The role of hypoxia and adenosine signaling in vaccine design

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Abstract of Dissertation

Germinal centers (GCs) are anatomic sites where B cells undergo secondary diversification to produce class switched antibodies in response to pathogens or vaccination. We hypothesized that proliferating B cells in GCs create a hypoxic and adenosine rich microenvironment that governs their further differentiation. Using molecular markers, we found GCs to be predominantly hypoxic. Compared to normoxia (21% O₂), hypoxic culture conditions (1% O₂) in vitro accelerated class switching and plasma cell formation and enhanced expression of GL-7 on B and CD4⁺ T cells. Reversal of GC hypoxia in vivo by breathing 60% O₂ during immunization resulted in reduced frequencies of GC B cells, T follicular helper (T<sub>FH</sub>) cells and plasmacytes, as well as lower expression of ICOS on T<sub>FH</sub>. Importantly, this reversal of GC hypoxia decreased antigen-specific serum IgG1 and reduced the frequency of IgG1⁺ B cells within the antigen specific GC. Taken together, these observations reveal a critical role for hypoxia in GC B cell differentiation.

Moreover, we observed that the hypoxic germinal center is an area that is likely rich in extracellular adenosine due to the upregulation of the adenosine generating hypoxia inducible CD73 ectoenzyme on IgG1 class switched GC B cells, TFH, and T follicular regulatory cells. In addition to Ig class switching, GCs are where B cells undergo stepwise somatic hypermutation and clonal selection in the process of affinity maturation to generate high affinity neutralizing antibodies. Mechanisms that
govern affinity maturation within the germinal center have remained elusive. Here we report that A2aR deficient mice have a significantly impaired ability to undergo affinity maturation when immunized with the classic immunogen NP-OVA/Alum. We observed a significant increase in the frequency of T follicular helper cells and ICOS expression level of and decreased frequency of T follicular regulatory cells and expression of suppressive CTLA-4. Moreover, stimulation with the A2aR agonist CGS21680 during immunization suppressed the germinal center reaction and led to the accelerated generation of high affinity antibody and accelerated affinity maturation.

In search for further adenosine mediated regulation of the GC response we found the A2b adenosine receptor (A2bR) to play a unique and opposite role of A2aR. We found A2bR gene deficient mice to display significant defects in concentration of serum IgG and IgA as well as frequencies of TFH and GCs. Importantly, stimulation of A2bR using Bay60-6583 during vaccination promoted the formation of TFH and GCs. A2bR agonist loaded nanoparticles may serve as a future tool to improve the early response to vaccine targets that are too weak to mount an efficient response.

Together we demonstrate the previously unidentified role of hypoxia and adenosine receptor signaling during the humoral immune response. These insights
could provide novel vaccination strategies or new oxygen based treatments in disease states such as systemic lupus erythematosus.
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List of Abbreviations

- A1R: Adenosine 1 Receptor
- A2aR: Adenosine 2a receptor
- A2bR: Adenosine 2b receptor
- Ado: Adenosine
- ADP: Adenosine diphosphate
- AFC: Antibody Forming Cell
- AID: Activation induced cytidine deaminase
- RPA: Replication protein A
- SHM: Somatic Hypermutation
- DSB: Double Stranded Breaks
- mRNA: Messenger Ribonucleic Acid
- DNA: Deoxyribonucleic Acid
- HCL: Hydrochloric Acid
- KOH: Potassium Hydroxide
- pO₂: Partial pressure of oxygen
- ROS: Reactive oxygen species
- Alum: Aluminum Hydroxide
- AMP: Adenosine monophosphate
- ATP: Adenosine triphosphate
- BCR: B cell receptor
- BSA: Bovine Serum Albumin
- BTK: Bruntons tyrosine kinase
- cAMP: Cyclic adenosine monophosphate
- CD40L: CD40 ligand
- CFSE: Carboxyfluorocine succinate Ester
- CSR: Class Switch Recombination
- DAG: Diacyl
- DMEM: Dulbecco’s modified eagle’s media
- IMDM: Iscove’s modified Dulbecco’s media
- GC: Germinal Center
- HIF: Hypoxia inducible factor
- IgA: Immunoglobulin Alpha
- IgG1: Immunoglobulin Gamma 1
- IgG2a: Immunoglobulin Gamma 2a
- IgG2b: Immunoglobulin Gamma 2b
- IgG2c: Immunoglobulin Gamma 2c
- IgG3: Immunoglobulin Gamma 3
- IgM: Immunoglobulin Mu
- IgE: Immunoglobulin epsilon
- IL-21: Interleukin 21
- IL-21R: Interleukin 21 receptor
- IL-4: Interleukin 4
- IL-7: Interleukin 7
- IMDM: Iscove’s modified dulbecco’s medium
- ITAM: Immunotyrosine activation motif
- ITIM: Immunotyrosine inhibitory motif
- LPS: Lipopolysachharide
- LLPC: Long lived plasma cell
- NP-CGG: (nitrophenyl)acetyl-chicken gamma globulin
- NP-OVA: (nitrophenyl)acetyl-ovalbumin
- PKA: protein kinase A
- PKC: protein kinase C
- SAP: Slam associated protein
- SLAM: Signalling leukocyte adhesion molecule
- TCR: T cell receptor
- TFH: T follicular helper
- TFR: T Follicular regulatory
- TGFβ: Transforming Growth Factor Beta
- TLR: Toll like receptor
- VEGF: Vascular endothelial growth factor
- VEGFR2: Vascular endothelial growth factor receptor 2
- CRE – Cyclic AMP response element
- FoxP3 – Forkhead box p3
- HBSS – Hanks Balanced Salt Solution
- PBS – Phosphate Buffered Saline
- HP-1 – Hypoxyprobe 1
- HRE – Hypoxia response element
- CREB – Cre response element binding
- BCL-6 – B cell lymphoma 6
- gMFI – geometric mean fluorescent intensity
- MHC – Major Histocompatibility Complex
- OD – Optical density
- PD-1 – Programmed death receptor 1
- CTLA-4 – Cytotoxic T lymphocyte associated protien 4
- A2aRKO – A2a receptor knockout mice
- A2bRKO – A2b receptor knockout mice
- DKO – A2a/A2b double knockout mice
- CD73KO – CD73 knockout mice.
- TCRβδKO – TCR beta delta knockout mice
- μMTKO – IgM knockout mice
- GCME – Germainal center microenvironment
- Foxp1 – Forkhead box p1
- PE – Phycoerythrin
- FITC – Fluoroecine isothiocyonate
- FCS – Fetal Calf Serum
The development of effective vaccines to combat disease is arguably the greatest medical advance of modern time. The process by which an individual can be exposed to a small part of a weakened or dead pathogen and then attain protection or immunity was pioneered in 1796 by Edward Jenner (2). Jenner cleverly noted that patients who had been stricken with the non-fatal disease of cowpox were protected from smallpox. He tested this observation by vaccinating individuals with cowpox and found that this process did protect them from smallpox. This ushered in the birth of vaccinology.

Advances in modern vaccinology in the 20th century paralleled the implementation of effective vaccines and eradication or near eradication of numerous diseases such as smallpox, polio, mumps, measles, rubella, influenza and tetanus. However, effective vaccines for certain pathogens remain elusive such as human immunodeficiency virus (HIV). It is likely that a new wave of understanding of the fundamental biological principles that govern the vaccine response may help in designing effective vaccines.
The germinal center reaction

The germinal center was first described by Walther Flemming in 1884 as a site of lymphocyte development. This turned out not to be the case as proposed and in a sense Flemming was not entirely incorrect. During ongoing immune responses the germinal center is the anatomical site in which new B cell clones are born through class switching of the constant region of Ig genes and subsequent somatic hypermutation of their Ig variable region genes. Both of these events are genetic events mediated by the enzyme activation induced cytidine deaminase (AID), which will be discussed in more detail later. Mutated GC B cells are then compete for survival in a T cell dependent manner over the weeks of immunization leading to selection of high affinity B cell clones that become either long lived plasma cells in the bone marrow or memory B cells (3, 4).

In an effective vaccine, these high affinity cells are ideally long lived and produce antibodies that efficiently neutralize the targeted pathogen. To understand affinity maturation, one must appreciate what affinity actually means. Affinity of an antibody is defined classically (5) by equilibrium dialysis between antibody and antigen followed by measurements free antigen, free antibody, and antigen/antibody complexes over time. The biochemical approach to measurement of affinity gives nanomolar concentrations of antibodies produced in response to antigen and represents how well the antibody binds antigen. The higher the affinity the antibody,
the lower concentration of antibody one needs to bind to an antigen and ideally neutralize a pathogen.

The germinal center consists of two anatomically distinct zones; the dark zone which consists primarily of centroblasts, and the light zone, which consists primarily of centrocytes. These anatomic zones were originally described by light microscopy in which centroblasts appeared “dark” as cells appeared to be rapidly dividing and heavily clustered, while antigen and a specialized dendritic cells called follicular dendritic cells (FDCs) appeared to be enriched in the light zone and were capable of retaining antigen (3, 4). Later it was shown that FDCs have a unique phenotype compared to conventional dendritic cells in that they lack cell intrinsic major histocompatibility complex class II (MHC II) (6), the ability to capture internalize and process antigen efficiently, bind high levels of immune complexes through complement receptors CD21 and CD35 (in mice CD21/35 is one receptor), and have a unique developmental pathway that is shared with mesenchymal cells and fibroblasts (7, 8). The dark zone and light zone have been shown to be regulated by chemokine gradients and two distinct chemokine receptors; CXCR4 and CXCR5. CXCR4 is principally expressed in the dark zone and binds to CXCL12 and CXCR5 which binds to CXCL13 and predominates in the light zone (9, 10). Relatively recent studies utilizing in vivo multiphoton microscopy has revealed that B cells readily cycle between these zones (with a preference of DZ→LZ migration) and have lengthy interactions
with T cells within the germinal center, supporting the cyclic re-entry hypothesis in which B cells undergo T cell mediated selection within the light zone and re-populate the dark zone after selection (11-13).

The early discovery of the germinal center and work of characterizing this anatomical site by light microscopy. Even with limited reagents compared to modern times it was known as early as 1957 (14) that the germinal center was a major site of gamma globulin formation, even though the enzyme responsible for class switch recombination (CSR) would not be discovered for more than 40 years. At nearly the same time it was shown that a single cell can produce a single antibody (15), and the clonal selection theory was postulated by Sir Frank Macfarlane Burnet which extended upon the natural selection hypothesis of Niels Kaj Jerne (16). While these studies and postulations laid the theoretical and experimental groundwork for the discovery of primary lymphocyte diversity via recombination activation genes (RAG)(17, 18), they also set the framework for modern theories of how the germinal center functions in a competitive and clonally selective manner (4).

Moreover, the process by which immunoglobulins undergo a progressive improvement in ability to bind antigen over the weeks that follow immunization (later termed “affinity maturation”) was observed in 1964 by Herman Eisen and Gregory Siskind in a landmark study (19), one year before the B cell was actually discovered by Max Cooper and colleagues in the bursa of Fabricious of chickens (hence the term B
cell) (20). This was the start of resolving questions arisen from the study of humoral and cellular immunology. These early detailed studies did give rise to several theories of how this anatomical site produced antibodies that were capable of neutralizing pathogens. Curiously, it was noted in the very first publication on affinity maturation (19) that this process was significantly impaired at high antigen doses, implying a competitive and selective nature to the process.

