkinson’s disease to antagonize A2AR in the brain, thus blocking dimerization with dopamine receptors. We have taken advantage of the powerful and selective antagonism of KW6002 to facilitate the immunotherapy of tumors established in the central nervous system.
Figure 3.13. IFN-γ production by adoptively transferred tumor-reactive T cells is increased by KW6002. Four days after adoptive transfer, donor Thy1.1⁺ MCA205 TDLN T cells from mice with or without KW6002 treatment were analyzed by flow cytometry for IFN-γ following a 4-hour stimulation with anti-CD3. The number in each quadrant represents the percentage of Thy1.1⁺ CD4 or CD8 cells. Images are representative of three independent experiments.
Mice with 7-day established MCA205 intracranial tumors received escalating doses of tumor-reactive T cells with or without A2AR-specific antagonistic treatment. As shown in Figure 3.14, adoptive T cell immunotherapy with $10 \times 10^6$ culture-activated TDLN T cells in conjunction with daily antagonistic treatment with KW6002 resulted in tumor rejection in 50% of mice with no signs of relapse up to 60 days. Although significant prolongations of survival compared to controls were observed in mice with the same number of transferred tumor-reactive T cells without antagonistic treatment, all tumor-bearing mice succumbed to early morbidity and progressive tumor growth (Fig. 3.14). Furthermore, mice given $20 \times 10^6$ T cells in combination with KW6002 treatment demonstrated 100% survival. These results indicate that adjuvant administration of the A2AR-specific antagonist significantly enhanced the therapeutic efficacy of adoptively transferred T cells regardless of anatomical tumor location.

Long-lasting tumor-specific immunological memory was analyzed in animals that were cured of MCA205 intracranial tumors as a consequence of antagonistic treatment and adoptive T cell immunotherapy. As shown in Figure 3.15, long-lasting immunity was established in mice that had previously rejected the primary intracranial tumor since these mice also survived re-challenging with MCA205 tumor cells. In contrast, the growth of the antigenically distinct MCA207 intracranial tumor was not affected by the initial rejection of the MCA205 tumor. These results indicate that the tumor eradication triggered by the combined treatment of tumor-reactive T cells and KW6002 established a long-lasting tumor-specific memory response.
Figure 3.14. KW6002 prevents the inhibition of tumor-reactive T cells and promotes survival in mice with 7-day established MCA205 intracranial tumors. Mice were injected intracranially with 1x10^5 MCA205 fibrosarcoma. On day 6, all mice were pre-treated with 100 mg/kg cyclophosphamide, one day before i.v. infusion of culture-activated MCA205 TDLN T cells. (A) KW6002 (20 mg/kg/daily) or (B) vehicle control was given i.p. for 10 consecutive days commencing on the day of cell transfer. Each group consisted of six mice. Therapeutic response was evaluated by survival time.
Figure 3.15. KW6002 treatment combined with adoptive immunotherapy provides long-term tumor specific immunological memory. Nine mice cured of intracranial MCA205 tumors after adoptive T cell and KW6002 therapy (Fig. 3.14) were followed out for 60 days then re-challenged intracranially with MCA205 (1x10⁵) or the antigenically distinct MCA207 (1x10⁵). Control, naïve mice received intracranial injection of same number of tumor cells.
3.3 Discussion

We have addressed the well-appreciated need to develop anti-TME hypoxia-directed therapies\textsuperscript{65,132,133}. We approached this goal by antagonizing the hypoxia-driven and A2AR-mediated immunosuppression. It is now well established that tissue hypoxia promotes the generation of extracellular adenosine and that T cells are inhibited by both HIF-1\(\alpha\) and A2AR-triggered immunosuppressive transcription in the TME\textsuperscript{9,12,34,43,99,132}.

The data presented here support our previous findings that implicate the A2AR in the inhibition of anti-tumor immunity against immunogenic class-I gene-transfected CL8-1 melanoma or RMA T lymphoma\textsuperscript{7}. In several studies, including anti-pathogenic inflammation, hypoxia-induced immunosuppression via A2AR was shown to protect normal tissues from self-antigen destruction\textsuperscript{9,77,134}. The TME may have adopted this host-derived suppression to inhibit anti-tumor immunity. The majority of tumor cells have a high manifestation of tumor-associated antigens (TAA) shared with low expression of normal tissue. Clinically controlled pharmacological targeting of the A2AR capable of enhancing T cell reactivity during ongoing cancerous disease without causing significant autoimmunity may improve the clinical applicability of adoptive immunotherapy.

