

Investigations in Immunology: TACI Localization in B Cells

Author: Keri Sanborn

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Investigations in Immunology:
TACI Localization in B Cells

Keri B. Sanborn

Dr. Thomas Chiles, Advisor

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Acknowledgements

I would like to thank my family and friends for supporting me throughout all of my college experience, and in particular during the extremely busy process of writing my thesis. Without their love and support, I would probably have spent many hours staring at a blank computer screen on more than one occasion during this year. Their support has been an inspiration and has shown me that I can achieve anything I set my heart on.

I would also like to thank Dr. Tom Chiles for his help and advice during my thesis-writing process. His guidance has taught me a lot about science and taught me to be self-reliant in my knowledge and my work.

Abstract

For ten weeks during the summer of 2005, I was a Summer Undergraduate Research Fellow in an immunology laboratory at the Mayo Clinic. My research focused on the BLyS/APRIL system and the receptor TACI on the surface of B cells. Going into my summer research, I had very little experience in immunology. Throughout the process of writing this thesis, I have sought to improve upon my knowledge of immunology, building a cohesive story that begins with basic biology and ends with the results of the summer's experiments.

The first part of this thesis covers topics in general immunology, and narrow down in focus to cover the function and development of lymphocytes and B cell maturation and activation. In the second part of the thesis, the background for my research is described in more detail, and topics such as autoimmunity and cancer, lipid rafts, cell polarization, the BLyS/APRIL system for B cell survival, and TACI are covered extensively. The final portion of this thesis discusses the experimental logic, a background on materials and methods, and the results of the experiments I conducted over the summer. By reading this thesis, anyone with a background in biology should become familiar with basic subjects in immunology, advanced concepts in the study of lymphocytes, the ligands BLyS and APRIL, and the receptor TACI in B cells.

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Preface: Notation

It is important to note that midway through this thesis, there is a shift from using textbook-based sources to using scientific papers. Because of this shift, the notation used for citing sources will also change.

In Part I, as well as the Preface to Part II, textbooks are the primary sources cited. In these sections, citation more typical of a humanities paper will be utilized, including footnotes and a Works Cited section at the end of the paper. This method of citation enables the reader to find the pages cited in each particular section of the paper.

In Part II and Part III, papers from scientific journals are the primary sources cited. In these sections, citation commonly used in scientific papers will be used. This includes a parenthetical reference for each citation, as well as a References section in the back of the paper.

This method of citation is intended to reflect the nature of sources used and the nature of scientific knowledge which each section is based upon.

Introduction: Purpose and Goals

In the summer of 2005, I had the privilege of being a student in the Summer Undergraduate Research Fellowship program at the Mayo Clinic in Rochester, Minnesota. For ten weeks, I worked full-time in the lab of Dr. Diane Jelinek, an immunologist who primarily concentrates on B cells. To be entirely honest, I wasn't sure I would enjoy myself that summer. I had applied to the Molecular Genetics



The Jelinek lab, my home and family for the summer.

program at Mayo, and instead, with no background or previous interest in immunology, I was asked to join the Immunology department instead. In my mind as I began the project, I remembered that it would be only ten weeks of my life, and that even if I didn't enjoy it, it would be a valuable experience. I began the summer unsure of my interest in immunology and even of my decision to pursue a career in research. What followed was possibly one of the best summers of my life. I left Mayo in August with a newfound passion for immunology and a renewed commitment to graduate school and the field of scientific research.

As I began my summer research project, I had no background in immunology, beyond the typical Intro Bio and Molecular Cell Biology courses in college. I didn't really know the difference between B cells and T cells, or the function of an antibody. I certainly didn't know anything about the B_{LYS}/APRIL system with which I would become very familiar over the next ten weeks. In research there is a very steep learning curve, and I learned the basics of the system I was working on and the techniques

necessary for my research very quickly. What I didn't have time to learn, due to the hectic nature of the summer, was the background behind my research. Knowing that detailed, intricate knowledge is nothing without a foundation to build it upon, I set out this year to build that foundation by beginning with the basics. While my summer research project is the inspiration for my thesis, in fact it is the end point of this work.

The purpose of this thesis is to begin at the beginning. It is to start with a broad



Celebrating at the end of the summer. You should feel this good when you finish reading this thesis.

understanding of immunology and work down to the details surrounding my summer research. As I began with very little knowledge of immunology, this thesis should provide all of the necessary background to bridge the gap from basic biology to the intricate details of my project, making it possible for anyone

with a basic background in biology to understand the material in the thesis. My goal is to provide anyone who reads this paper from cover to cover with the knowledge necessary to understand all of the intricacies of the B_{LYS}/APRIL system and TACI in B cells, as well as the concepts of receptor localization and lipid rafts and how these concepts relate to my research.

To anyone who picks up this thesis, I hope you enjoy learning about the world of B cells as much as I have enjoyed researching it. Through this window into a small piece of B cell research, perhaps you will come to appreciate the role of TACI in the fascinatingly complex system of B cell survival and regulation.

Part I: Basic B Cell Biology

Section I: A Brief Overview of the Human Body's Defenses

Throughout our lives, we come into contact with many disease-causing viruses and bacteria, as well as other environmental pathogens. When our bodies are faced with these invaders, they employ both nonspecific and specific types of defense.

The body's nonspecific line of defense is both external and internal. In order to enter the body, a pathogen must first penetrate the physical and chemical barriers of the skin or the mucous membrane linings of the respiratory, digestive, and urogenital tracts. After these barriers have been surmounted, the pathogen will face many nonspecific internal defenses, including various types of phagocytic cells such as macrophages, eosinophils, and neutrophils; natural killer (NK) cells; and the many chemicals that create an inflammatory response. These defenses do not distinguish among pathogens, but they are often very effective at limiting the scope of an infection or preventing infection altogether. They can also be useful in activating the body's specific defenses.