More definitive evidence for somatic mutation and clonal selection within the germinal center came in the 1980s and 1990s with the advent of new molecular techniques. Irradiation chimeras gave evidence that germinal centers arise from just a few B cell clones (termed “oligoclonal”), implying that the structure is formed from clonal expansion of antigen activated cells (21). Pioneering studies of micro-dissected germinal centers and subsequent DNA sequencing of variably region genes provided direct evidence that this anatomical site facilitates clonal evolution of the antibody repertoire over time and enrichment of high affinity clones (22, 23). Further studies by Kelsoe and Jacob and colleagues developed a protein hapten based system solidifying the evidence for the clonal evolution of the germinal center response (24-26) and providing the current gold standard system for evaluating the process.

The molecular mechanism by which these processes occurred was unknown until 2000, in which Dr. Tasuku Honjo discovered this was due to the deamination of adenine groups by the enzyme activation induced cytidine deaminase (AID) which
was found to be expressed in germinal centers, was previously thought to be strictly an RNA editing enzyme (27-30). AID was originally found to be required for class switch recombination and defect in AID leads to hyper-IgM syndrome in humans (28). Later studies showed that fibroblasts transfected with AID could undergo hypermutation (31) and E. coli transformed with AID gain a mutation prone phenotype (32). Extensive studies have delved into the regulation of AID by post transcriptional modifications, revealing that phosphorylation affects class switching and somatic hypermutating ability of AID (33-35). Two distinct AID phosphorylation sites that have functional impacts have been identified, serine 38 and tyrosine 140, of which serine 38 is critical for both CSR and SHM, while tyrosine 140 appears to be more exclusively required for SHM (36).

Discussing the germinal center reaction would not be complete without mentioning T follicular helper cells, which are a specialized subtype of CD4 T cells that reside within the B cell follicle and germinal center and are primed to deliver help signals to appropriate B cell clones that are formed through SHM. As early as 1968 it was observed through cell transfer experiments of thymocytes and bone marrow cells into irradiated mice that co-transfer of these cells was required to develop a strong antibody response during immunization (37). Major pathways of how T cells provide B cell help were subsequently elucidated and include IL-4 (38), CD40 ligand (36), Inducible Co-stimulator (ICOS) (39) and IL-21 (40). A new stake in the ground to
define T follicular helper cells came with the identification of BCL-6 as the master regulator of this T cell program (41). Most recently, a T follicular regulatory cells, which co-express FoxP3 and BCL-6 (and quite curiously BLIMP-1 as this has been shown to be antagonistic of BCL-6) (42, 43) have been identified and shown to suppress the germinal center reaction through mechanisms that are still being defined but include CD86 independent functions of CTLA-4 (44, 45).

Less well studied and more esoteric members of the germinal center microenvironment are tangible body macrophages (TBM) which were originally observed by Flemming in 1885 and sparsely and sporadically studied since, but were found to contain significant amounts of cellular debris by electron microscopy (46). Recently exciting studies have revealed that TBMs may play a role in preventing autoimmunity in germinal centers through FDC secreted milk fat globule 8 (MFG8) activation of TBMs and Mer receptor tyrosine kinase (47-50). Additionally, a unique kind of CD8+ T cell that is Qa-1 restricted has been shown to suppress the germinal center reaction through competition for ICOS on TFH and a perforin mediated mechanism (51, 52).

In summation, the germinal center is a complex microenvironment with multiple constituents that is exquisitely designed to facilitate the birth and selection of new B cell clones that are capable to neutralize pathogens.
Purinergic receptors in inflammation

There are two major classes of purinergic receptors, the P1 and P2 receptors.

P2 purinergic receptors principally have an affinity for phosphorylated adenosine, namely ATP and ADP and consist of seven P2X and eight P2Y receptors which are known (53). P2X and P2Y receptors differ in that P2X receptors are ATP-gated ion channels and P2Y receptors are known to be metabotropic g protein coupled receptors (54). P2 receptors, in contrast to P1 receptors, have been shown to act as damage associated molecular patterns (DAMPs) during inflammation. During inflammation, ATP can be released from both necrotic cells in an uncontrolled fashion, but also from apoptotic and stressed cells through classical secretory pathways including pannexin and connexin channels as well as vesicular release (55).

P1 receptors principally have an affinity for the nucleoside adenosine and consist of the four known adenosine receptor subtypes; A2a adenosine receptor (A2aR), A2b adenosine receptor (A2bR), A1 adenosine receptor (A1R), and A3 adenosine receptor (A3R). Classically, evidence for the likely existence of adenosine receptors was born out of the neurobiological field principally using pharmacological studies in the late 1960s and early 1970s. These studies were among the earliest to show that the addition of exogenous adenosine to either neurological tissue slices or neuroblastoma cells caused a potent increase in intracellular cyclic AMP (56-59). Moreover, the addition of methylxanthine based compounds such as theophylline to
cell cultures blocked the rise in cAMP which was observed in cultures in which exogenous adenosine was added.

Later this was postulated and shown to be competitive antagonism by theophylline of surface adenosine receptors (60). The identification of adenosine receptors and the concept of P1 vs P2 receptors did not come until the late 1970s and 1980s, and was pioneered by Geoffrey Burnstock using pharmacological means, and centered around ATP and adenosine being mediators of neurotransmission (61-63). A major advance forward in studying adenosine receptors came in 1989 (64) when the sequences for A1 and what would later to be known as the A2a adenosine receptor were cloned, although they were only known to be g protein coupled receptors titled RCD7 and RCD8 at the time. A fervor of studies in the early 1990s confirmed these to be functional adenosine receptors and clones from several species were sequenced by several groups (65-74). It was postulated in the early 1980s that there is a likely existence of at least two subclasses of A2 adenosine receptors based on high and low affinity for ligands. Almost 10 years later in 1992, the A2b receptor was defined by molecular cloning and characterized by generating cAMP in response to the nonspecific adenosine receptor agonist NECA, while failing to generate cAMP response to the A2a specific agonist CGS-21680 (75, 76). The same year also marked the identification and molecular cloning of the A3 adenosine receptor (77).
Since the identification of the four known adenosine receptors, it has become evident that the four subtypes are distinct in both function and cell type distribution. In regulation of inflammation, the A2a adenosine receptor has been the most widely studied. The A2a adenosine receptor is a Gs protein coupled receptor that is known to activated adenyl cyclase and stimulate cyclic AMP formation and downstream PKA activation (78). Among the adenosine receptors, A2aR is unique in size due to an exceptionally long intracellular tail - 412 AAs compared to 332AA (A2bR), 318AA (A3R), and 326AA (A1R). Early studies in the late 1990s involved the use of specific A2aR agonist CGS21680 in identifying the role of A2aR in attenuation of T cell activation and postulating that A2aR mediates the deleterious effects of ADA deficiency and possibly could lead to immunosuppression in hypoxic tumors (79, 80). A major step forward came in 2001 with a landmark study which showed that gene deficient mice for A2aR (A2aR−/−) showed exaggerated inflammation in a model of concanavalin A induced liver injury, providing the first in vivo evidence for the role of A2aR in limiting inflammation (81). Follow on studies showed A2aR immunosuppression to regulate tumor infiltrating T cells and A2aR−/− mice showed greater tumor rejection which could also be achieved by pharmacologic anatagonism of the receptor (82). This suppressive effect of A2aR signaling was also found to be true on anti-myeloma specific human T cells (83). A2aR immunosuppression has also become evident in a variety of other conditions such as the enhancement of T
regulatory cell suppressive capacity (84, 85), suppression of NKT cell activation (86-88) and limiting ischemia reperfusion injuries (89-91).

The A2b adenosine receptor history in inflammation is not quite as straightforward as that of A2aR. First, the A2b adenosine receptor has a not as well defined signaling pathway as that of A2aR. Although A2b is considered to be a low affinity receptor for stimulating gs proteins and activation of adenylyl cyclase, the relative affinity for A2b in signaling through its’ alternate gq coupled pathway has not been widely studied. A2b adenosine receptors are expressed on lymphocytes, but are also known to be highly expressed on innate cells such as dendritic cells and mast cells (92). In inflammation, studies of the A2b adenosine receptor have revealed differing results based on the experimental setting highlighting the complexity of A2bR signaling pathway. For example, genetic deletion of A2bR in mice resulted in increased leukocyte adhesion and TNF-α secretion in response to LPS injection(93), suggesting that signaling through A2bR works to limit inflammation like its’ A2aR kin. Conversely, A2b receptors have been shown to play a pro-inflammatory role in mast cell cytokine production of Il-13 and VEGF (94) as well as dendritic cell priming and Th17 cell formation through production of IL-6 (95) (96). Furthermore, mast cells activated through A2bR were capable of stimulating B cells to undergo class switching to IgE (97).
The A1 adenosine receptor and A3 adenosine receptor are much less studied in the regulation of inflammation, although A3 receptors have been shown to be important in regulating mast cell degranulation and asthma (98, 99). Further studies will be needed to elucidate the roles of these receptors in B cell biology.
Sources of extracellular adenosine and adenosine metabolism

The accumulation of extracellular adenosine is common in inflamed and cancerous tissues. The mechanisms by which this accumulation and regulation occurs is diverse however. During inflammation, metabolically active cells produce adenosine intracellularly by hydrolysis of adenosine monophosphate (AMP) by enzymes such as cytosolic 5’ nucleotidase or by the transmethylation pathway by catabolism of S-adenosyl-L-homocysteine by SAH-hydrolase. Intercellular levels of ATP in cells is high and can range between 5-8 µM, providing confidence that there is rarely a physiological condition in which lack of substrate for generation of signaling through P1 and P2 receptors. Once adenosine has formed in the intercellular space by hydrolysis, it is targeted to one of three primary fates; 1) re-phosphorylation to AMP by adenosine kinase, 2) exportation to the extracellular space by equilibrative nucleotide transporters (ENTs) and concentrative nucleotide transporters (CNTs), or 3) degradation to inosine by adenosine deaminase.

It has been shown that the metabolism of adenosine is critical to lymphocyte function as failure to degrade adenosine is toxic to cells and is most aptly displayed by adenosine deaminase (ADA) deficiency, in which patients are severely immunocompromised and often die in childhood. This deficiency is corrected by administration of pegylated adenosine deaminase or by transfection of autologous CD34+ bone marrow cells with the ADA gene (100). At the cellular level it has been
shown that ADA deficient mice have normal B cell development, but have severe
defect in ability to form germinal centers and principally have an extra follicular
plasmablast response in response to antigen (101). Moreover, T cell deficits are also
apparent in ADA deficiency in that ADA−/− mice have severe impairment in T cell
development that results in altered T cell receptor signaling and increased rates of
apoptosis in thymocytes (102).

Extracellular adenosine can also accumulate via the release of 5’ ATP, ADP, and
AMP via calcium sensitive vesicular release, transporters, pannexin channels or
necrosis (78) and subsequent dephosphorylation by a collection of ectoenzymes of the
NTPDase family and subsequent 5’ nucleotidase family. The NTPDase family
consists of eight separate enzymes which catalyze the degradation of ATP or ADP to
AMP. Of the eight known NTPDases, only 1, 2, 3 and 8 are expressed on the
extracellular side and are able to contribute to extracellular AMP generation (103),
however some NTPDases can be shed and released into the extracellular space and
contribute in certain circumstances. The most well studied of the NTPDases is
NTPDase1, also known as CD39 (103), which kinetically is very efficient at
converting ATP directly to AMP compared to other NTPDases. Curiously CD39 was
first purified from activated B cells (although not germinal center B cells) as well as T
and NK cells and was noted to be an adhesion molecule that was independent of
integrins (104). Mice deficient in CD39 ectoenzyme display increased levels of
extracellular ATP and ADP (with decreased levels of extracellular adenosine) which contributes to an increased susceptibility to overwhelming inflammation during disease states (78, 105) such as ischemia-reperfusion injury (106), acute lung injury (107), and liver inflammation (108).