The data presented here were motivated by the need to evaluate the mechanisms and anti-tumor effects of genetic and pharmacological targeting of the cAMP-elevating A2AR and A2BR to prevent the inhibition of activated T cells in hypoxic, inflamed and cancerous tissues\textsuperscript{7,9}. The data from gene-deficient A2A and/or A2B receptor knock-out mice with progressively growing weakly immunogenic MCA205 fibrosarcoma (Fig. 3.1) identified the A2AR as a key mechanistic player involved in breaking CD8 effector T cell anergy and preventing immunosuppression, thus allowing tumor regression. Importantly, significant enhancement of
tumor regression was also observed after adoptive immunotherapy using culture-activated MCA205 TDLN T cells lacking the A2AR (Fig. 3.6)

Since genetic targeting of A2AR \textit{in vivo} is not yet feasible in the clinic, the use of small molecules to antagonize the A2AR remains the only option, though an appealing one given the well-established safety profiles of these drugs\textsuperscript{135}. Until now, natural (e.g. 1,3,7-trimethylxanthine, a.k.a. caffeine) or commercially available synthetic A2AR antagonists have only been able to delay tumor growth without significant improvement in mouse survival\textsuperscript{7}. Therefore, we improved the method of synthesis of the selective A2AR antagonist KW6002\textsuperscript{83}, which was initially developed for Parkinson’s disease, and used it in our studies of anti-tumor immunity.

Of potential clinical significance, treatment with the selective synthetic A2AR antagonist KW6002 mimicked gene deletion in adoptive T cell immunotherapy against pulmonary metastasis (Figure 3.8 and Figure 3.9). The therapeutic benefit of pharmacological targeting of the A2AR by antagonism was independent of the anatomical site of tumor growth since the anti-tumor reactivity eradicated tumors in mice with established lung and intracranial tumors. In mechanistic studies, the blockade of the A2AR on adoptively transferred T cells did not interfere with the selective accumulation of cells within tumors or with their proliferation in the TME, but did prevent the inhibition of anti-tumor effector functions as reflected by higher levels of IFN-\(\gamma\) secretion by TME-infiltrating anti-tumor CD8 T cells. Interestingly, IFN-\(\gamma\) secretion by CD4 T cells was not significantly affected, even though both CD4 and CD8 transferred T cell populations express the A2AR, suggesting that tumor-induced immunosuppression may interfere dissimilarly on transferred tumor-reactive CD4 and CD8 T cell subsets recruited to the TME. The disparity between the suppression of CD4 and CD8 T cells could be accounted for by
differential regulation by other negative regulators such as CTLA-4 or PD-1 or suppressor cells such as T regs or myeloid derived suppressors cells.

Our data using MCA205 intracranial inoculation also implicated A2AR antagonism in mouse survival and the subsequent development of tumor-specific long-term memory. MCA205 and MCA207 tumors are chemically induced and characteristically similar, but display distinct tumor antigens recognized only by specific T cells\textsuperscript{51,53}. This allowed us to demonstrate the ability of anti-A2AR treatment to enable immunological memory against the previously rejected MCA205 tumor, but not against MCA207.

Importantly, these data may have clinical implications in designing immunotherapy protocols. We show that it is the deficiency of A2AR, but not A2BR, in T cells that is critical in enabling anti-tumor T cells to reject tumors. It is therapeutically promising that the prevention of inhibition of anti-tumor T cells in the TME could be accomplished by eliminating only A2AR since clinical trials of A2AR antagonists have shown favorable safety profiles\textsuperscript{136}. Additional targeting of the A2BR could cause undesirable side effects due to potential effects on the cardiovascular system.

Taken together, these studies justify the importance of a novel pharmacological immunotherapy protocol that combines the liberation of anti-tumor immunity from hypoxia-adenosinergic immunosuppression in the TME with T cell-based immunotherapy. While adenosine receptor antagonists such as caffeine and theophylline are well studied and have a long history of safe use, they are non-specific and exhibit a brief half-life in patients. We have shown here for the first time that KW6002 is the more efficacious antagonist; capable of inducing tumor rejection, survival and long term memory in mice when combined with adoptively transferred T cells. Our data suggest that tumor hypoxia and A2AR should join the list of immunological
negative regulators$^{1,2,4,5,5,8,8,137}$ as potential targets for elimination in the enhancement of anti-tumor immunity.