The body's more specific type of defense is primarily based on lymphocytes, a subset of white blood cells which are designed to protect the body against foreign invaders. Lymphocytes, which include several types of B and T cells, are responsible for secreting antibodies against specific pathogens and for recognizing and killing infected cells. They are also responsible for protecting the body against cancer by killing cells which have become cancerous. The responses of lymphocytes to a pathogen are specific to the pathogen, yet lymphocytes are able to provide an enormous amount of diversity because each cell recognizes a different antigen. Through a system that combines specificity with diversity, lymphocytes make it possible for the body to combat virtually any pathogen it encounters.

While many connections between the body's two types of defense will be made, this thesis will primarily focus on lymphocytes and the body's more specific defenses against pathogens, particularly B lymphocytes and their role in the immune system.¹

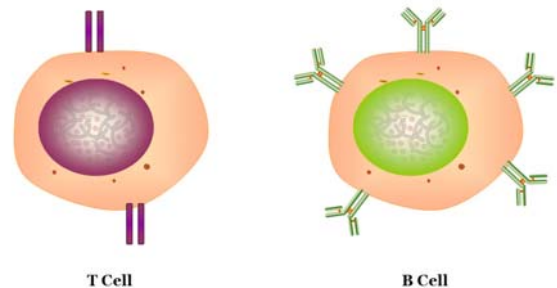
¹ Parts of section are summarized from Campbell & Reece, *Biology*.

Section II: The Cells of the Immune System

The body's specific and non-specific defenses are both provided by leukocytes, or white blood cells. Along with red blood cells, leukocytes arise from hematopoietic stem cells in the bone marrow, which mature to form more limited stem cells of many types.

Hematopoietic stem cells can form the myeloid progenitor, which is the precursor cell type for most of the cells that make up the innate (non-specific) immune system. The myeloid progenitor gives rise to macrophages, which are phagocytic cells that circulate through tissues to engulf invaders; specialized antigen presenting cells called dendritic cells; mast cells, which have a key role in allergic responses and may also protect the body's mucosal surfaces; and granulocytes, including neutrophils, eosinophils, and basophils, which assist in fighting bacterial and parasitic infections as well as stimulating an allergic response.

The lymphoid progenitor, which also arises from hematopoietic stem cells, is the precursor cell type for lymphocytes, the cells which make up the adaptive (specific) immune system, as well as natural killer cells and some dendritic cells. Two types of lymphocytes exist in the body: T cells and B cells. T cells, which leave the bone marrow to complete maturation in the thymus, differentiate into both cytotoxic cells, which kill infected cells, and helper cells, which activate other lymphocytes and macrophages. B cells, which complete their maturation in the bone marrow, differentiate into plasma cells, which secrete antibodies that fight



A T cell and B cell. Note the difference between the TCR and BCR. Figure created using VisiScience ScienceSlides graphics.

foreign molecules in the body, and memory cells, which boost the immune response upon a second exposure to an antigen.

Lymphocytes must encounter an antigen and, in some cases, co-stimulatory molecules in order to become activated and differentiate. Each lymphocyte has specificity for a unique antigen, created through the modular design of the genes for the T cell receptor (TCR) and B cell receptor (BCR). While the T cell receptor is always membrane-bound, the B cell receptor is a membrane-bound form of the antibody molecule, or immunoglobulin (Ig), that is secreted by activated plasma B cells.

Along with lymphocytes, the lymphoid progenitor also produces natural killer (NK) cells. NK cells are active in the innate immune system and play a key role in recognizing abnormal cells in the body such as tumor cells. Some dendritic cells are also produced by the lymphoid progenitor, but these dendritic cells appear identical to those produced by the myeloid progenitor, and the majority of the body's dendritic cells are of myeloid origin.

The cells of the immune system work together, combining innate and adaptive immunity in many different ways to effectively fight off pathogens and guard against cell abnormalities and cancer.²

² Parts of section summarized from Sompayrac, *How the Immune System Works*.

Section III: Adaptive and Innate Immunity

The first line of defense when the body is invaded by a pathogen is its innate immunity. Innate immunity is relatively nonspecific, designed to target common elements of pathogens, such as cell surface molecules. Because the adaptive immune response can take four to seven days to build, the body's innate response is critical in fighting pathogens immediately after they have been introduced into the body, and it can even prevent the need for an adaptive response altogether.³

Immediately upon encountering a pathogen, the cells that provide the body's innate immunity recognize the pathogen as non-self through structures, such as unmethylated CpG DNA, that are common to many different antigens.⁴ The first cells to encounter a pathogen are typically the macrophages, which circulate in the blood and tissues and engulf foreign molecules through phagocytosis. When a macrophage comes into contact with a surface molecule on a pathogen, it engulfs the invader and begins to secrete cytokines and chemokines, which begin the inflammation process. When the infection site becomes inflamed, the blood vessels near the area dilate to allow more cells to come into contact with the pathogens, causing redness, pain, heat, and swelling. Additionally, the endothelium of the blood vessels becomes more adhesive, causing more leukocytes to stick to the endothelium and migrate to the site of infection. Inflammation increases the flow of the lymph, which contains antigen presenting cells that can become activated by encountering an antigen and go on to activate lymphocytes.