Resulting 5’ AMP can signal through P2 family receptors, be re-uptaken and rephosphorylated to ADP and ATP by the cell, or continue through the degradation pathway through nucleotidases such as 5’ nucleotidase (CD73) which has a membrane bound and secreted form. In addition to CD73, tissue nonspecific alkalkine phosphatase (TNAP) and prostatic acid phosphatase (109) have been shown to catalyze the degradation of 5’AMP to adenosine. Importantly it was observed that this conversion of 5’AMP to adenosine was noted through TNAP in the brains of mice genetically lacking CD73 (CD73−/−), but not in wild type mice, indicating that TNAP can be a redundant pathway for adenosine generation in the absence of CD73.

Taken together the study of the physiological function of adenosine ectoenzymes is certainly complicated and one must not forget that genetic deletion or pharmacological inhibition of these enzymes by definition has bi-directional effects that cannot be excluded. More precisely removal of a member of the enzymatic pathway may decrease the generation of extracellular adenosine which would decrease signaling through adenosine receptors, but also potentially increase signaling through
P2 receptors. This must be accounted for in designing experiments to tease apart the physiological role of these enzymes.
Hypoxia and hypoxia inducible factors in immune regulation

Tissue hypoxia has been long studied in the world of cancer research as tumors develop hypoxic microenvironments which facilitate growth of tumors, alter metabolism and suppress ongoing immune responses (110-112). Inhaled oxygen tension consists of 21% oxygen (partial pressure of ~160mm/hg at sea level) and subsequently declines when distributed through blood vasculature in tissues. In the blood, oxygen tensions range widely from ~104mm/hg (~13.6% O₂) in the lung, to ~40mm/hg (~5.2% O₂) in the venous blood (113). In normal healthy tissues can be as low as 4-5% oxygen. Tissue hypoxia is often considered to be less than 3% oxygen, whereby hypoxia inducible factors (HIFs) are stabilized.

Oxygen itself is transported primarily bound to hemoglobin within red blood cells as the gas in the O₂ form is non-charged and poorly water soluble, therefore poorly soluble in blood plasma. This hemoglobin bound O₂ transport accounts for ~97% of all oxygen delivered by the blood (5ml O₂ delivered per 100ml/blood) compared to 3% of O₂ delivered by dissolved oxygen (0.17ml O₂ delivered per 100ml/blood) (113). Inspiration of hyperbaric oxygen or gas mixtures containing high oxygen can dramatically increase the amount of oxygen dissolved in blood plasma, thereby increasing total oxygen delivered to tissues (113). Although hyperbaric oxygen can lead to oxygen toxicity, oxygen gas mixtures as high as 60% can be tolerated without signs of oxygen toxicity (113, 114). This provides a valuable tool to modulate
oxygen tensions in a safe manner, which can then directly influence affected pathways such as the hypoxia adenosinergic pathway (discussed in next section).

Major advances in the study of the role of local oxygen tensions in immune regulation came from early studies that first identified HIF-1α in 1992 and 1993 in the search for factors that induce erythropoiesis (115, 116). This was followed by the identification of two other HIFs, HIF-2α, and HIF-3α (117, 118). These early studies helped to pave the way in terms of discovery of the molecular machinery that allows cells to undergo metabolic shifts and adapt to hypoxic environments (119). HIF-1α has been come to known as a master regulator of the metabolic program in hypoxia. Under low oxygen tensions (e.g. less than 3% oxygen), HIF-1, a heterodimeric protein complex consisting of the constitutively expressed HIF-1β and stabilized HIF-1α (or HIF-2α, HIF-3α) is formed. Contrastingly, under normal physiological oxygen tensions (termed “physioxia”), HIF-1α is targeted for proteasomal degredation by prolyl hydroxylation at P402 and P564, and subsequently binds to the E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) for degradation. (120). In low oxygen settings, prolyl hydroxylases act as oxygen sensors as the lack of prolyl hydroxylation of the HIF isoforms leads to escape from the proteasome (120).

Once HIFs are stabilized, a variety of genes such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), and glucose transporters (GLUT-1, GLUT-4) are turned on by binding of the HIF heterodimer to hypoxia response
elements within enhancer regions of these genes (120). Curiously, HIF-1α and HIF-2α have unique gene targets and several studies have shown that HIF-1α appears to principally mediate the acute response to hypoxia while HIF-2α is more active under both milder hypoxia and chronic hypoxia (120). Also worth noting is that HIF-2α and HIF-1α are not always expressed in the same tissues and that HIF-2α can be rendered inactive within the cell by means of cytosolic sequestration (121). To further complicate the matter, HIF-3α, which has little sequence homology to its’ two predecessors, was found to actually bind to and inhibit HIF-1α (122). Further research is required to full tease out the tissue distribution, timing and interaction of the three known HIF subclasses.

It is important to note, however, that HIFs can be stabilized in normoxia by pathophysiological states such as gene deficiency in pVHL or instances conditions which cells attain TCA cycle defects that can prevent degradation of HIFs (123). Furthermore, providing more exceptions to the rule is the fact that activation of the T cell receptor can induce strong accumulation of HIF-1α in Th17 cells that is not oxygen dependent, but rather relies on a mechanism involving STAT3 (124, 125).

In regards to the regulation of inflammation, the role of HIFs is somewhat controversial, and in many studies is cell type dependent. It has been shown that the HIF-1α and the I.1 isoform plays a role in the negative regulation of T cells by suppressing IFNγ and TNFα secretion (126). Similarly it was shown that deletion of
the HIF-1α I.1 isoform in T cells potentiates the pro-inflammatory cytokine profile and increases survival of mice in the cecal ligation and puncture model of bacterial sepsis (127).

In contrast, HIF-1α has been shown to bind FoxP3 directly and regulate the balance of Th17/T regulatory cells in favor of promoting Th17 action while being involved in inhibiting the function of T regulatory cells (125). More recently it has been shown that pVHL is critical in regulating this pathway and maintaining the suppressive function of T regulatory cells by degrading HIF-1α (128). Additionally, Hif-1α has been shown to promote pro-inflammatory signals in innate cells such as neutrophils and macrophages (129). Such signals include the induction of toll like receptors (TLRs) (130) and induction of MHC-II, CD80 and CD86 in antigen presenting cells (131).

Future studies will help to resolve the context and timing dependent roles of HIFs in the regulation of inflammation.
The Hypoxia Adenosinergic Pathway

The hypoxia adenosinergic pathway has been shown to be a critical and evolutionarily conserved mechanism by which inflamed tissue microenvironments (e.g. cancerous tumors, sepsis) develop processes by which extracellular adenosine is upregulated and suppress or redirect immune responses (132-136). The hypoxia adenosinergic pathway lies at the intersection of hypoxia field and metabolic signaling field.

This process is mitigated by several distinct components that have been elucidated to date. One major example of this is the transcriptional activation and subsequent upregulation of ectoenzymes within the adenosine generating pathway. For example, NTPDase1 (CD39), which surface expressed and catalyzes the hydrolysis of ATP to AMP, has been shown to be upregulated in a hypoxia-induced sphingosine 1 phosphate manner (78, 106, 137). Additionally, other members of the adenosine generating cascade like CD73 have been shown to be responsive to hypoxia and HIF-1α (138, 139). One must be scrupulous in interpreting these responses of cells in terms of upregulation of ectoenzymes in hypoxic states, as direct evidence for HIF-1α induction of CD73 on lymphocytes has yet to be shown and the original publication showed this effect on endothelial cells (139). That being said, oxygenation during tumor development by respiratory hyperoxia has shown suppression of tumor development, lowering of intratumoral extracellular adenosine levels, and reduced
levels of CD73 and CD39 among T cells (140, 141), lending credence to the hypothesis that in some states these ectoenzymes can be under direct control by oxygen tensions.

Moreover, other members of the adenosine signaling and metabolism cascade have been shown to be regulated by hypoxia or HIFs. Namely, the inhibition of adenosine kinase in hypoxic conditions with regard to cardiomyocytes has been shown (142), upregulation of A2b adenosine receptor by HIF-1α (143), upregulation of A2a adenosine receptor by HIF-2α (144, 145), repression of reuptake of adenosine by ENTs during ischemia (146) and HIF-1α specific repression of ENT2 during mucosal inflammation (147). Taken together these studies support the fact that hypoxia promotes the accumulation of extracellular adenosine through multiple mechanisms ensuring some level of redundancy at this step of the pathway.
Hypoxia and adenosine in the context of B cell biology

To date there has been relatively few number of studies that have systemically addressed the role of adenosine receptors and hypoxia in terms of the germinal center reaction or humoral mediated immunity for that matter. Early physiological studies in the 1970s and 1980s surrounded the immunological adaptation to humans and mice in high altitude environment. Early experiments in 1975 found a tendency that humans living at high altitude and had an increased presence of class switch IgA and IgG in the blood when compared to matched cohorts (148). Although this early study is tantalizing, given the focus of this dissertation, studies in hypoxic treated mice in 1978 showed reduced capacity for splenocytes to form antibody forming cells when compared to normoxic controls (149). Solidified evidence of the importance of hypoxia and HIFs did not come until 2002 where it was shown that HIF-1α was critical for normal B cell development (150). In a cre-lox system targeted to B cells through CD19, it was also shown in 2003 that Hif1α regulates cell cycle arrest in hypoxia (151). Follow on studies have been sparse as there has been little follow up in the last ten years of the role of hypoxia and HIFs on B cells.

In regards to the history of the study of adenosine signaling in B cell biology, there is nearly equally limited in the frequency of systemic studies. The earliest studies of relevance, which predated the postulation and pursuit of adenosine receptors, centered around the identification of CD73 being located on the surface of mouse
lymphocytes (152), and the lack of adenosine generating capacity on human B cells in patients afflicted with primary hypogammaglobulinemia (153, 154). CD73 was also considered to be a marker of human B cell maturation (155, 156). In regards to relation with the germinal center, early histochemical studies detected CD73 within parts of the GC in rats (157), and later studies showed that CD73 can mediate the adhesion of GC B cells to FDCs (158). However, no studies to date have addressed the role of any adenosine receptor signaling within the germinal center.
Adenosine receptors

Fig 1.1: The four known adenosine receptors (1)
Fig 1.2: Sources of extracellular adenosine generation. 1) ATP is released by multiple mechanisms including vesicular release, pannexin channels, connexin channels, and necrosis. 2) ATP can signal through P2X or P2Y receptors, or be degraded to ADP by NTPDases (1, 2, 3, 8 expressed on the surface, NTPDase 1 is CD39 (NTPD), alkaline phosphatase (AP), tartate resistant alkaline phosphatase (TRAP), nucleotide pyrophosphatase/phosphodiesterase (NPP). 3) ADP can then signal through P2Y receptors or continued degradation to AMP by enzymes mentioned in previous step as well as calcium activated nucleotidase (CAN). 4) Alternate pathways through enzymes such as CD38 and CD157 can generate AMP from ADP ribose in conjugation with NPP. 5) AMP can signal through P2 receptors or be further degraded to adenosine by ectoenzymes such as 5’ nucleotidase (CD73), AP, or transmembrane bound prostatic acid phosphatase (PAPtm). 6) Adenosine can be further degraded to inosine by adenosine deaminase extracellularly which is bound to CD26, reuptaken by equilibrative nucleotide transporters, or signal through the four known P1 receptors (A1, A2a, A2b, A3).
Fig 1.3: The hypoxia adenosinergic pathway at the cellular level.
Chapter 2
Germinal center hypoxia potentiates immunoglobulin class switch recombination

2.1 Introduction

Development of effective vaccination strategies requires a detailed understanding of the mechanisms that govern adaptive immunity. The germinal center (GC) is the anatomical site where antigen-activated B- and T-lymphocytes interact, initiating immunoglobulin class switch recombination (CSR), somatic hypermutation, and the affinity maturation associated with effective antibody responses (3). However, surprisingly little is known about the physiological mechanisms within the GC microenvironment that regulate these processes.