It will be interesting to determine whether A2AR/A2BR antagonism will further stimulate the anti-tumor response in other immune-based therapies such as cancer vaccines and CTLA-4 blockade. Another appealing option may be to combine the therapeutic benefit of A2AR antagonism with hyperoxic breathing. Our recent findings have demonstrated that breathing 60% oxygen can induce tumor regression through alleviation of hypoxia-adenosinergic immunosuppression. Since we suspect the reversal of hypoxia to eliminate some, but not all of the adenosine in the tumor microenvironment, it seems likely that A2AR antagonism will further the therapeutic potential of high oxygen therapy.
3.4 Materials and Methods

Animals
Female C57BL/6N (B6) mice, 9-12 weeks old, were purchased from Charles River Laboratories. A2AR<sup>−/−</sup>, A2BR<sup>−/−</sup> or A2AR<sup>−/−</sup>/A2BR<sup>−/−</sup> knockout mice on a mixed genetic C57BL/6 background were routinely maintained as breeding colonies at Northeastern University and housed in a specific pathogen-free environment according to National Institutes of Health guidelines.

Tumors
The MCA205 and MCA207 fibrosarcomas are 3-methylcholanthrene-induced tumors of B6 origin. These tumor cells were maintained in culture in complete medium (CM). CM consisted of RPMI-1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 50 mg/ml gentamicin, and 0.5 mg/ml fungizone (all from Lonza Biologics), and 5x10<sup>−5</sup> M 2-mercaptoethanol (Sigma). Cultured tumor cells were harvested after a short incubation at 37°C with a solution containing 0.25% trypsin and 0.02% EDTA (Lonza Biologics). The tumor cells were washed and resuspended in HBSS for animal inoculation.

Depletion of T cell subsets
Two days prior to intradermal (i.d) injection of 1x10<sup>5</sup> MCA205 fibrosarcoma cells, mice were depleted of CD4 or CD8 T cells with intraperitoneal injections of 300 mg/mouse of GK1.5 or YTS 169 (BioXcell). Control mice were injected with 300 mg of rat IgG. Depletion with mAb was repeated on a weekly interval.
**Tumor draining LN cells**

Wild type, A2AR\(^{-/-}\), A2BR\(^{-/-}\) or A2AR\(^{-/-}/A2BR^{-/-}\) knockout B6 mice were inoculated subcutaneously with 1x10\(^6\) MCA205 tumor cells in both flanks. Twelve days later, tumor draining inguinal LNs were harvested, and single cell suspensions were prepared mechanically as described previously\(^{53,111}\). Tumor draining LN cells (TDLN) were activated with anti-CD3 mAb (145-2C11) immobilized on 24-well tissue culture plates at 4x10\(^6\) cells/2 ml of CM for 2 days. After anti-CD3 activation, cells were harvested, washed and further cultured in gas-permeable culture bags (Baxter Healthcare, Deerfield, IL) at 3x10\(^5\) cells/ml of CM supplemented with 10 U/ml IL-2. Four days later, culture-activated TDLN T cells were harvested, washed and resuspended in HBSS for adoptive immunotherapy.

**Measurements of functional expression of A2AR and A2BR by cAMP**

Stimulation of intracellular cAMP production and measurement of cAMP levels were performed as described previously\(^{7,138}\). Initially, CD8 or CD4 T cells from culture-activated TDLN cells were isolated using Lyt2 or L3T4 MACS-coated mouse mAb respectively (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The production of cAMP from each subtype of T cells (2x10\(^5\)) was induced by NECA (A2 non-specific agonist), CGS21680 (A2AR-specific agonist; from Tocris, Ellisville, MO) or forskolin (adenylate cyclase activator; from Sigma). The concentrations of cAMP inducers were 5 or 10 µM. The cells were incubated for 15 min at 37°C, and the reaction was stopped by addition of 1N hydrochloric acid. cAMP levels were determined by ELISA (GE Healthcare).
Cytotoxicity assay