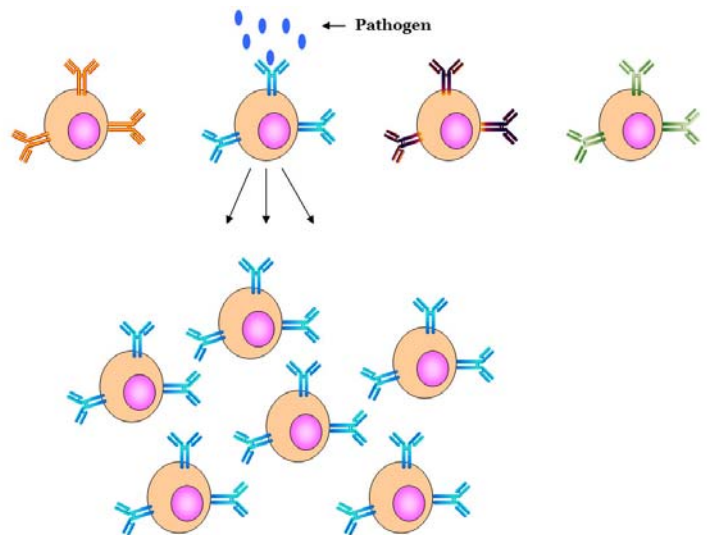
³ Janeway, Travers, Walport, & Shlomchik, *Immunobiology*, 12.

⁴ Janeway, 15.

Upon secretion of cytokines by macrophages and an increase in the flow of lymph, dendritic cells that have been recruited to the site of infection come into contact with a pathogen. In a similar manner to macrophages, the dendritic cells ingest an antigen through phagocytosis, causing them to become activated and mature into antigen presenting cells (APCs). They then migrate to the lymph nodes, where they present the antigen in order to activate T cells.

Following the activation of T cells by dendritic cells, the adaptive immune response begins to play an active role in fighting the pathogens that are invading the body.

Because of the genetic mechanism that creates the T cell receptor and B cell receptor, each lymphocyte recognizes a different antigen. B cells are able to recognize free-floating, whole antigen molecules, while T cells recognize only smaller, fragmented peptide antigens presented on MHC I or MHC II



Clonal expansion.

Image created using VisiScience ScienceSlides program.

molecules. When a lymphocyte recognizes its specific antigen, it becomes activated, stops migrating, and enlarges. The activated cell, called a lymphoblast, divides approximately four times every 24 hours for 3-5 days. Through this process, called clonal expansion, the lymphoblast produces many cells with the same antigenic specificity. These cells differentiate into their respective effector cells – cytotoxic

lymphocytes in the case of T cells, or plasma cells in the case of B cells – and their cell-adhesion molecules change, allowing them to migrate into tissues to fight infections.

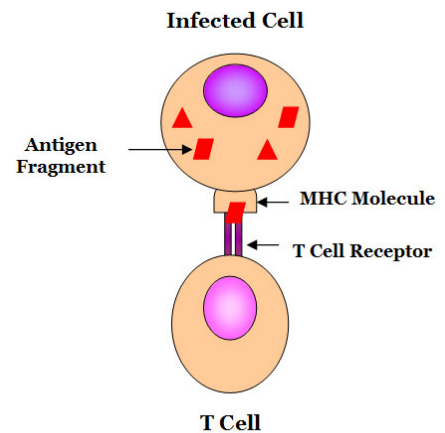
When T cells and B cells enter infected tissues, they help the body fight against pathogens in unique ways. While cytotoxic lymphocytes, or “killer” T cells, kill infected cells by inducing apoptosis, B cells protect the body from free antigen molecules by secreting antibodies. These antibodies mark (opsonize) antigens for ingestion by macrophages and prevent bacteria and viruses from entering new cells by blocking their receptor-binding sites (neutralization). In addition, antibodies also activate the complement system, which is helpful to the body during bacterial infections. Through the combination of cytotoxic T cells and antibody-secreting B cells, the body is typically able to combat pathogens and prevent further infection.

After an antigen has been eliminated, the effector cells which were generated to help fight the infection undergo apoptosis. A subset of the antigen-specific B cells and T cells remain, however, and create the body’s immunological memory. These cells, known as memory cells, persist in larger numbers than naïve lymphocytes and are easier to activate, helping the body to quickly fight off a secondary infection with the same antigen. In addition, the secondary antibody response reaches a much higher level than the primary response and produces antibodies with a higher affinity to the antigen.

Section IV: Lymphocytes

The key players in the adaptive immune response are T cells and B cells, collectively known as lymphocytes. T cells, the lymphocytes that mature in the thymus, are activated through a system known as antigen presentation, the process of one cell activating another cell by presenting a protein fragment to it.

Antigen presentation is done via two different molecules, major histocompatibility complex (MHC) class I molecules and MHC class II molecules. MHC class I molecules are used by all of the body's cells to display proteins from inside the cell, which have traveled through the proteasome to be cleaved into fragments small enough to fit into the MHC I molecule. This type of antigen presentation is used to show the cytotoxic lymphocytes the internal environment of the cell in order to signal the immune system to kill infected cells. In contrast, MHC II



Antigen Presentation

molecules are used by the cells of the immune system to display proteins brought in from the extracellular environment. Presentation by MHC II molecules tells the immune system, in particular helper T cells, that something is wrong in another part of the body, causing the activation and proliferation of naive T cells. Specialized antigen presenting cells, particularly dendritic cells, are instrumental in presentation using MHC II molecules. Overall, antigen presentation is an important mechanism to ensure that cytotoxic lymphocytes are well regulated, as T cells are only able to recognize an antigen if it is properly presented in an MHC molecule.

All T cells have a T cell receptor (TCR), composed of two different proteins, that recognizes a specific antigen. While approximately 5% of the body's T cells contain a $\gamma\delta$ receptor and do not express either CD4 or CD8 molecules, the vast majority of T cells contains an $\alpha\beta$ TCR and express CD4 or CD8 on their cell surface.⁵ These cells recognize a small peptide antigen presented by an MHC molecule on the surface of an infected somatic cell or activated immune cell. Within the two classes of $\alpha\beta$ T cells, helper T cells express the CD4 molecule and recognize antigen presented on an MHC II molecule, and cytotoxic T cells express the CD8 molecule and recognize antigen presented on an MHC I molecule. The CD4 and CD8 molecules act as co-receptors and signaling molecules, assisting in antigen recognition and telling the cell which actions to take in response to activation.