It has become evident that local oxygen tension and the physiological response to hypoxia play a role in regulating inflammation (159) through synergistic and independent mechanisms including, but not limited to, hypoxia inducible factors (e.g. HIF-1α, HIF-2α and HIF-3α), NF-κB, mammalian target of rapamycin kinase (mTOR), and the unfolded protein response (UPR) (119, 159, 160). Moreover, the cellular response to tissue hypoxia often results in metabolites in the extracellular space, which have diverse signaling capacities that can facilitate both pro and anti-inflammatory responses (78, 159). Indications that hypoxia may be important in B
cell physiology originally came from experiments that revealed HIF-1α is required for B cell development and prevention of autoimmunity (150). Furthermore, HIF-1α has also been detected in human tonsillar GCs (161). Fortuitously, studies have revealed numerous hypoxia-associated (162-164) pathways including the PD-1 - PD-L1 axis (165), CXCR4-CXCL12 signaling (9), and the sphingosine-1-phosphate (S1P) S1P receptor pathway (166) to be critical for GC function.

We hypothesized that rapidly proliferating B cells within GCs develop a hypoxic microenvironment that promotes CSR. We show that GCs contain hypoxic regions linked to accelerated class switching, plasma cell development and antibody secretion. Following vaccination, administration of clinically relevant (114) respiratory hyperoxia (60% O₂) via supplemental oxygen dramatically pressed the GC response and subsequent antibody production, revealing a previously unappreciated functional role of hypoxia within the GC microenvironment. Supplemental oxygenation is generally considered to be benign when not exceeding 60% O₂ in healthy subjects (18), although it may be clinically over utilized in patients who have normal pulse oximetry readings (167). This new understanding of GC physiology may suggest improved vaccination guidelines for patients receiving supplemental oxygen and stimulate new therapies for autoimmune diseases such as systemic lupus erythematosus (SLE).
2.2 Results

To ascertain whether the GC microenvironment is hypoxic, we immunized female C57BL/6J mice with the hapten-protein antigen, (nitrophenyl) acetyl-ovalbumin (NP-OVA) in aluminum hydroxide (alum) adjuvant. Identification of areas of tissue hypoxia or cells in hypoxic areas was determined by immunohistology using a well-documented molecular marker of tissue hypoxia (<1.3% O₂) in vivo, Hypoxyprobe (pimonidazole) (168). Injected intravenously, Hypoxyprobe creates thiol adducts with proteins under hypoxic conditions, which are then specifically recognized by a monoclonal antibody. To assess GC hypoxia in situ, we studied Hypoxyprobe labeling of splenic GCs (Figure 2.1). These histologic studies indicated that GCs are enriched for hypoxic areas (Figure 2.1).

We subsequently confirmed Hypoxyprobe labeling of GC B cells by multi-color flow cytometric analysis of GC B cells from spleen (Figure 2.1), Peyer’s Patch (Figure 2.1) and mesenteric lymph node (Figure 2.1) of immunized and naïve mice. To a lesser extent, GC associated T<sub>FH</sub> cells (CD4<sup>+</sup>CXCR5<sup>+</sup>Bcl-6<sup>+</sup>GL-7<sup>+</sup>) showed increased Hypoxyprobe staining compared to respective controls (Figure 2.1), indicating hypoxia. In support of this demonstration of GC B- and T-cell hypoxia, we injected fluorescently-labeled tomato lectin that binds to blood vessel walls and then used fluorescence microscopy to measure the relative distances between GC B cells and the splenic blood vasculature (Figure 2.2). We confirmed vasculature staining by
tomato lectin by using the endothelial marker CD31 and found GCs to be poorly perfused (Figure 2.2).

Due to the fact that earlier studies have shown that pO$_2$ can drop to hypoxic levels 30-40 μm from blood vessels (169), this distance was used to evaluate the likelihood of a hypoxic environment at GC sites. We found that 8 days after NP-OVA immunization, the substantial majority of GC area lies ≥40 μm from the nearest blood vessel (Figure 2.3). Although the majority of GCs were located ≥40 μm from the nearest blood vessel, occasional GCs were adjacent to or even surrounded tomato lectin stained vessels (data not shown). However, in the three dimensional space of lymphoid tissue, GCs are preferentially sited in hypoxic regions (Figure 2.2, Figure 2,3).

To assess whether GC hypoxia has any functional role, we used *in vitro* experiments with defined gas mixtures. Briefly, resting B cells (CD43$^-$GL7$^-$) were isolated by magnetic depletion and stimulated in cultures containing anti-CD40 and anti-IgM antibodies in normoxic (21%O$_2$) or hypoxic (1% O$_2$) incubators. We found that the GL-7 activation marker was upregulated on a greater fraction of hypoxic B cells and to higher levels than their normoxic controls (Figure 2.4). Although GL-7 expression can be upregulated on B- and T cells *in vitro* (170), it is widely used to identify GC B cells *in vivo* [1]. The GL-7 carbohydrate epitope may be influenced by oxygen levels directly, as it depends on the repression of CMP-Neu5Ac hydroxylase.
Since GL-7 was recently shown to mark a subset of GC associated T_{FH} (172), we assessed the effect of hypoxia on GL-7 up-regulation on CD4^{+} T cells in vitro. Upon stimulation with anti-CD3 antibody, we observed that hypoxic culture conditions increased the frequency of GL-7 expressing CD4^{+} T cells and also increased the expression level of GL-7 in CD4^{+} T cells when compared to normoxic controls (Figure 2.4).

To identify any additional consequences of hypoxia and increased GL-7 expression by B cells activated in hypoxic cultures, we determined the frequency of cells exhibiting IgM^{+}→IgG1^{+} class switch recombination after stimulating resting B cells with anti-CD40 antibody and IL-4 (173). We then placed matched cohorts under normoxic (21%O_{2}) or hypoxic (1%O_{2}) conditions for four days to determine the numbers of class switched, IgG1^{+}, B cells by flow cytometry. Interestingly, hypoxic culture conditions resulted in acceleration of IgM^{+}→IgG1^{+} class switch kinetics on day 3, while reducing the numbers of IgG1^{+} B cells on day 4 (Figure 2.5). Furthermore, by CFSE labeling B cells at the start of culture, we assessed if IgG1^{+} B cells in hypoxic chambers proliferated comparably to normoxic controls. We found that the early increase in IgG1^{+} B cells in hypoxic cultures does not represent biased proliferation (Figure 2.6), but rather increased rates of class switch recombination (IgM to IgG1) determined by the increase in IgM excision circles (Figure 2.5), which are hallmarks of CSR (174). Interestingly, the rate of B cell division in hypoxic
chambers was accelerated on day 3 but slowed significantly and specifically in the IgG1+ compartment on day 4 (Figure 2.6), suggesting hypoxia may have a further role in regulating proliferation of class switched B cells. In support of this regulatory notion of hypoxia, we observed that B cells on day 4 in culture displayed lower surface levels of IgM and IgG1 (Figure 2.5).

Plasma cell differentiation, as determined by the appearance of CD138+B220- cells in hypoxic and normoxic cultures, was dramatically increased under conditions of low oxygen tension (Figure 2.8), suggesting that hypoxia accelerates both class switch recombination and differentiation to plasmacytes. In support of this we observed higher levels of CD43 expression and CD138 expression among plasmacytes (Figure 2.8). This interpretation is further supported by the observation that on day 4, hypoxic cultures had an increased frequency of IgG1+ antibody secreting cells (ASC) but not IgM+ ASCs as determined by ELISPOT (Figure 2.7). Taken together, the accelerated plasma cell differentiation and $\sim 50\%$ increase in total IgG1+ ASC in hypoxic cultures may partially account for the $\sim 50\%$ reduction of IgG1+ B cells in these cultures on day 4 (Figure 2.8).

If hypoxia modulates B cell differentiation during GC responses, we predicted that reversing tissue hypoxia during immunization would press the GC reaction. To test this prediction, we systemically weakened tissue hypoxia, as done clinically, by placing mice in chambers containing 60% O$_2$ to induce respiratory hyperoxia (114).
Female C57BL/6J mice immunized with NP-OVA or NP-chicken γ-globulin (NP-CGG) in alum and held under conditions of respiratory hyperoxia exhibited dramatic suppressions of total and NP-specific GC responses 8 days after immunization (Figure 2.9). Similarly, but to a lesser extent, T_{FH} and GC associated T_{FH}, defined as CD4^{+}B220^{-}CXCR5^{+}PD1^{+}GL7^{-} and CD4^{+}B220^{-}CXCR5^{+}PD1^{+}GL7^{+}, respectively, were reduced in frequency by hyperoxia (Figure 2.10). Moreover, administration of respiratory hyperoxia in mice resulted in lower ICOS expression by both T_{FH} and GC associated T_{FH} (Figure 2.10).

Functional impairment of the GC response by respiratory hyperoxia was also manifested by fewer IgM^{+} and IgG1^{+} (nitroiodophenyl)acetyl-bovine serum albumin (NIP-BSA) specific ASCs (Figure 2.11). Since ASCs do not discriminate between either T_{FH} and GC associated T_{FH}, we identified plasma cells using flow cytometry (CD138^{+}B220^{-}) and found that hyperoxia significantly pressed plasma cell formation (Figure 2.12). This was in excellent correlation with our in vitro finding that hypoxic cultures accelerated plasmacytic differentiation (Figure 2.8).

Functionally, serum concentrations of NIP-BSA-specific IgM and IgG1 antibody were significantly reduced in mice treated with respiratory hyperoxia (Figure 2.12). Moreover, among NP-specific GC B cells, hyperoxia significantly reduced the frequency of class switched IgG1^{+} GC B cells whereas the proportion of IgM^{+} GC B
cells was unchanged (Figure 2.13). We subsequently confirmed the reduction in IgG1$^+$ GC B cells by respiratory hyperoxia histologically (Figure 2.13). Taken together, the reduction in IgG1$^+$ NP-binding GC B cells and diminished serum NP-specific IgG1 (Figs. 4G-I) suggests that respiratory hyperoxia likely has a more robust effect on Ig class switching than on initial, extrafollicular interactions between activated T and B cells.
Fig 2.2: The germinal center is poorly vascularized. A) Tissue histology of a splenic germinal center from an NP-OVA immunized mouse perfused with tomato lectin binding perfused vasculature. B, C) Tissue histology of GC containing follicle or control follicle showing endothelial marker CD31.
Fig 2.3: The germinal center is distant from local vasculature. A) Strategy for measuring local distance from blood vasculature B) Example masking strategy C) Quantitative difference of GC versus B cell follicles of areas within 40 micrometers.
Fig 2.4: GL-7 expression is upregulated in hypoxic culture conditions on both B and CD4 T cells. A) Representative flow cytometric plots of B cells stimulated in hypoxic or normoxic incubators. B,C) Quantification of GL-7 expression of B cells in A. D) Representative flow cytometric plots of CD4 T cells stimulated in hypoxic or normoxic incubators. E,F) Quantification of GL-7 expression of T cells in D.
Fig 2.5: Hypoxic culture conditions accelerates class switch recombination. A) representative flow cytometric plots of B cells stimulated to undergo CSR in hypoxic or normoxic incubators. B) Class switch kinetics of B cells depicted in A. C) Surface expression of IgG1 and IgM on IgG1+ or IgM+ B cells on day 4. D) Looped out circle transcripts for IgG1 12 and 24 hours after culture stimulation.
Fig 2.6: Hypoxic culture conditions slows proliferation of IgG1 cells on day 4. A) Live cells of hypoxic or normoxic B cell cultures enumerated by trypan blue exclusion on a hemocytometer B) Frequency of IgM+ B cells in hypoxic or normoxic cultures as described in fig 2.5. C) Proliferation of B cells in hypoxic or normoxic cultures determined by CFSE. D,E) Division index calculated from C.
Fig 2.7: Hypoxic culture conditions increase the frequency of IgG1 antibody secreting cells on d4. A) Representative IgG1 or IgM ELISPOTs on day 4 of culture in hypoxic or normoxic incubators. B) Quantification of ELISPOTs in A.
Fig 2.8: Hypoxic culture conditions accelerates plasma cell formation. A) Flow cytometric analysis of plasma cell formation in hypoxic or normoxic culture on day 4. B) Kinetics of plasma cell formation in same cultures. C) CD43 expression on plasma cells on day 4 of culture. D) Expression of Cd138 level (top) and CD43 level (bottom) of plasma cells on day 4.
Fig 2.9: Respiratory hyperoxia during vaccination suppresses GC frequencies.
Fig 2.10: Respiratory hyperoxia during vaccination suppresses TFH frequencies and ICOS expression.
Fig 2.11: Respiratory hyperoxia during vaccination suppresses IgM and IgG1 ASC formation.
Fig 2.12: Respiratory hyperoxia during vaccination suppresses plasma cell frequencies and antigen specific serum IgM and IgG1.
Fig 2.13: Respiratory hyperoxia during vaccination suppresses class switching within the germinal center. A) Gating strategy for determination of IgG1 and IgM NP-PE binding GC B cells on day 8 following NP-OVA/Alum immunization. B) Quantification of flow compartments depicted in A. C) Tissue histology of splenic GC B cells of mice treated as in A.
2.3 Discussion