Cytotoxicity of culture-activated TDLN T cells against MCA205 or MCA207 fibrosarcomas was determined by $^{51}$Cr release assay. Initially, tumor cells (2x$10^6$) were incubated with 100 $\mu$Ci $[^{51}\text{Cr}]$ sodium chromate (Perkin Elmer, Boston, MA) for 1 hour at 37°C. The cells were washed three times to remove excess radioactivity. The labeled tumor cells (1x$10^4$ cells) were mixed with the effector T cells in a volume of 150 $\mu$l. Effector-target ratio was between 2.5:1 and 20:1. Sedimentation of the cells in a v-bottom 96-well plate by brief centrifugation was followed by incubation at 37°C. After 4 hours, $^{51}$Cr release in the supernatant from target cells was measured using $\gamma$-radiation counter. Spontaneous $^{51}$Cr release was measured by culturing target cells alone. Maximum $^{51}$Cr release was measured by adding 1N hydrochloric acid to the same number of target cells. Cytotoxicity was calculated as percentage cell lysis when spontaneous and maximum $^{51}$Cr release were set to 0 % and 100 %, respectively.

Cytokine release assay

Culture-activated MCA205 TDLN T cells (2x$10^6$/ml) derived from wild type, A2AR$^{-/-}$, A2BR$^{-/-}$ or A2AR$^{+/+}$/A2BR$^{+/+}$ knockout B6 mice were stimulated with mitomycin C-treated MCA205 or MCA207 tumors at a 1:1 ratio or with immobilized anti-CD3 mAb for 24 hours in 200 ml of CM in 96-well plates at 37°C. Selected samples were incubated with or without 5-10 $\mu$M NECA or CGS21680 agonists. Supernatants were harvested and the concentration of IFN-γ was measured by ELISA using paired mAb and standard purchased from BD Pharmingen.
Tumor inoculation and adoptive immunotherapy

B6 mice were given intradermal (i.d.) injections with $1 \times 10^5$ MCA205 tumor cells suspended in 100 ml of HBSS to initiate tumor growth. The diameters of the tumors were measured twice weekly with a Vernier caliper, and size was recorded as an average of perpendicular measurements and presented as the mean of a group. Mice were sacrificed when the dermal tumor reached a size greater than 20 mm. To establish intracranial tumors, B6 mice were anesthetized with an intraperitoneal injection of pentobarbital (0.8 mg) and inoculated with 10 ml of tumor cell suspension ($2 \times 10^6$/ml) transcranially using a 27-gauge needle and glass tuberculin syringe (Perfectum; Popper & Sons, Inc, New Hyde Park, NY). The needle insertion was perpendicular to the skull and in line with the anterior margin of the ear and the medial half of the right eye. The depth of insertion was controlled by placement of electric wire insulation as a collar over the needle with exposure of the terminal 4 mm. To establish pulmonary metastases, mice were given intravenous injections of $3 \times 10^5$ MCA205 tumor cells suspended in 200 ml of HBSS. On day 21, mice were sacrificed and metastatic tumor nodules on the surface of lungs enumerated after counterstaining with India ink. Lungs with greater than 250 metastatic nodules were assigned $\geq 250$, as this is the maximum number of metastases that can be reliably counted.

Therapeutic efficacy of culture-activated TDLN T cells was assessed in the treatment of 3-day or 11-day established pulmonary metastases or 7-day intracranial tumors. For adoptive T cell immunotherapy of mice with either 11-day established pulmonary metastases or 7-day intracranial tumors, all tumor-bearing mice were pretreated with 100 mg/kg of cyclophosphamide 24 hours before intravenous infusion of culture-activated T cells. Antagonists (20 mg/kg/day) were given by intraperitoneal injection commencing on the day of T cell transfer.
**Trafficking and proliferation of CFSE-labeled adoptive transferred T cell**

For *in vivo* trafficking studies of fluorochrome-labeled T cells, culture-activated TDLN T cells were washed and resuspended at 1x10^7/ml in HBSS containing 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, Inc., OR). Following incubation at 37°C for 10 min, the labeling was stopped by the addition of ice-cold HBSS, and washed twice with HBSS containing 5% FCS before being resuspended in HBSS prior to adoptive transfer. Forty-eight hours after intravenous transfer of 5x10^6 CFSE-labeled cells into tumor-bearing mice, samples of organs and tissues were harvested and fixed in 4% formalin for 24 hours, before being placed in 30% sucrose for an additional 24 hours. The tissues were snap-frozen in *n*-hexane at -70°C, and 8-mm cryosections were prepared from 10–20 different cutting surfaces. The sections were dried and examined under a fluorescent microscope (Olympus, New Hyde Park, NY) equipped with a filter combination of band-pass 490 for CFSE detection. In the sections, the area of tumor tissue was identified and the number of labeled cells in the tumor was counted using a 40x objective and a reticle containing 100 squares. Afterward, the sections were counterstained with Meyer’s hematoxylin to confirm the presence of metastases by light microscopy.