Following antigen recognition, the cell's T cell receptors cluster together, or cross-link, to allow the cell to reach the signaling threshold necessary to activate the cell. This cross-linking often occurs through signaling platforms in the cell surface called lipid rafts, which bring together TCRs and other associated signaling molecules in order to generate and amplify a signal. A T cell activated through its cross-linked TCRs will proliferate and upregulate growth factor receptors on its surface. This activation also requires additional co-stimulatory molecules, such as B7 on an antigen-presenting cell.

CD4⁺ cells, or helper T cells, help to activate B cells and other T cells by secreting cytokines. A naïve helper T cell (T_{H0}) usually secretes only IL-2, but upon activation, it will generally differentiate to secrete one of two subsets of cytokines, depending on the signals it receives from dendritic cells. Although the rules of cytokine secretion do not appear to be strict, a T_{H1} cell usually secretes cytokines such as IL-1, IFN- γ , and tumor

⁵ Sompayrac, *How the Immune System Works*, 56.

necrosis factor (TNF), while a T_H2 cell secretes IL-4, IL-5, and IL-10.⁶ These signals help to activate various cells of the immune system during the fight against a pathogen.

CD8⁺ cells, or cytotoxic lymphocytes (CTLs), fight pathogens by killing infected cells to stop the spread of the infection. The activation of CTLs requires antigen presentation on an MHC I molecule, as well as signals from a helper T cell. Upon activation, a CTL proliferates, then enters the bloodstream to search for cells infected by its cognate antigen, which it will recognize via MHC I. Using weapons such as perforin, which creates holes in the cell membrane, or FasL, which binds to a receptor on the cell surface, the T cell induces programmed cell death (apoptosis) in infected cells. While most T cells die within a few days of activation (a phenomenon termed “activation induced cell death”), some are kept alive as memory cells and are easily re-activated upon a second exposure to the pathogen.

In contrast to the action-hero profile of T cells, B lymphocytes have a somewhat less glamorous existence. While this means that B cells are not as frequently studied in recent studies of the immune system, they are in fact crucial in the fight against some pathogens. B cells, like T cells, originate in the bone marrow, but unlike T cells, they do not migrate to another location to complete their maturation. The B cell contains a B cell receptor (BCR) which, much like the TCR, arises from the rearrangement of gene segments and is composed of two different proteins. In the case of the BCR, the proteins are the heavy chain (Hc) and light chain (Lc), which come together in a dimer of dimers. The BCR is identical to the antibody which is secreted by the B cell, with the exception that the BCR contains a short segment of amino acids which anchors it to the cell membrane. Through the Hc-associated proteins $Ig\alpha$ and $Ig\beta$, the BCR signals to the

⁶ Sompayrac, 60-61.

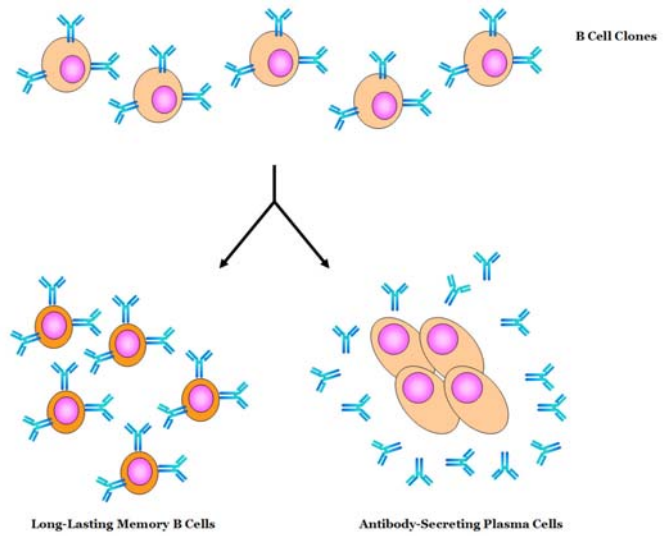
cell when it encounters its cognate antigen. In a similar manner to the TCR, BCRs on the surface of B cells cross-link to generate a signal sufficient to begin an enzymatic cascade within the cell. B cells also contain complement receptors, which cross-link with the BCR to amplify the signal.

B cell activation is comprised of two components. First, the BCR must recognize its cognate antigen. Unlike T cells, which can only recognize properly presented peptide antigens, B cells are able to recognize whole antigen molecules by binding to epitopes on their surfaces. While B cells do not require the presentation of antigen on MHC molecules, they do require a secondary signal, which can be obtained through numerous pathways. The most common method of secondary activation is T cell dependent activation, which requires the interaction of a CD40L molecule on the surface of a T helper cell with the CD40 receptor on B cells. Some cells may be activated without the help of a T cell, however, and can undergo T cell independent activation or polyclonal activation. The mechanism of T cell independent response helps the body recognize unique molecules on the surface of invaders that would not typically be recognized by T cells. Once a B cell is activated through one of these methods, the cell begins to proliferate, but it will not secrete antibodies until it receives a cytokine signal.

Upon activation, a B cell chooses during maturation to become either a plasma cell or a memory cell. Plasma cells, also called antibody-secreting cells, are generally located in the spleen or bone marrow. From these locations, they secrete antibodies at a rate of 2,000 antibodies per second for several days.⁷ This effort is strenuous, however, and plasma cells are typically short-lived, dying after only a few days of secreting antibodies. In contrast to plasma cells, memory cells retain their membrane-bound B

⁷ Sompayrac, 39.

cell receptors and continue to survive in the bloodstream for longer periods of time, conferring a quick immune response to the organism upon second exposure to an antigen. Due to various genetic mechanisms, the secondary response to an antigen is faster, stronger, and has a higher affinity than the primary response. Memory cells are typically formed in immune responses that require T cell help.



The fate of B cells. Once a cell is activated and proliferates, its clones must choose to become plasma cells or memory cells.