In summary, we have described a previously unappreciated role for hypoxia in GCs that acts on both B- and T cells and promotes class switch recombination as well as plasmacyte differentiation. Our observations of the GC microenvironment offer new avenues of research into the role of hypoxia and hypoxia-induced immune-regulatory pathways during vaccination. Functional hypoxia within the GC raises many new questions as to the role of hypoxia in setting the stage for a competitive and possibly hostile environment for GC B cells that may facilitate the necessary clonal competition to develop somatically mutated high affinity B cells. Furthermore, it would be interesting to test if the hypoxic microenvironment within the germinal center acts as a signal to facilitate mobilization of AID from switch regions to V regions and bring about somatic hypermutation.

Our study suggests the possibility of unintended, negative consequences of supplemental oxygenation following vaccination or during ongoing humoral responses to infection. These data show that administration of clinically relevant (8) 60% O₂ dramatically presses the GC reaction and production of IgG1⁺ antibody. We view this study as pointing to the importance of further investigations of the functional role of hypoxia within the GC microenvironment.
Chapter 3

The A2a adenosine receptor drives affinity maturation in the germinal center.

3.1 Introduction

The generation of high affinity neutralizing antibodies following vaccination is the ultimate goal of any rational vaccine. The germinal center (GC) is the anatomical site in which antigen activated B cells undergo class switch recombination, somatic hypermutation and T cell mediated clonal selection in response to T dependent antigens (4). Affinity maturation was first described over 50 years ago as a stepwise increase in antibody affinity over the course of immunization (19).

T follicular helper cells (T<sub>FH</sub>) are a specialized subset of CD4+ T cells have been shown to be crucially important in providing help signals to B cells within the GC. T<sub>FH</sub> are CXCR5+, PD-1+ through multiple distinct mechanisms including but not limited to ICOS, CD40L, OX-40, CD84, II-4, II-21, and SLAM (175). Follicular regulatory T cells (T<sub>FR</sub>) which are CXCR5+, PD-1+ CD4+ T cells that co-express BCL-6 and FoxP3 (42, 43). T<sub>FR</sub> have more recently been shown to limit T and B cells within the germinal center at least partially due to the action of CTLA-4 (44).

The A2a adenosine receptor has been shown to be a non-redundant cyclic AMP elevating gs protein coupled receptor that is responsible for limiting
inflammation in innate and cellular mediated immunity(78). However, the role of A2aR in humoral mediated immunity has yet to be studied.

We hypothesized that the germinal center develops a microenvironment is hypoxic and adenosine rich which signals through A2aR to suppress the germinal center response. Indeed we found that A2aR deficiency leads to impaired resolution of the GC reaction and augmented TFH/TFR balance and function. This impaired resolution was linked with significantly retarded affinity maturation in A2aR deficient mice. Recruitment of A2aR signaling during vaccination importantly led to the acceleration of affinity maturation, providing a brand new mechanism to alter the affinity of the vaccine response.
3.2 Results

We previously determined that the germinal center microenvironment is hypoxic based on pimonidazole staining and measurements of local vasculature (Chapter 2). Henceforth, we postulated that the hypoxic microenvironment in the germinal center is also rich in extracellular adenosine, which is known to occur in hypoxic environments and contribute to immunosuppression/immunoredirection. We hypothesized that this adenosine rich microenvironment develops in the germinal center and works to resolve the reaction and lead to affinity matured B cell clones.

To assess the likelihood of the presence of extracellular adenosine within the germinal center, and technical inability to measure adenosine levels by equilibrium dialysis directly, we used detection of 5’nucleotidase (CD73) as a surrogate marker for likelihood of adenosine signaling. Within the B cell compartment we found that CD73 expression develops over time in the germinal center and is particularly enriched on IgG1 class switched B cells. In the T cell compartment, we found that T follicular helper cells and T follicular regulatory cells express nearly tenfold more CD73 than GC B cells and are among the highest expressers of CD73 among detected lymphocytes (Figure 3.1). This observation gave us good impetus that extracellular adenosine signaling plays a role in regulating the GC response.
We chose to directly assess the role of CD73 in the GC response by immunizing mice genetically deficient in CD73 (CD73\(^{-/-}\)) with the classic hapten NP-OVA in Alum. Surprisingly, in CD73\(^{-/-}\) animals we found only mild defects in the germinal center reaction in preliminary experiments (data not shown). However, we did note that these mice had specifically reduced levels of total IgG in the serum (Figure 3.2). One explanation of these mild defects may lie in the fact that CD73\(^{-/-}\) mice have been shown to compensate for lack of CD73 expression by upregulating TNAP, raising the possibility that these mice have sufficient compensatory extracellular adenosine production with respect to the germinal center microenvironment. Preliminarily lending promise to this idea, we immunized wild type mice with NP-OVA/Alum and injected an enzymatic inhibitor of CD73, APCP, from days 3-7 and found a small but statistically significant increase in GC frequencies on day 7 (data not shown).

We hypothesized that extracellular adenosine within the germinal center would resolve the reaction through the A2a adenosine receptor, which has been shown to critically negatively regulate T cells. Unlike CD73, the A2a adenosine receptor is a non-redundant member of the purinergic signaling cascade. We next immunized mice genetically deficient in the A2a adenosine receptor (A2aR\(^{-/-}\)) with NP-OVA in Alum and assessed the functional product of the GC reaction, that being the development of high affinity antibody. Strikingly, we found that A2aR\(^{-/-}\) mice have a significant
impaired in the ability to generate high affinity NIP-5 IgG and undergo affinity maturation (Figure 3.3). We investigated as to whether these gene deficient mice exhibited any defects in B cell development and found that B cell compartments within the spleen appear grossly normal and there is normal levels of serum immunoglobulins in the blood as well as presence of both kappa and lambda light chain. This gave us credence that the impaired affinity maturation we were observing in A2aRKO mice was not due to a developmental block but represented a physiological mechanisms that develops concurrently with immunization. We also chose to investigate if IL-7 receptor signaling on CD4 T cells was perturbed on A2aRKO mice following immunization due to the fact that one group has shown that A2aR impairs IL-7 receptor signaling in thymocytes and may regulate T cell homeostasis in peripheral tissues (176). In our system we did not find deficiencies in IL-7 receptor on CD4 T cells following immunization (Figure 3.5).

We next assessed which cell types that could be responsible for impaired affinity maturation expressed A2a adenosine receptors. Due to lack of available specific monoclonal antibody for mouse A2aR, detection of functional A2aRs by purification of cells and stimulation with A2aR agonist followed by measurement of cAMP induction was done. After fluorescence activated cell sorting (FACS) we purified T follicular cells to >99% purity and found that when stimulated with CGS21680, these cells did induce cAMP, indicating they express functional A2aR
(Figure 3.6). Future studies using A2aR/GFP or A2aR/RFP mice would be required to delineate if these cells have differential functional A2aR expression, as the T follicular gate contains both cell types. Next we tried to purify germinal center B cells by FACS and detect functional A2aR as we did on T follicular cells, however several attempts did not yield respondent cells.

Therefore we changed our approach to a magnetic depletion coupled with positive selection to achieve 93% GC purity. This gentler cell purification process yielded respondent cells, showing that GCs indeed express functional A2aR as indicated by cAMP induction (Figure 3.7).

Due to the fact that the germinal center in the principle site in which B cells undergo somatic hypermutation and clonal selection and since we hypothesized that the GC is adenosine rich and regulated by A2aR, we decided to investigate if A2aR knockout mice had perturbed GC dynamics following immunization. We found that at the peak of the GC reaction, there is little difference in total GC frequencies in A2aR knockout mice, although they tend to be increased (Figure 3.8). Later time points during immunization revealed that A2aR knockout mice fail to resolve the GC reaction when compared to wild type mice (Figure 3.8).

Next we chose to assess if germinal center architecture was affected by A2aR deletion possibly contributing to the defect in affinity maturation we have observed. Curiously, we found at the peak of the germinal center that A2aR knockout mice have
an increased frequency of dark zone B cells within the GC, as defined by CXCR4 and CD86 staining by flow cytometry and confirmed histologically by CD21/35 staining (Figure 3.9).

Since A2aR has been shown to be involved in cell survival we chose to assess if rates of apoptosis within the GC are elevated in A2aRKO mice. Preliminarily, we found at near the peak of the reaction following immunization that rates of apoptosis as defined by presence of activated Caspase 3 was increased in A2aRKO mice (Figure 3.10A). We also postulated that T cells may be providing more T cell help in the germinal center, possibly leading to this phenomenon and outgrowth of low affinity or non-dominant antigenic epitopes and found that A2aRKO B cells tend to have fewer frequencies of NP-binding cells among total GCs but an increased frequency of IgG1+ cells (Figure 3.10 C,D). Moreover, we also assessed if Apoptotic rates within A2aRKO mice were elevated in other cell types than GC B cells during immunization and found these rates to be comparable (Figure 3.11), suggesting that A2aR protection from apoptosis may only be distinct in highly metabolically active cells.

To ascertain possibly molecular and cellular contributions to the increased frequencies of GC B cells in A2aRKO mice and slightly elevated rates of GC apoptosis, we investigated if A2aR signaling was capable of suppressing phosphorylation events downstream of BCR ligation. Although it is known that A2aR stimulation suppresses phosphorylation events triggered in T cells by TCR ligation, no
studies to date have directly studied the effects of A2aR signaling in BCR mediated events. We found that when purified resting B cells (by CD43 depletion) are stimulated with anti-IgM Fab₂ and cocultured with A2aR agonist CGS-21680, both early and late events in BCR signaling are attenuated (Figure 3.12). This could suggest that potentially A2aRKO GC B cells have elevated BCR signaling that may contribute to activation induced cell death. BCR signaling within the GC, which although generally thought to be strongly dampened within the GC to favor T cell mediated selection processes, has been recently challenged using an in vivo reporter system (177). Further work in determining actual phosphorylation events within the GC of A2aR knockout mice will help elucidate this possibility.