For assessment of *in vivo* proliferation, donor culture-activated T cells derived from B6/Thy1.1 congenic mice were labeled with CFSE as described previously^54_. These CFSE-labeled cells (5x10^6/mouse) were given intravenously to B6/Thy1.2 tumor-bearing recipients. Four days after adoptive transfer, lungs and spleens were harvested and single cell suspensions were prepared by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 U/ml hyaluronidase (all from Sigma, St. Louis, MO) for 4 hours at room temperature. After being washed in phosphate-buffered saline solution containing 5% FCS, cells were stained with PE-conjugated anti-Thy1.1
and CyChrome-conjugated anti-CD4 or anti-CD8 for identification of each subset of donor T cells. By three-color analysis, double positive CD4⁺Thy1.1⁺ or CD8⁺Thy1.1⁺ events (2000–4000) were collected within the live lymphoid gate as determined by forward and side scatter, and the decay in intensity of CFSE was recorded as separated peaks on fluorescein-activated cell sorter (FACS) histograms.

**IFN-γ production of adoptively transferred TDLN T cells**

Cytokine production from culture-activated TDLN T cells was analyzed by intracellular staining of donor cells four days after adoptive transfer into tumor-bearing mice. Single cell suspension of spleens and tumor-bearing lungs were prepared by digestion as described above. After a brief stimulation with immobilized anti-CD3 for 2 hours, cells were further incubated in the presence of brefeldin A (10 µg ml⁻¹) for an additional 2 hours. Cells were fixed with 4% paraformaldehyde-PBS for 15 min, permeabilized with permeabilizing buffer (50 mM NaCl, 5 mM EDTA, 0.02 % NaN₃, 0.5 % Triton X-100, 10 mM Tris-HCl, pH 7.5) for 15 min, and stained with fluorescein-labeled anti-IFN-γ mAb. IFN-γ-producing CD8⁺ or CD4⁺ T cells were identified by cell surface staining using anti-CD8 mAb and anti-CD4 mAb. All antibodies were obtained from BD Biosciences.

**Statistical analysis**

The significance of differences in numbers of pulmonary metastases between groups was analyzed by the Wilcoxon rank sum test. Differences in numbers of cells infiltrating lung tumor tissues, cAMP and IFN-γ ELISA, tumor sizes and cytotoxicity results were analyzed by the Student t test. A two-tailed p value of <0.05 was considered significant.
Chapter 4

Concluding remarks and future directions

Reversing hypoxia-adenosinergic immunosuppression

Here, I have demonstrated the feasibility of our approach of using moderately high supplemental oxygen (60% oxygen) to improve the immunotherapy of cancer by disengaging immunosuppressive hypoxia-adenosinergic signaling. We have shown that breathing 60% oxygen is sufficient to i) alleviate tumor hypoxia and reduce intratumoral adenosine levels, ii) prevent the inhibition of infiltration and/or proliferation of endogenous or adoptively transferred tumor-reactive T cells, iii) promote the increase in the levels of pro-inflammatory cytokines while decreasing negative regulators of the immune response and iv) induce dramatic pulmonary tumor regression mediated by T- and NK cells. We have focused on the elimination of lung tumors since they are likely to be the most susceptible to oxygenation. While we aim to rigorously test our proposed treatment in tumors of other anatomical locations as well as in transgenic tumor models, we believe this finding to be of significant medical interest given the prevalence and high mortality rates of lung cancers.