As the experiment described in the remainder of this thesis involves only B cells, this section is the furthest extent to which T cells will be covered. While T cells are important to the immune response, they are not a major component of the experiments performed to study the receptor TACI, and as such, they will not be discussed in detail in the rest of this thesis. The next section will describe, in greater detail, the process of B cell maturation and generation of diversity.

Section V: B Cell Maturation and Mechanisms of B Cell Diversity

B cells begin their existence as hematopoietic stem cells in the bone marrow of an organism. The cell differentiation process takes B cells through a series of stages, each of which possesses unique markers. It should be noted, however, that while some surface markers change, after a stem cell becomes a precursor of a B cell, it possesses the cell surface marker CD19.

After a lymphoid progenitor makes the decision to become a B cell (thus becoming a committed lymphocyte progenitor), it first becomes a pro-B cell, which expresses the molecules B220 and CD43 on its surface. Following this change, the pro-B cell becomes a pre-B cell, which expresses B220 on its surface but does not express CD43. The pre-B cell then develops into an immature B cell, which has the surface markers B220 and IgM. While all of the stages up until the immature B cell stage are marked by a rearrangement of genes on the B cell receptor, the immature B cell possesses a fully rearranged heavy chain and light chain. Immature B cells further develop into mature naïve B cells, which possess the surface markers B220, IgM, and IgD. Mature naïve B cells are similar to effector B cells, but as they have not yet encountered antigen, they do not possess all of the characteristics of either plasma or memory cells. It is important to note that upon maturation, mature naïve B cells leave the bone marrow and enter the periphery, where they will be activated by encountering an antigen for which they have specificity.⁸

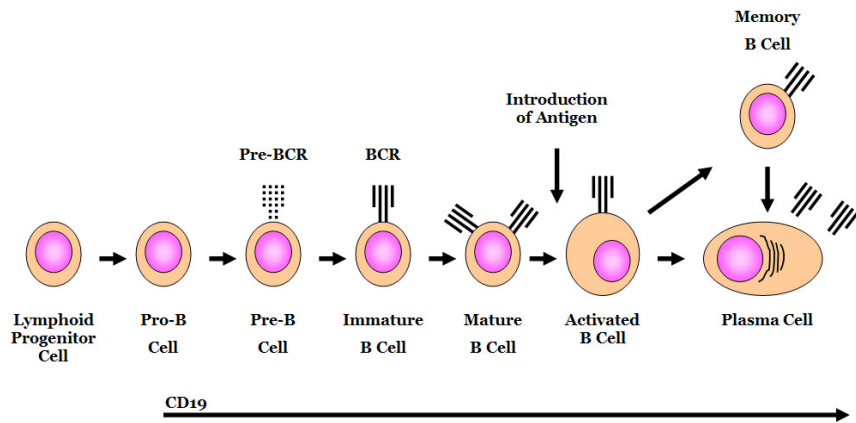
Upon activation, a B cell becomes a lymphoblast and must make a career decision to become either a plasma cell or a memory cell. Memory cells, which express B220, MHCII, IgG, and IgA, lay in wait for a secondary exposure to an antigen. Plasma cells,

⁸ Janeway, 310.

which additionally express CD135 and CD38, return to the bone marrow and secrete large quantities of antibody molecules to fight an infection.⁹

Throughout the maturation process, diversity is generated between B cells as the B cell receptor (or antibody) molecule

undergoes a series of changes. These changes happen on the level of the genes encoding the receptor, and take place sequentially as the cell



Normal human B cell maturation.

matures. Both the heavy chain and the light chain are encoded by a collection of gene segments called V, J, and C genes; additionally, the heavy chain genes also include D genes. These gene segments are rearranged through recombination to produce a complete antibody molecule. First, during the transition from stem cell to large pre-B cell, the heavy chain of the B cell receptor is arranged by the joining of a D and a J segment followed by V-DJ joining. The VDJ segment is then joined to a C region. C_{μ} is the first C region to connect, but isotype switching may occur to connect the VDJ segment to different C regions later. Following the construction of the heavy chain, the light chain is constructed in a similar manner, beginning with V-J joining and ending with the VJ segment connecting to a C_{κ} or C_{λ} gene.¹⁰

⁹ Janeway y, 310.

¹⁰ Janeway, 310.

Because many possibilities exist for each gene segment that is chosen, there are a multitude of possible antibody molecules that can be created through this rearrangement alone. The areas of greatest diversity in antibody molecules are called the hypervariable regions, and it is these regions that determine which antigen the antibody molecule will bind to. In addition to simple rearrangement of gene segments, hypervariable regions are given even greater diversity through the action of proteins called RAG-1 and RAG-2, which randomly cut at the recombination signal sequences (RSS) to create slightly differential splicing even between antibody molecules with the same gene segments. The variability in gene segments creates many different possible antibody molecules, each capable of binding a different epitope on an antigen.¹¹

In addition to the diversity generated by the initial rearrangement of genes for B cell receptors, a phenomenon called isotype switching (or class switching) amplifies the number of possible antibodies that can be produced. To complete the initial arrangement of the heavy chain, the VDJ segment is initially joined to a C segment called C_μ, which encodes antibody molecules of the class IgM. In some cells, C_δ is joined instead, and the antibody molecules are of the class IgD. Later in the development of the cell, the B cell receptor may change its C region to C_γ, C_α, or C_ε, encoding IgG, IgA, or IgE, respectively. The isotype switching process generates antibodies to the same epitopes that may be of a different class from other antibodies that are produced. Because each class of antibodies has a different function, this enables the immune system to fight an infection in many ways.