Next we chose to assess if T cell help within the germinal center was increased, as this could explain the greater GC frequencies we observe and subsequent failure of affinity maturation in A2aR knockout mice. Indeed, we found that T follicular helper cell frequencies as defined by CXCR5 and PD-1 staining as well as BCL-6 staining is increased both at peak of the germinal center reaction and at later time points (Figure 3.13). We also noted increased absolute frequencies of GC associated TFH cells in A2aR KO mice as defined by GL-7 staining within the TFH compartment (data not shown), but due to the fact that this demarcation is not definitive in designating TFH cells to be resident within the GC, we chose to assess T cell frequencies histologically. We did find that GCs in A2aRKO mice contained greater frequencies of T cells.
within the GC compared to wild type (Figure 3.14), providing evidence that A2aR not only plays a role in DZ/LZ balance but also in regulating the ratio of T to B cells within the GC.

Due to the fact that the T follicular designation of CD4 T cells contains both T follicular helper and T follicular regulatory cells, we chose to investigate if the ratio and function of these cells was perturbed. We did find that A2aR knockout mice had less T follicular regulatory cells and more T follicular helper cells within this compartment (Figure 3.15), suggesting that A2aR deficiency impairs the ability of T follicular regulatory cells to adequately suppress the GC reaction. Functional markers on these cells support this notion as A2aR knockout TFH have higher levels of ICOS costimulatory while TFR cells express lower levels of CTLA-4 which has been shown to be a critical component of TFR mediated suppression (44). Lending support to this notion is the observation that FoxP3 levels are also reduced in TFR cells of A2aRKO mice (Figure 3.15), which has been correlated with suppressive ability of T regulatory cells.

Curiously, IL-21 signaling axis may be perturbed in A2aR knockout mice afflicting the balance of TFH to TFR cells. This was evidenced by lower expression levels of IL-21R on both TFH and TFR in A2aRKO mice (Figure 3.15). It has been shown that high levels of IL-21 can select and enhance TFH cell phenotype and tip the balance to a TFH vs TFR phenotype (178). IL-21R receptor downregulation in
A2aRKO mice may be a result of receptor internalization following IL-21 ligation. Future work will have to be done to elucidate direct IL-21 levels within these mice.

Even though we did not observe any gross defects in lymphocyte development and IL-7 receptor expression during immunization, we wanted to ensure that the germinal center perturbations we were observing were not due to the possibility that T cells in A2aRKO mice are more activated at baseline, as dysregulation of T cell homeostasis has been observed in these mice (176). To do this, we bred A2aRKO mice with OTII mice, which are transgenic for a TCR that is specific to ovalbumin (OVA)(179), which would largely bypass the possibility of increased baseline TCR triggering through endogenously encountered antigens. We did find that when we immunized these mice with NP-OVA, many of the phenotypes we had observed in A2aRKO mice were recapitulated including the tendency for GC and T follicular frequencies to be increased, GCs to be dark zone shifted, and TFRegs to be impaired in number and FoxP3 level (Figure 3.17). This finding supports the notion that the effects of A2aR deletion on the GC reaction are independent of baseline inflammatory status as well as TCR diversity, specificity, affinity or avidity for pMHC complexes.

We next chose to investigate if the T cell deficits we observed in TFH and TFR in A2aR gene deficient mice in response to NP-OVA immunization were T cell intrinsic or extrinsic. To do this we employed the use of a cell transfer system, in
which we purified CD4 T cells by magnetic depletion to greater than 96% purity and injected these cells intraperitoneally into mice genetically lacking T cells as they lack genes for the beta and delta chain of TCR. Resulting mice are chimeric in that only the CD4 T cells lack A2aR. We found that upon immunization of these mice splenic CD4 T cells grafted equally between groups and constituted ~5% of splenocytes when assessed by flow cytometry (data not shown). Importantly, we found that many of the observed deficits in T cells in total A2aRKO mice were recapitulated in this chimeric system such as the reduced TFR to TFH ratio and CTLA-4 expression level both acute and chronically (Figure 3.18, 3.19). Lending credence to the hypothesis that A2aR deficiency impairs TFR suppression of the germinal center reaction, we found that GC frequencies were increased on day 12 following immunization with NP-OVA. Curiously there were increased NP-PE binding GC B cells that exhibited increased frequencies of class switch to IgG1. This is in partial alignment with data from A2aR total knockout mice in that greater frequencies of IgG1 are observed, but differs in the fact that NP-PE binding compartment is higher in the transfer system. This could be due to the role of A2aR on other cell types that should be further investigated.

Curiously, CD44 expression was also observed to be slightly upregulated on TFR cells on day 42 following immunization, which may be signs of a compensatory pathway to increase TFR function in A2aR gene deficient mice as it has been shown
that CD44 expression can enhance function of natural T regs (180). Moreover, BCL-6 expression in TFH, TFR, and GC B cells was reduced in A2aRKO mice (Figure 3.19), suggesting A2aR on T cells has multiple pathways in altering the GC microenvironment and may play a role in IL-21 signaling as it has been shown that IL-21 can directly influence GC B cell levels of BCL-6 (181). Future work will have to be done to delineate if this is indeed the case.

To further assess if the phenotypes we were observing in A2aR gene deficiency specifically on T cells was independent of TCR triggering or basal activation state as discussed before, we transferred OTII or OTII/A2aRKO CD4 T cells into TCRβδ knockout mice and subsequently immunized these chimeric mice with NP-OVA. We were shocked to find that on day 8 following immunization there was no detectable differences between GC frequencies or T follicular frequencies. Quite curiously, we found that in this system, nearly 100% of the T follicular response is of TFH and TFR fail to develop to any appreciable quantity (Figure 3.20). This provides some level of indirect evidence that the T cell intrinsic deficits caused by A2aR gene deficiency are more heavily linked to deficits in TFR rather than deficits in TFH directly. Future studies using FoxP3 Cre and A2aRfloxed mice may help elucidate the role of A2aR on TFR directly.

Moving on, since we did detect that there was functional A2aR expression on GC B cells (Figure 3.7), and long ago postulated that A2aR could play a role in PKA
mediated phosphorylation of AID which may contribute to rate of somatic mutation in GC B cells, we chose to assess the role of A2aR deficiency on B cells in a cell transfer system by which we injected wild type or A2aR knockout B cells intravenously into mice genetically lacking mature B cells by way of disruption of the IgM gene (μMT mice).

We found that following immunization of these chimeric mice that the phenotype observed was relatively mild and failed to recapitulate results seen in total knockout mice (figure 3.21). However, within this system we did not observed normal expression of dark zone and light zone markers CXCR4 and CD86 implying that under these conditions normal GC architecture fails to develop. In support of this is the known fact that these recipient mice fail to develop FDC, as the development of these cells is dependent upon lymphotoxin provided by B cells. Curiously, however, we did notice that CD4 T cell frequencies tended to be increased while B cell proportions were equal. This observation taken with the furthering observation that TFH and TFR exhibited lower levels of apoptosis defined by Caspase 3 imply that there is a functional signal potentiated by A2aR on B cells that directly affects the T follicular compartment. Whether this is a signal that directly induces apoptosis, or stimulates activation of these cells that can lead to greater rates of apoptosis remains to be determined. Future studies using better systems such as irradiation chimeras or
transfer into BCR knockin mice with normal splenic architecture may more clearly elucidate the role of A2aR on B cells within the GC.

Since we observed that A2aR is critical for affinity maturation within the GC, likely in a heavily TFR mediated fashion, we chose to assess if we could manipulate the GC compartment and affinity maturation by administration of A2aR agonist CGS21680 during immunization. We chose to assess both the acute effects of A2aR agonist treatment and long term effects of A2aR agonist treatment during immunization by giving the drug 2x daily from days 8-10 or from days 8-35 following NP-OVA/alum immunization (Figure 3.22A). We found that both acute and chronic administration of A2aR agonist resulted in dramatic reductions in GC B cell frequencies (Figure 3.22), in excellent correlation with data observed in A2aR knockout mice. Moreover, we also observed that the T follicular compartment was reduced when A2aR agonist was administered both acutely and chronically (figure 3.23). In further alignment we found that after chronic administration with A2aR agonist, TFR cell frequencies were increased as well as the likelihood of suppressive capacity of these cells as indicated by increased FoxP3 expression level and CTLA-4 expression level while concomitant ICOS costimulatory levels on TFH cells were reduced (figure 3.24).

Quite curiously, however, acute administration of A2aR agonist did not significantly affect TFH/TFR frequencies or ICOS levels (figure 3.24 and data not
shown), but did manage to significantly reduce GC B cell frequencies (figure 3.22). This implies that there is the possibility that acute administration of A2aR agonist facilitates GC suppression through a T cell independent manner, and possibly on GC B cells directly. Future studies will have to be conducted to figure this out.

To confirm that our observations regarding injection of A2aR agonist CGS21680 indeed represent specific effects through A2aR and not non-specific activation of other adenosine receptors, we immunized A2aR gene deficient mice with NP-OVA/Alum and injected them 2x daily with CGS21680 from days 8-35 as we did with wild type mice, and observed that none of the A2aR agonist induced perturbations of the GC reaction we had observed in wild type mice were visible in A2aR gene deficient mice. This exquisite control provides very strong evidence that manipulation of A2aR directly controls these effects.

Lastly, and most importantly, we chose to assess if administration of A2aR agonist CGS21680 over the course of immunization with NP-OVA/Alum could improve the serum antibody response. We found that chronic administration of CGS21680 facilitated the accelerated development of high affinity NIP-5 IgG and may contribute to accelerating affinity maturation (figure 3.26).
Fig 3.1: The germinal center microenvironment is permissive to extracellular adenosine generation.
Fig 3.2: CD73 knockout mice have impaired baseline IgG but normal IgM and IgA.
Fig 3.3: A2aR knockout mice have an impaired affinity maturation. A) concentration of high affinity NIP-5 IgG antibody or B) total NIP-25 IgG antibody in response to NP-OVA/Alum immunization. Normalized to H33L γ1. Representative dilution curves shown beneath each plot. Middle points were averaged from duplicate plates. C) Calculated NIP5/25 ratio from A and B.
Fig 3.4: A2aR KO mice have developmentally normal B cells. A) Splenic B cells stained for IgM and IgD. B) Baseline Igs C) Baseline Lambda and kappa light chain IgG.
Fig 3.5: IL-7 receptor expression appears normal in A2aR KO mice on CD4 T cells on day 8 following NP-OVA/Alum immunization.
Fig 3.6: T Follicular cells express functional A2aR.
Fig 3.7: Germinal center B cells express functional A2aR.
Fig 3.8: The resolution of the germinal center reaction is impaired in A2aR KO mice following NP-OVA/Alum immunization.
Fig 3.9: A2aR knockout mice have a dark zone shifted GC response following immunization with NP-OVA/Alum.
Fig 3.10: Prelimarily: A2aR knockout mice have increased apoptosis and reduced NP Binding GC B cells on day 12 following NP-OVA/Alum immunization.
Fig 3.11: A2aRKO mice have similar rates of apoptosis in total CD4 T cells, B cells and T follicular cells on day 12 following immunization with NP-OVA/Alum.
Fig 3.12: Preliminary: Early and late BCR phosphorylation events are attenuated upon administration of A2aR agonist.
Fig 3.13: A2aR knockout mice have increased T follicular cells following immunization with NP-OVA/Alum.
Fig 3.14: A2aR knockout mice have increased T cells in the germinal center 12 days following immunization with NP-OVA/Alum.
Fig 3.15: A2aR Knockout mice have impaired TFH/TFReg ratio following immunization with NP-OVA/Alum.
Fig 3.16: A2aR KO mice have increased TFH ICOS levels and decreased CTLA-4 on TFR.
Fig 3.17: A2aR knockout mice with transgenic TCR show similar differences in GC and TFH phenotype to conventional A2aR knockout mice.
Fig 3.18: A2aR deficiency on T cells partially recapitulates total knockout phenotype at the peak of the GC reaction following NP-OVA/Alum immunization.
Fig 3.19: A2aR deficiency on T cells partially recapitulates total knockout phenotype on day 42.
Fig 3.20: A2aR deficiency on OTII T cells fails to recapitulate results and development of TFRegs.
Fig 3.21: A2aR deficiency on B cells does not perturb GC frequencies at peak but reduces T follicular cell apoptosis.
Fig 3.22: Acute and chronic A2aR agonist therapy suppresses germinal center frequencies.
Fig 3.23: Administration of A2aR agonist CGS21680 suppresses T Follicular phenotype.
Fig 3.24: Administration of A2aR agonist CGS21680 decreases ICOS expression and increases ratio of TFR/TFH.
Fig 3.25: A2aR agonist CGS21680 acts specifically through A2aR and fails to affect GC reaction after chronic administration to A2aRKO mice.
Fig 3.26: Chronic A2aR agonist therapy can accelerate the development of high affinity antibody following immunization.
3.3 Discussion