We propose that our treatment will be most effective when combined with immune-stimulating agents or other immunotherapy protocols, i.e. cancer vaccines. Current work is being done at the University of Miami Sylvester Cancer Center in preparation for clinical trials to use a heat shock protein gp96-Ig-secreting tumor cell-vaccine in combination with suppression of adenosinergic pathways by supplemental oxygen (40%-60%) and A2 receptor antagonism in patients with non-small-cell lung cancer (NSCLC). Combining hyperoxic breathing with A2AR antagonist treatment remains an appealing option to minimize A2AR signaling and thereby
maximize immune-enhancement. In contrast to this hypothesis, preliminary studies have indicated that co-administration of KW6002 may not be capable of further enhancing the tumor-regressing effects of hyperoxia in the lung metastasis model (Fig. 4.1). This finding is parallel to our previous observations in A2AR−/− mice demonstrating that hyperoxia and A2AR signaling are operating upstream and downstream of the same tumor-protecting pathway (Fig. 2.8). However, our recent studies have demonstrated that hyperoxia has only marginal tumor-regressing effects in solid, intradermal MCA205 tumors (Figure 2.22). Moreover, our analysis of Hypoxyprobe-1 in intradermal tumors was in agreement with findings by others suggesting that tumor hypoxia in a solid tumor is spatially and temporally heterogeneous65,102 (Figure 2.21). It seems likely that breathing 60% oxygen may only partially affect solid tumor hypoxia, allowing hypoxia-adenosinergic suppression to persist in some areas of the tumor. From this perspective, combinatorial treatment of hyperoxia and KW6002 may synergize to break tumor-induced T cell tolerance more completely in a solid tumor model.

An interesting finding of this research was the demonstration of the critical necessity of NK cells in the hyperoxia-induced tumor regression. NK cells appear to be highly susceptible to hypoxia-adenosinergic immunosuppression. In agreement, complementary studies by Raskovalova et al. established that activated NK cells express high levels of A2AR and that adenosine and its analogues could prevent both perforin- and Fas ligand-mediated cytotoxic pathways against lung carcinoma cells in vitro8. Preliminary studies in our lab have confirmed these findings. Figure 4.2 demonstrates that NK cells not only express high levels of A2 adenosine receptors, but that the NECA-induced accumulation of cAMP can be prevented by treatment with KW6002.
Figure 4.1. Pulmonary tumor regression in mice breathing 60% oxygen is not significantly enhanced by A2AR antagonism. Mice with 11-day established MCA205 pulmonary metastases were placed in either 21% or 60% oxygen and given daily intraperitoneal injections of KW6002 (20 mg/kg) or vehicle control. On day 21, lungs were harvested and metastases were enumerated.
Figure 4.2. *In vitro* expanded NK cells are highly susceptible to hypoxia-adenosinergic immunosuppression. NK cells derived from splenocytes in naïve mice were expanded *in vitro* with high dose IL-2 (6000 U/ml) as was done previously \(^{139,141}\) and analyzed in a cAMP assay. The production of cAMP by the non-specific agonist NECA (10 µm) can be attributed to A2AR and A2BR signaling. The majority of the NECA-induced cAMP production was suppressed by the selective A2AR antagonist KW6002 (1 µM). The remaining cAMP levels are most likely due to NECA-induced A2BR signaling.
To further establish the effects of breathing 60% oxygen on anti-tumor NK cell reactivity, we employed a model of adoptive NK cell transfer. *In vitro* cultures of lymphocytes supplemented with high dose IL-2 (6000 U/ml) mature into highly cytotoxic lymphokine activated killer (LAK) cells, comprised of a large number of NK cells\textsuperscript{139}. A subpopulation of these NK cells are adherent to the culture flask and can be isolated for expansion of activated NK (A-NK) cells, possessing the NK-phenotype\textsuperscript{139}. To investigate the effect of hyperoxia on the therapeutic efficacy of A-NK cells, we adoptively transferred A-NK cells into mice bearing 3-day established pulmonary metastases and placed them in 60% oxygen units. Since NK cells are non-MHC restricted, we made use of the poorly immunogenic B16 melanoma tumor model. Figure 4.3 demonstrates the enhanced regression of B16 metastases in mice breathing 60% oxygen in conjunction with A-NK cell transfer. Importantly, the tumor-regressing effects of A-NK cells and hyperoxia did not require exogenous administration of IL-2, which has been shown in previous studies to be necessary for the therapeutic efficacy of A-NK cells. Hyperoxic treatment may provide an alternative method of sustaining A-NK cell activity *in vivo*, since high dose IL-2 causes vascular leak syndrome in mice and humans\textsuperscript{140}.