As if VDJ rearrangement and isotype switching were not enough to generate a diverse B cell repertoire, an additional mechanism exists for creating an even greater

¹¹ Janeway, 269.

variety of B cells. Upon encountering an antigen and becoming activated, a B cell may rearrange amino acids in the hypervariable regions of its antibody molecules. This process, termed somatic hypermutation, involves an enzyme called activation-induced cytidine deaminase, or AID. During somatic hypermutation, point mutations are introduced into the V regions of the heavy and light chains, in the region where the antibody molecule contacts the antigen. AID recognizes a specific motif in the nucleotide sequence of the DNA and selectively deaminates cytosine residues, which are then excised by another enzyme and randomly replaced. Somatic hypermutation can have one of three effects: there can be no change in the antibody's affinity for the epitope; the antibody can lose affinity for the epitope; or the antibody could increase in affinity for the epitope. The final option, termed affinity maturation, is the reason that somatic hypermutation occurs. After the B cell is activated, affinity maturation helps to create antibodies that have even higher affinity for the antigen, resulting in a more effective secondary response to a pathogen.¹²

The processes of VDJ rearrangement, isotype switching, and somatic hypermutation all introduce a large amount of diversity to the B cell repertoire, both before and after B cell activation. Some of the diversity that is generated is not desirable, however, as B cells may become reactive toward self-generated substances. This reactivity toward self, termed autoimmunity, can cause serious disease. In order to curb autoimmunity, the development of lymphocytes encourages self-tolerance through negative selection mechanisms. Throughout various stages of B cell (and T cell) development, those cells that react too strongly with self-antigens are deleted, via

¹² Janeway, 146-147.

apoptosis, from the lymphocyte repertoire. Autoimmunity and self-tolerance mechanisms will be covered more in depth in Section VIII of the thesis.

Section VI: T Cell Dependent and T Cell Independent Activation

In order for B cells to carry out their effector functions, they must first be activated by encountering the pathogen for which they are specific. Antigen recognition is not the only necessary thing for activation, however; in most cases, B cells must also receive a signal from T cells, and in other cases, they must receive a special kind of signal from the pathogen. These two types of responses are termed thymus-dependent (TD) and thymus-independent (TI) humoral responses. While the TD response is more typical, TI responses are also important to the ability of the organism to fight pathogens.¹³

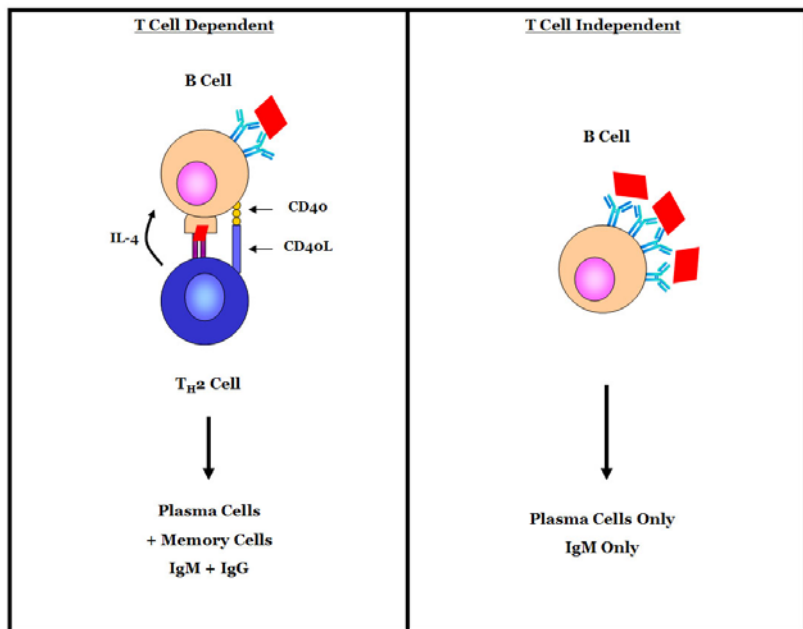
In the thymus-dependent response, an antibody response by B cells requires help from T cells which recognize the same antigen. This critical interaction of a B cell and T cell specific for the same antigen is termed linked recognition. The B cell and T cell need not recognize the same epitope on the antigen; they must only recognize epitopes on the same antigen molecule. In order for a B cell to be fully activated, it must receive help from an activated helper T (T_H) cell. A T_{H2} cell, which activates B cells, can be activated by any antigen-presenting cell displaying its cognate antigen on an MHC class II molecule, including B cells that have internalized a pathogen and presented it. Upon recognizing an antigen, a B cell will travel to the border between the T cell zone and B cell zone of the lymph node, where it will attempt to encounter a T_{H2} cell with the same specificity. If the endeavor is successful, a T_{H2} cell will activate a B cell via the interaction between the receptor CD40 on the B cell and the ligand CD40L (also called CD154) on the T cell. The activation also involves the secretion of cytokines such as IL-4. The T cell-B cell pair then begins to proliferate and differentiate, forming a structure

¹³ Janeway, 369.

called the germinal center. From the germinal center, cells are released that become plasma cells, traveling through the periphery to the bone marrow and spleen to secrete antibody molecules. In the model of a TD humoral response, helper T cells are necessary to create an effective antibody response to a pathogen.¹⁴

Although the thymus-dependent response is the typical mechanism of humoral response, in some cases B cells do not require the help of T cells in order to become activated. The thymus-

independent (TI) response often occurs in the case of components of bacteria and other microbes, such as bacterial polysaccharides and polymeric proteins. These antigens are termed TI antigens because of their ability to elicit a TI humoral response. In addition to the



Typical T cell dependent and T cell independent responses. Note the difference in the resulting cells and antibodies produced. Figure created using VisiScience ScienceSlides program.

recognition of TI antigens, a B cell will receive a second signal either from the recognition of a high concentration of a common microbial component, or by excessive cross-linking of the BCR. The difference in the second signal for activation is what distinguishes TI-I from TI-II responses.¹⁵

¹⁴ Janeway, 369-376.