Taken together, these data reveal a critical and previously unappreciated role of A2aR in driving affinity maturation within the germinal center. We observed that deletion of A2aR results in significant impairments in the development of high affinity antibodies following immunization with the classic hapten NP-OVA/Alum. Furthermore, we found through our observations that multiple aspects of the GC reaction are perturbed including impaired T cell regulation by defects in TFR and increased apoptosis rates in GC B cells of A2aR deficient mice. Germinal center architecture was also significantly altered facilitating increased frequencies of T cells and increased frequencies of dark zone GC B cells.

Cell transfer experiments partially recapitulated the observed phenotypes suggesting that A2aR deficiency on T cells contributes to many of the observed phenotypes. B cell transfer experiments have yet to provide clarity however on the role of A2aR on germinal center B cells. Most crucially, we were able to demonstrate that administration of A2aR agonist during vaccination can suppress the germinal center response and facilitate the development of high affinity antibody. Perhaps this treatment could be used in the future to help develop novel vaccination strategies to develop effective vaccines for elusive viruses such as HIV.

In summation it appears affinity maturation occurs through suppression of the germinal center reaction mediated through A2aR. One could postulate that affinity
maturation itself is the natural byproduct of the gradual resolution of the germinal center and co-evolved with immune tolerance mechanisms.
Chapter 4: The A2b adenosine receptor potentiates T follicular helper cell formation and the GC reaction.

4.1 Introduction

The A2b adenosine receptor has been shown to have multiple roles in regulating and promoting tissue inflammation in a variety of settings. The role of A2b receptor signaling in regulation of humoral mediated immunity however, has yet to be investigated. In the previous chapter we discussed the critical role of A2aR in resolving the GC reaction and being required for affinity maturation. Although A2b receptors can signal through a shared pathway of A2aR, they are generally considered to be low affinity for stimulating this cAMP response. A2b receptors do also stimulate responses through gq coupled pathway through a yet to be determined affinity, but some evidence suggests it is much higher affinity in this pathway. A2b receptors have also been shown to be quite strongly inducible in hypoxic conditions, lending credence to the possibility they might be upregulated in GCs which we found to be hypoxic.

We hypothesized that A2b receptors act through alternate signaling pathway to promote the development of TFH and potentiate the germinal center response.
4.2 Results

To evaluate the role of A2b adenosine receptors on humoral mediated immunity we employed the use of A2bR gene deficient mice (A2bRKO) which were generated on the C57Bl/6 background. Baseline phenotyping of these mice revealed dramatic defects in serum IgG and IgA while IgM was unaffected (Figure 4.1). We determined by flow cytometry that lymphocyte development is grossly normal and proportions of T and B cells are nearly equal (data not shown). We did note, however that there were particular defects in the frequencies of GC B cells and TFH (Figures 4.2 and 4.3) giving evidence that A2bR plays a positive role in regulating the germinal center response.

Upon further investigation we found that the A2bR is expressed abundantly within the light zone of the GC (Figure 4.4), possibly on FDC as dendritic and myeloid cells are known to be high expressers of A2bR (78). Next, we chose to investigate the role of A2bR during antigen specific challenge by immunization with NP-OVA in Alum and found that total the T follicular compartment on day 8 following immunization was relatively unaffected, but there was a dramatic shift toward a TFR phenotype within this compartment and impaired TFH frequencies (Figure 4.5). Correspondingly, we observed reduced frequencies of NP binding germinal center B cells (Figure 4.5). Curiously, upon analysis of serum IgG for high affinity and total NIP binding IgG, we found that there was a slight but statistically
significant acceleration of affinity maturation (Figure 4.5). This raises the possibility that A2bR may play a role in survival or retention of GC B cells within the germinal center. This will have to be investigated more intensely in the future.

From our original findings we postulated that A2bR may be acting through multiple cell types to promote the germinal center reaction. One potential mechanism of TFH induction could be that A2bR stimulation on APCs facilitates the release of IL-6, which is known to promote TFH differentiation. We chose to assess if we could facilitate enhanced TFH formation by recruiting A2bR signaling in the developmental phase of the GC response. We achieved this by giving the A2bR selective agonist Bay60-6583 1x daily from days 0-8 during NP-OVA/Alum immunization and found that this increased the T follicular compartment substantially (Figure 4.6). Further studies, however, are needed to define if this is an IL-6 mediated mechanism and to which cell types A2bR signaling plays a dominant role in facilitating the generation of T follicular cells.

The utility of nanoparticle drug carriers has become yet another major advance in vaccine design. We chose to investigate the utility of employing such a system for delivery of adenosine modulating drugs, such as Bay60-6583. This delivery method provides several distinct advantages in the context of delivery of this A2bR agonist. First, it ameliorates the need for harsh methods to dissolve very hydrophobic drugs like Bay60-6583 (Figure 4.7), and secondly it can help target the drug to the lymphatic
system and avoid known cardiac side effects of A2bR signaling. We found that a PLGA based nanoparticle system is feasible to carry BAY60-6583 to lymphoid tissues in a vaccine formulation (Figures 4.7, 4.8).

Clearly future studies are needed to fully elucidate the role of A2bR signaling throughout the time course of the GC response as well as development of nanoparticle carriers for delivery of these hydrophobic drugs.
Fig 4.1 A2b knockout mice have normal IgM and reduced serum IgG and IgA at baseline.
Fig 4.2 A2b knockout mice have reduced GC frequencies at baseline (spleen shown).
Fig 4.3 A2b knockout mice have impaired T Follicular frequencies at baseline (spleen shown).
Fig 4.4 A2b receptor is expressed strongly in the light zone of the germinal center.
Fig 4.5 Immunization of A2bR knockout mice results in impaired TFH frequencies, increased TFR frequencies, reduced GCs and accelerated affinity kinetics.
Fig 4.6 Stimulation of the A2bR with agonist Bay60-6583 facilitates the development of the T follicular compartment.
Fig 4.7 A2b agonist is a good candidate for nanoparticle delivery.
Fig 4.8 PLGA based nanoparticle system is efficient at tracking and persisting in lymphoid tissues.
4.3 Discussion

The A2b receptor offers an exciting new development in the role of adenosine signaling in the GC reaction. This finding was somewhat surprising, in that A2b receptor signaling is often discussed solely as a low affinity adenosine receptor for inducing cyclic AMP and in several contexts has been shown to limit inflammation (93). This postulating ends up in several conundrums as to what the true role of the A2b receptor actually is. It truly is an oversimplification to claim that A2b receptors only serve as low affinity cyclic AMP inducing adenosine receptor. This confusingly implies that the non-redundant A2a adenosine receptor, is actually possibly redundant in yet to be found conditions of very high extracellular adenosine.

Clearly, in the germinal center response A2b adenosine receptors have unique signaling profile that distinguishes it from A2a receptor signaling. In nearly every instance observed with respect to the germinal center reaction, A2b receptor deficient mice exhibit opposite effects of A2aR. Mice doubly deficient in A2a and A2b help to prove the counteracting roles of the two receptors as they exhibit nearly normal phenotypes within the GC. This discrepancy between A2a and A2b signaling within the GC begs the obvious question, if they act in opposite manners then what is the role of endogenously generated adenosine within the GC? Hopefully detailed studies as to the expressional distribution and timing of expression of these receptors during the vaccine response may help to answer this question in the years to come.
5 Conclusions and Future Directions

Here we have identified that the germinal center, the site in which B cells undergo CSR and SHM to generate potent antibodies to neutralize pathogens, creates a hypoxic microenvironment. This hypoxic microenvironment promotes secondary diversification of B cells through potentiating CSR. Future studies of how hypoxia affects the molecular mechanisms of SHM and development of disease states such as systemic lupus erythematosus would be of particular interest.

Moreover we found that this hypoxic microenvironment also develops complex signaling pathways through A2a and A2b adenosine receptors. While we found the A2a adenosine receptor to drive affinity maturation within the GC, we found the A2b receptor appears to do the opposite, while promoting increases in T follicular helper cells.

More simplistically, it appears that A2aR appears to act in resolving inflammation in the GC, leading to development of high affinity antibodies. Conversely, deletion of the A2b receptor appears to significantly impair the formation of the GC and results in accelerated disappearance of GCs, resulting in what appears to be accelerated affinity maturation. Future studies are clearly needed to fully elucidate the timing depend induction and signaling through these receptors. It would be interesting to identify how these two receptor mediate clonal evolution within the GC, and experiments are currently underway to address this question.
One can postulate however, that modulating signaling over the course of immunization with altered cycles of A2a and A2b signaling may lead to not only the acceleration of affinity maturation of an intended vaccine, but possibly break beyond known affinity limits for vaccination for a particular antigen.
Materials and Methods

Mice

10-12 week old Female C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor ME) and were acclimated to cages and environment at Northeastern University for at least one week prior to any experiments. A2aR knockout mice were originally generated as described (81). A2bR knockout mice were generated as described (182).

Immunization Protocol

Mice were immunized intraperitoneally with 200ul (in PBS) of co-precipitated Alum hydroxide / NP(6)-OVA that was injected at a dose of Alum per mouse of 5mg/mouse and NP-OVA of 1ug/mouse. Briefly, 10% Alum sulfate (Sigma) solution was mixed at a 1:1 ratio with PBS (Gibco) with NP(8)-OVA or (NP11-CGG) (Biosearch technologies / produced in-house) in a 50ml conical. Solution was then precipitated in a drop-wise manner using 1M KOH to a pH of 6.5, as tested by pH test strip paper. Solution was then washed 3x at 2500rpm 10 minutes 4 degrees with 30ml of sterile PBS. Final solution was resuspended for an injection volume of 200ul.

60% oxygen therapy
To treat with 60% O\(_2\) Mice were immunized and then placed in custom made hyperoxic chambers where an oxygen concentrator (AirSep) pumped room air into the chamber to a maintained level of 60% oxygen as monitored by oxygen sensors. Mice were housed in wire top cages and CO\(_2\) buildup was prevented by the addition of sodasorb to the chamber.