Further investigation is necessary to reveal the mechanisms by which hyperoxia affects NK cell tumor reactivity. It seems possible that the reduction of hypoxia-adenosinergic suppression in the TME could affect the expression of adhesion molecules and activation receptors, the production of chemokines and cytokines or the tumor-infiltration of NK cells. Some studies have indicated that close cell-to-cell contact is a requirement of NK cell-mediated tumor elimination\textsuperscript{141,142}. Future studies with fluorescently labeled NK cells will determine whether oxygen enhances tumor-infiltration, thereby increasing the number of NK – tumor cell interactions.
Figure 4.3. Breathing 60% oxygen improves the therapeutic outcome of adoptively transferred A-NK cells. A-NK cells were generated from lymphocytes in the spleen of naïve mice and expanded with high dose IL-2 (6000 U/ml) as was done previously. Mice bearing 3-day established B16 melanoma metastases were infused with 5x10⁶ culture-activated A-NK cells or maintained as control. On the same day, mice were placed in either 21% or 60% oxygen. Tumor-bearing lungs were harvested on day 21 and the metastases were enumerated.
**Targeting hypoxia-adenosinergic immunosuppression**

We have shown using the more selective and longer lasting A2AR antagonist KW6002, the feasibility of targeting hypoxia-adenosinergic suppression by A2AR blockade in the TME. Our data has revealed that treatment with KW6002 improves tumor regression and mimics A2AR gene-deletion in the adoptive immunotherapy of MCA205 pulmonary metastases by enhancing the cytokine production of tumor-reactive T cells. While our previous findings have described the anti-tumor properties of non-selective A2AR antagonists, the data shown here demonstrate the superiority of KW6002 in promoting tumor regression. Importantly, the safety profile of KW6002 has also been thoroughly tested in clinical trials for Parkinson’s disease.

A potential caveat of KW6002 is the poor solubility of the drug. Due to its hydrophobicity, KW6002 requires a relatively high concentration of organic solvents to dissolve, which can be toxic. While our pre-clinical studies have shown that a daily regimen of KW6002 is well tolerated in mice, it is possible that the immune-suppressing effects of the organic solvents counteract the immune-enhancing effects of KW6002. Another potential complication of the clinical use of KW6002 is the unintended antagonism of A2AR in the brain. The effects of KW6002 as a behavioral drug are likely to be similar to caffeine, although the selectivity of KW6002 will minimize A1- or A2B receptor antagonism. To address both caveats, our collaborators in the Jones Lab have synthesized PEGylated KW6002 (KW$_{PEG}$). The covalent attachment of polyethylene glycol can provide water solubility to hydrophobic drugs, increase circulatory time and drug stability and reduce crossover into the brain via the blood-brain barrier (BBB). Preliminary assays have demonstrated a similar capacity of KW and KW$_{PEG}$ (8-chain) to inhibit NECA- and CGS-induced cAMP production *in vitro* (Fig. 4.4).
Figure 4.4. Comparative analysis of KW and KW\textsubscript{PEG} reveals similar efficacy in antagonizing A2AR \textit{in vitro}. Splenocytes were isolated from naïve mice and incubated with CGS or NECA (10 µM). The ability of KW or KW\textsubscript{PEG} to antagonize A2AR was measured by the suppression of agonist-induced cAMP production. KW and KW\textsubscript{PEG} (1 µM) were capable of preventing cAMP production to a similar extent.
Future studies will be essential to determine whether KW\textsubscript{PEG} will be as effective as KW6002 in targeting hypoxia-adenosinergic immunosuppression \textit{in vivo}.