¹⁵ Janeway, 383-384

In a TI-I response, the pathogen possesses the ability to directly induce the activation and proliferation of B cells. One example of a TI-I antigen is LPS, a component of gram-negative bacterial cell walls. At high concentrations of this type of antigen, many B cells will be activated and induced to proliferate regardless of their antigen specificity. This type of activation is called polyclonal activation. At low concentrations of the same antigen, however, only B cells which are specific for a surface molecule of the pathogen will be activated.

In contrast to a TI-I antigen, TI-II antigens contain no intrinsic ability to induce proliferation in B cells. TI-II antigens are typically bacterial polysaccharides which are extremely repetitive on the surface of the pathogen. These antigens can activate only mature B cells, and are able to activate the cells through a large amount of BCR cross-linking. For this type of antigen, a critical density is necessary: at concentrations that are too low, the antigen is unable to create enough BCR cross-linking to activate the B cell, and at concentrations that are too high, the cell becomes so highly activated that it commits suicide via apoptosis. The most instrumental B cell types in TI-II responses are the less typical types, B-1 cells and marginal zone (MZ) B cells.¹⁶

It should be noted that while TI antigens are efficient at activating B cells and may do so more quickly than TD antigens because of the lack of requirement for T cells, TI responses are largely inefficient at inducing B cell memory. This means that TI responses are instrumental in primary infections, but while the same TI response may be induced upon a secondary introduction of a pathogen, the secondary response to the pathogen will be no quicker or stronger than the primary response.¹⁷

¹⁶ Janeway, 385-386.

¹⁷ Janeway, 369.

In examining the reactions of B cells to certain types of activation, it is critical to remember the difference between thymus-dependent and thymus-independent responses. These responses take place through different pathways and are useful for different types of infection. Later in this thesis, we will return to the theme of TD vs. TI response as we encounter various types of activation for B cells.

Section VII: Summary of B Cell Functions

Most B cells begin their development as hematopoietic stem cells in the bone marrow of an organism and differentiate through many stages. Each stage of development is marked by a rearrangement of part of the genes that encode the B cell receptor, and can be followed by monitoring the markers expressed by the cell. After many transitional stages, a hematopoietic stem cell differentiates to become an immature B cell and leaves the bone marrow. In the periphery, the cell encounters antigen and becomes activated. The recognition of an antigen by a B cell depends on the antigen being at least partially intact, so that the epitope that is recognized by the cell is preserved. Upon activation by antigen recognition and by a second signal, a B cell will stop migrating, grow, and proliferate.

The mature B cells that arise from the activation of one cell have a major career decision to make: they must become plasma cells, which secrete antibody, or memory cells, which undergo somatic hypermutation and respond strongly upon a second introduction of a pathogen into the organism. Both types of cell are extremely helpful to the body in combating certain types of pathogen.

In general, B cells are most useful in controlling invasions by extracellular pathogens. This is only logical, as antibodies can not reach pathogens inside cells. Because T cells can not recognize whole, unrepresented antigen molecules, B cells are instrumental in fighting pathogens that spend at least a significant portion of their life cycle outside of the cell. Antibodies secreted by plasma cells have three primary functions in creating the humoral immune response. First, antibodies can neutralize pathogens such as viruses and intracellular bacteria by binding to the cell surface and making the pathogen unable to enter the body's cells. Neutralization is also helpful in

preventing bacterial toxins from entering cells. Second, antibody molecules can mark a pathogen for destruction by phagocytosis, a phenomenon called opsonization. Finally, antibodies can activate the complement protein system, causing the proteins to bind to the pathogen and opsonize the pathogen.¹⁸

Because the antibody response takes time to form, it is typically more helpful during a secondary response than a primary response. While other mechanisms of immunity may be more effective in clearing the pathogen during a primary response, antibodies from memory B cells are extremely effective in stopping an infection upon a second introduction of a pathogen.

¹⁸ Janeway, 367-368.

Part II: Relevant Topics in Immunology

Section VIII: Autoimmunity & Cancer: When the Immune System Goes Wrong

While the immune system is typically highly regulated and specifically targeted, there are cases in which some form of regulation can be turned off, or changed, so that the immune system is no longer as finely tuned as it normally is. When this occurs, part or all of the immune system can become extremely dangerous to the individual. Two cases in which dysregulation of the immune response is harmful include autoimmunity and cancer.

Because many infections can become serious if not effectively cleared, the immune system must by necessity contain many potent methods for combating pathogens. The immune response is not necessarily limited to fighting pathogens, however; if not properly regulated, it can just as easily be directed against the body itself. Luckily, in most individuals, the cells of the immune system are highly regulated and contain mechanisms for ensuring self-tolerance. Autoimmunity is not an extremely rare phenomenon, however; estimates indicate that approximately 5% of Americans suffer from some sort of autoimmune disease.¹⁹

An autoimmune response can be characterized as either organ-specific, concentrated primarily within one organ, or systemic, spread throughout the body. Some examples of organ-specific autoimmune disease include Graves' disease, type I diabetes mellitus, multiple sclerosis, and myasthenia gravis. Some examples of systemic autoimmune disease are systemic lupus erythematosus (SLE), Sjögren's syndrome, and rheumatoid arthritis. In addition to the distinction between organ-specific and systemic disease, autoimmune disorders can be primarily antibody-mediated, T-cell-mediated, or

¹⁹ Sompayrac, 100.

both, although nearly all autoimmune disorders require both B cells and T cells to fully develop the disease.²⁰