Low Oxygen Incubator

For cell culture experiments Napco 7000 series incubators were used to control oxygen concentration. Oxygen and CO\(_2\) levels were calibrated using Fyrite device. Injected Nitrogen was used to achieve 1% oxygen concentration. In all hypoxic in vitro experiments, unless stated otherwise, media was de-gassed of oxygen for at least 8 hours in hypoxic incubator (5ml of media per well of 6 well plate). Control media was “gassed” in 21% oxygen incubator for equal time. 5% CO\(_2\) was maintained for pH and humidity was monitored and normalized between hypoxic and normoxic incubators.

Flow cytometry

Single cell suspensions of splenocytes were prepared by manually dissecting the spleen and dissociating lymphocytes by gentle mashing with the blunt end of a 5ml syringe and then suspension was passed through a 70 micron nylon mesh filter. Red blood cells were lysed with ACK lysis (Gibco) buffer for 2 minutes and reaction was stopped with FACS Buffer. Cells were enumerated with a hemocytometer and 1x10\(^8\) cells were stained for various panels in 1.2 ml cluster tubes (Costar) in a final volume.
of 100µl. Cells were first FC blocked for 10 minutes at 4 degrees, and primary and secondary stains were each 20-30 minutes at 4 degrees. Cells were washed twice with 1ml of FACS buffer and fixed in 200 µl of fixation buffer in the eBioscience FoxP3 kit. For hypoxyprobe staining, hypoxyprobe antibody (4.1.1.3) was diluted 1:50 in 1x permeabilization buffer of FoxP3 kit (eBioscience) and added to cells for 1 hour at 4 degrees. Cells in fixation buffer were washed twice before and twice after hypoxyprobe staining. FACS Buffer consisted of 1xPBS with 5% FCS. The full list of antibodies and conjugations are listed in supplement. Cells were acquired on a cytek DxP8 FACSCalibur upgrade equipped with digital electronics (18bit) and 3 lasers (405,488,640). Singlets were excluded by width parameter on scatter. At least one negative gate was included in all panels to avoid non-specific binding. All analysis was completed on Flowjo (treestar).

**In Vitro Class Switch Assay**

Briefly, splenocytes from a CB57/B6 mouse (Jackson Labs) were harvested and separated by Ficoll separation (GE Healthcare) and then prepared in gassed or de-gassed media. Resting B cells were purified by CD43 depletion. Purified lymphocytes were stained for 20 minutes at 4°C with 5µl αCD43 FITC mAB (clone S7, BD Biosciences) in a volume of 250µl in a 15ml Falcon tube. Cells were washed with 10mL of sterile FACS Buffer and stained with 25µl αFITC Microbeads (Miltenyi Biotec) in a volume of 250µl for 20 minutes at 4°C. Cells were then washed again.
and depleted through an AutoMACS (program depleteS250). Purity was checked by
flow cytometry before every experiment and was greater than 99%. Media consisted
of IMDM supplemented with 25mM HEPES, 10% FCS, Glutamine, 55mM-2-ME,
and 100U/ml Penicillin/Streptomycin. Media was prepared fresh weekly for all in
vitro experiments. Cells were stimulated with αCD40 monoclonal antibody (clone
1C10) at a concentration of 2.5µg/ml (eBioscience and Biolegend) and recombinant
mouse IL-4 at 10ng/ml (R&D Systems). Cells were cultured in a 24 well plate (Costar
#3474) in a volume of 1mL at a seeding density of 0.1x 10⁶ cells per well.

**CD4 T cell Stimulation In Vitro**

Briefly, splenocytes from a C57BL/6J mouse (Jackson Labs) were harvested
and separated by Ficoll separation (GE Healthcare) and then prepared in gassed or
de-gassed media. GL-7 and CD8 expressing cells were depleted. Purified lymphocytes
were stained for 20 minutes at 4°C with 5µl αGL-7 FITC (clone GL7, BD
Biosciences) and 5µl of αCD8 FITC (BD Biosciences, Clone 53-6.7) in a volume of
250µl in a 15ml Falcon tube. Cells were washed with 10mL of sterile FACS Buffer
and stained with 25µl αFITC Microbeads (Miltenyi Biotec) in a volume of 250µl for
20 minutes at 4°C. Cells were then washed again and depleted through an
AutoMACS (program depleteS250). Purity was checked by flow cytometry before
every experiment and was greater than 99%. Media consisted of IMDM
supplemented with 25mM HEPES, 10% FCS, Glutamine, 55mM-2-ME, and
100U/ml Penicillin/Streptomycin. Media was prepared fresh weekly for all in vitro experiments. Cells were stimulated with αCD3 monoclonal antibody (clone 2c11, BD Biosciences) at a concentration of 1µg/ml (BD Biosciences). Cells were cultured in a 24 well plate (Costar #3474) in a volume of 1mL at a seeding density of .5x 10^6 cells per well.

**B cell Stimulation In Vitro**

Briefly, splenocytes from a CB57/b6 mouse (Jackson Labs) were harvested and separated by Ficoll separation (GE Healthcare) and then prepared in gassed or degassed media. GL-7 and CD43 expressing cells were depleted. Purified lymphocytes were stained for 20 minutes at 4°C with 5µl αGL-7 FITC (clone GL7, BD Biosciences) and 5µl of αCD43 FITC (BD Biosciences, Clone S7) in a volume of 250µl in a 15ml Falcon tube. Cells were washed with 10mL of sterile FACS Buffer and stained with 25µl αFITC Microbeads (Miltenyi Biotec) in a volume of 250µl for 20 minutes at 4°C. Cells were then washed again and depleted through an AutoMACS (program depleteS250). Purity was checked by flow cytometry before every experiment and was greater than 99%. Media consisted of IMDM supplemented with 25mM HEPES, 10% FCS, Glutamine, 55mM-2-ME, and 100U/ml Penicillin/Streptomycin. Media was prepared fresh weekly for all in vitro experiments. Cells were stimulated with αCD40 monoclonal antibody (clone 1C10, Biolegend) at a concentration of 2.5µg/ml and αIgM Fab₂ at a concentration of
6µg/ml (Jackson Immunoresearch). Cells were cultured in a 24 well plate (Costar #3474) in a volume of 1mL at a seeding density of .5x 10^6 cells per well.

*Fluorescent labeling with Tomato Lectin*

Immunized mice were injected 10-30 minutes before sacrificing with Dylight 594 conjugated Tomato Lectin (Vector Labs) to label endothelium for visualization of germinal center and B cell follicle vasculature. Each mouse was injected with 200ul total volume consisting of 150ug of tomato lectin diluted with sterile saline. Once mice were sacrificed, they were perfused with 30ml of 1% paraformaldehyde through the left ventricle and then perfused with 30ml of PBS. Spleens were harvested and frozen for tissue sectioning and microscopy.

*Histological staining and Microscopy*

Frozen slides were prepared by cryo sectioning at a thickness of 5µm. Slides were then air dried and fixed in 1:1 mixture of Acetone and Methanol at -20 degrees for 10 minutes. Slides were then dried and stored at -20 ºC until staining. For staining procedure, slides were warmed to room temperature and then Pap Pen was applied (vector) to slides and dried. Slides were then re-hydrated in staining buffer (0.1% Tween 20, 0.5% BSA in 1xPBS) for 20 minutes. Slides were then blocked with FC Block for 20 minutes. Slides were dabbed dry and Stained with primary antibodies for B220, Hypoxyprobe, and GL-7 for 3 hours in humidified chamber in the dark. Slides were then washed 3x and stained with secondary anti-FITC, Oregon Green.
(Invitrogen) for 1 hour. Slides were then washed 3x again and mounted with Fluormount G for further analysis. Images were acquired either on a Nikon epifluorescence microscope at 20x power, or a Zeiss LSM 710 equipped with 8 laser lines and full spectrum PMT.

**Histology, flow cytometry, microscopy, lectin staining, hypoxyprobe staining, Serum ELISA**

For tissue histology, in general tissues were frozen in OCT compound and cut at 5 micrometer thickness and fixed in acetone/methanol. Fluorescence imaging was conducted on Zeiss LSM 710 or Nikon e80i. For flow cytometric analysis, single cell preparations were stained with antibodies in PBS with 5% FCS and analyzed on a DxP 8 FACSCalibur (Cytek). Flow cytometry analysis was conducted in Flowjo X (treestar). Hypoxyprobe was injected intravenously at 100mg/kg in HBSS and circulated for 60 minutes. Tomato lectin was injected intravenously at 6mg/kg. Analysis of histological samples was completed in Image J. For detection of antigen specific IgG, plates were coated with 2µg/ml NIP 25- BSA. Middle points of dilution curves were taken and values were calculated relative to monoclonal standards (B-18 and H33lγ1). For more detail see supplement.

*In Vitro low oxygen assays*
Resting B cells were purified by Magnetic depletion with antibodies against GL-7 and CD43. B cells were then stimulated with anti-CD40 (2.5µg/ml). For the class switching assay, resting B cells were purified by CD43 and then stimulated with anti-CD40 (2.5µg/ml) and IL-4 (10ng/ml). For T cell stimulation, CD8 T cells were depleted by magnetic separation and then CD4 were stimulated with anti-CD3 antibody (1µg/ml). All in vitro experiments were done in a volume of 1ml in complete IMDM media. Napco 7000 incubators were used to control oxygen level which was calibrated. Media was pre-gassed in normoxic or hypoxic incubators for at least 6 hours prior to experiments.

**RT-PCR for IgG1 circle transcripts**

Cells were isolated for IgG1 circle transcripts and re-suspended in RLT buffer and frozen at -80°C until RNA extraction. RNA extraction was carried out using the RNeasy mini kit (Qiagen cat no 74106) according to the manufacturer’s protocol. cDNA was made using Superscript III first strand synthesis (Invitrogen 11752-050) according to the manufacturer protocol. For RT-PCR there was an initial denaturing step of 95°C for 9 minutes followed by 40 cycles of PCR(94°C 30s, 58°C 1min) by using RT2 SYBR green PCR master mix (Life technologies cat no 4309155). All RT PCR was done on an Applied Biosciences 7300 PCR machine. Primers were ordered from Eurofins and were specific for IgM reverse (CμR, 5′-AAT GGT GCT GGG CAG GAA GT-3′) and IgG1 forward (Iγ1F, 5′-GGC CCT TCC AGA TCT TTG AG-3′) as
described in reference 15. PCR was: initial denaturation cycles at The house-keeping gene ribosomal protein L32 (Qiagen cat no PPM03300B-200) was used as internal control and to normalize expression.

**ELISPOTS**

Immobilon-P membrane high protein binding plates (Millipore cat no S2EM004M99) were coated overnight with NIP-25 BSA at 2µg/ml in carbonate buffer (pH 9.5). Plates were washed 3x with wash buffer containing 0.5% BSA and 0.1% Tween 20 in PBS. Plates were blocked in same buffer for one hour and washed 3x. RPMI media was used to wash once and cells at appropriate concentrations were serially diluted and added to the plate. The plate was incubated for 3 hours in humidified incubator containing 5% CO2. Plate was washed with DI and left in wash buffer overnight. The following day appropriate alkaline phosphatase conjugated antibodies to IgG1 or IgM (Southern Biotech) were added. Plates were developed using Sigma fast BCIP reagent (Sigma Aldrich). For in vitro detection of ELISPOTS detection was carried out at 21% oxygen tension for the 3 hour time period on day 4.

**Image J analysis**

ImageJ FIJI was used to quantify the germinal center and follicular area that laid within 40 microns of perfused vessels in the spleen. Binary mask files were created for germinal centers (GL7+), follicles (B220+), and vasculature (tomato lectin+). To estimate the extent of tissue oxygenation, the vasculature mask was
expanded radially by 40 microns. The percent area of germinal center and follicle that is covered by the expanded vasculature mask was then calculated.
References


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