Since the current use of KW6002 is restricted to clinical trials for Parkinson’s disease, it will be important to further characterize the effects of other A2AR antagonists. Unbiased epidemiological studies of more than 250,000 people have shown statistically strong association of caffeine consumption with a decreased incidence of melanoma, colon cancer, liver cancer, nasopharyngeal cancer, endometrial and breast cancer\textsuperscript{143-150}. Theophylline, found in tea, is a non-selective A2AR antagonist with structural and pharmacological similarities to caffeine and is currently available for clinical use in the treatment of respiratory disorders. Our recent studies have examined the effectiveness of theophylline in targeting hypoxia-adenosinergic immunosuppression in the TME of 11-day established pulmonary metastases in a model of adoptive immunotherapy. Figure 4.5 A demonstrates the improved therapeutic efficacy of transferred T cells when combined with theophylline. Significant tumor regression was observed when high doses of theophylline (20 mg/kg) were given twice a day commencing immediately after T cell transfer. In comparative assays, theophylline was shown to be similar to caffeine in the ability to improve adoptive immunotherapy (Fig. 4.5 B). Promisingly, these findings have led to the consideration of the use of theophylline in the gp96-Ig-secreting tumor cell-vaccine clinical trials due to its well established and favorable safety profile.
Figure 4.5. The non-selective A2AR antagonist theophylline is capable of enhancing the therapeutic efficacy of adoptively transferred tumor-reactive T cells. (A) Mice with 11-day established pulmonary metastases were infused with culture-activated TDLN T cells and given twice-daily intraperitoneal injections of theophylline. At day 21, lungs were harvested and metastases were enumerated. At higher doses (20mg/kg), theophylline significantly enhanced the efficacy of transferred T cells. (B) In comparative assays, mice with 11-day established metastases were infused with culture-activated TDLN T cells and given twice-daily injections of theophylline or caffeine (20mg/kg). Both treatments were equally effective at enhancing transferred T cells and inducing significant tumor regression.
A final note: possible consequences of the unintended elimination of the hypoxia-adenosinergic pathway

The focus of this thesis has been on rescuing powerful inflammatory mechanisms from negative regulation to boost the anti-tumor immune response. However, in the case of autoimmunity, it is important to highlight the possibility of the unintended exacerbation of inflammation as a result of weakening the tissue-protecting, hypoxia-adenosinergic pathway. The etiology of autoimmune diseases is often unclear, but it is understood that the balance of pro-inflammatory and anti-inflammatory mechanisms may play an important role in rheumatoid arthritis, multiple sclerosis and other diseases, where pathogenesis could be traced to dysregulated pro-inflammatory cytokines. In this regard, local tissue hypoxia caused by inflammatory damage may actually be beneficial in preventing a prolonged inflammatory assault on healthy tissues by triggering the hypoxia-adenosinergic pathway. Thus, the elimination of hypoxia by inspiring high oxygen-containing gas mixtures and/or the weakening of A2AR signaling as a result of pharmacological antagonism by synthetic drugs or natural compounds such as caffeine, may relieve immunosuppression, resulting in the unintentional exacerbation of inflammatory damage.

The potentially dangerous effects of inspiring high oxygen-containing gas mixtures have been demonstrated in animal studies that model acute respiratory distress syndrome (ARDS). Patients with ARDS usually require symptomatic, supportive therapy by inspiring supplemental high oxygen concentrations. It was demonstrated that oxygenation (100% oxygen) is capable of weakening A2AR-mediated anti-inflammatory mechanisms. This resulted in the exacerbation of bacterial-induced inflammatory lung injury and accelerated mortality. Similarly, treatment with A2AR antagonists was also capable of exacerbating lung injury by increasing neutrophil infiltration and protein leakage. Since the molecular mechanism of this potential iatrogenic
complication was shown to be A2AR-mediated, the authors offered simple preventative measures. In patients who must receive supplemental oxygen, it may be possible to compensate for the oxygen-associated loss of extracellular adenosine in inflamed lung tissues by inhalation of a selective A2AR agonist CGS. This drug prevented the exacerbation of inflammation by oxygen in mice and improved mouse survival.

Additionally, there is a possibility of disease exacerbation as a result of recreational caffeine habits. Caffeine is widely consumed as a behavioral drug, as its psychoactive effects are mediated largely by its antagonistic action on A2AR in the brain. Experiments to test whether the caffeine consumed during an acute inflammation episode would lead to the exacerbation of immune-mediated tissue damage established that caffeine (10 and 20 mg/kg) strongly increased acute liver damage and increased levels of pro-inflammatory cytokines in wild type mice but not in A2AR-deficient mice. This confirmed that the exacerbation of liver inflammation was a result of caffeine-mediated antagonism of the A2AR-mediated tissue-protecting mechanism. Interestingly, caffeine increased liver damage when consumed chronically, but effects were even stronger in naïve mice that had never consumed caffeine.

Thus, the pharmacological recruitment the hypoxia-adenosinergic immunosuppressive pathway could be beneficial when the goal is to prevent inflammatory tissue damage during pathogenesis of autoimmune diseases. Conversely, this very same mechanism should be eliminated when it protects cancerous tissues or prevents the development of an anti-pathogen immune response. Importantly, the use of drugs in different clinical applications should be re-evaluated if the drug under consideration may interfere with the hypoxia-adenosinergic mechanism.
References


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