During the development of lymphocytes, cells which react strongly to self-antigens undergo a negative selection, resulting in the apoptosis of these cells. This negative selection ensures that surviving lymphocytes will be tolerant of self antigens. While the distinction between self and non-self is still being studied and the specifics are not yet clear, results thus far indicate that the distinction is made both by the stage of development at which the cell encounters an antigen and by the concentration levels of the antigen.²¹ In most individuals, this self-tolerance mechanism works very well, with only a few autoreactive cells escaping negative selection. In some individuals, however, genetic defects cause problems with the mechanisms that control lymphocytes. In these individuals, many autoreactive cells escape self-tolerance mechanisms, and autoimmunity almost certainly develops. Although autoimmunity frequently arises in individuals with a genetic predisposition, it is possible for genotypically normal individuals to develop an autoimmune disease because even functional self-tolerance mechanisms are not 100% effective in eliminating autoreactive cells.²²

In individuals who are not genetically predisposed to autoimmune disorders, autoimmunity typically results from a change in lymphocytes with a low affinity for self antigen. Low-affinity lymphocytes will not be activated by self antigen, however, they can be activated by stimulation with a high amount of co-stimulatory molecules such as cytokines or by stimuli similar to the self-antigen, such as DNA from pathogenic bacteria. Once these lymphocytes are activated, they can become more highly reactive

²⁰ Janeway, 557-560.

²¹ Janeway, 558-559.

²² Sompayrac, 100.

against self antigens, either through a high level of activation and proliferation or through somatic hypermutation. In accordance with the idea that many autoimmune reactions are initially directed at non-self pathogens, a large percentage of autoreactive antibodies are directed at DNA. In addition, toll-like receptors (TLRs), which are typically involved in innate and innate-like immune recognition of common microbial components, have been implicated in the mediation of autoimmune disease.²³

Once an autoimmune reaction begins, it can easily become a chronic disorder. Because self antigens are frequently ubiquitous and difficult or impossible to clear from the system, an autoimmune response can be hard to stop once it has begun. In addition, an autoimmune response can be directed toward more than one epitope on a self antigen or other proteins in a tissue, a phenomenon called epitope spreading. Autoimmunity can also be fueled by the consequences of an immune reaction in a tissue, including inflammatory cytokines and the new availability of self antigens as a result of cell death. Because of these possibilities, most autoimmune disorders are chronic, and many worsen as the disorder persists.²⁴

In addition to autoimmunity, cancer can also result if the cells of the immune system are not properly regulated. In order for a normal cell to become cancerous, many changes must occur. The cell must lose the property of regulation of proliferation, usually because of mutations in cell-regulatory genes. In addition, tumor cells must be able to generate their own blood vessels, a property known as angiogenesis. Cancerous cells also lose the dependency upon contact for growth, causing them to be able to grow

²³ Janeway, 569-570.

²⁴ Janeway, 564-570.

on top of other cells and also be able to metastasize, or detach and move to other locations.²⁵

If blood cells such as lymphocytes are affected by the changes that cause cancer, blood cancers such as lymphomas and leukemias result. In cases of leukemia, immature lymphocytes fill up the bone marrow, where they prevent other cells from maturing; in lymphoma, clusters of cells that resemble tumors form in the lymph nodes. Leukemia and lymphoma can both result from mutations in either B cells or T cells, and are typically serious diseases.²⁶

In B cells, malignancies can occur at any stage of development. Precursor B cell neoplasms such as lymphoblastic lymphoma (LBL) and B-cell acute lymphoblastic leukemia (B-ALL) occur predominantly in children and young adults and are signified by the lack of Ig expression in the cancerous cells. Peripheral mature B cell neoplasms such as B cell lymphomas and B cell chronic lymphocytic leukemia (B-CLL) occur primarily in older individuals. B-CLL is signified by a large amount of small, round B cells with a low surface expression of Ig but a high expression of CD19 and CD5. These cells express surface markers that come from different stages of development, making it difficult to determine which cell stage is the precursor to the cancerous cells.²⁷

B cell lymphomas are typically characterized as either representing Hodgkin's disease or being part of a class of diseases termed non-Hodgkin's lymphomas (NHL). Until recently, Hodgkin's disease was thought to derive from T cells or some other precursor, but after further investigation, it is currently accepted that the precursor cell of Hodgkin's disease is the Reed-Sternberg (RS) cell, which derives from post-germinal

²⁵ Parts of this paragraph are summarized from Campbell & Reece, *Biology*.

²⁶ Janeway, 305.

²⁷ Jelinek & Darce, "Human B Lymphocyte Malignancies: Exploitation of BLyS and APRIL and Their Receptors," 268-269.

center B cells that have rearranged their Ig molecules but do not express them on the cell surface. Non-Hodgkin's lymphoma encompasses many different types of lymphoma, including diffuse large B cell lymphoma (DLBCL, the most common type of non-Hodgkin's lymphoma), follicular lymphomas, Burkitt's lymphoma, and marginal zone lymphoma. Each type of lymphoma is characterized by a different type of precursor cell and manifests itself differently in affected individuals.²⁸

In addition to leukemias and lymphomas, other types of plasma cell transformations also occur. Multiple myeloma (MM) is a monoclonal expansion of plasma cells, resulting in a high number of malignant plasma cells found in the bone marrow and sometimes in the periphery, as well as a large volume of secreted Ig, typically found in the serum and urine of affected individuals. The regulation of cell growth and expansion seems to be extremely complex in MM. Other plasma cell transformations include lymphoblastic lymphoma, which affects a variety of B lineage cells at different stages, and B cell related amyloidosis, which results in amyloid deposits of light chains from Ig molecules.²⁹

Many different methods of targeting and controlling tumors are currently being investigated by researchers or in use as therapy. Most of these involve targeting what are termed tumor rejection antigens, specific markers that are only present on tumor cells. Tumor rejection antigens can be targeted by antibodies specific for the antigen or by a cytotoxic T cell response, or both. Ideally, targeting these markers should ensure that only tumor cells are killed and that the body's normal cells are not harmed in the immune response. In addition to targeting these cells for destruction, methods such as

²⁸ Jelinek & Darce, 270-272.

²⁹ Jelinek & Darce, 272-273.

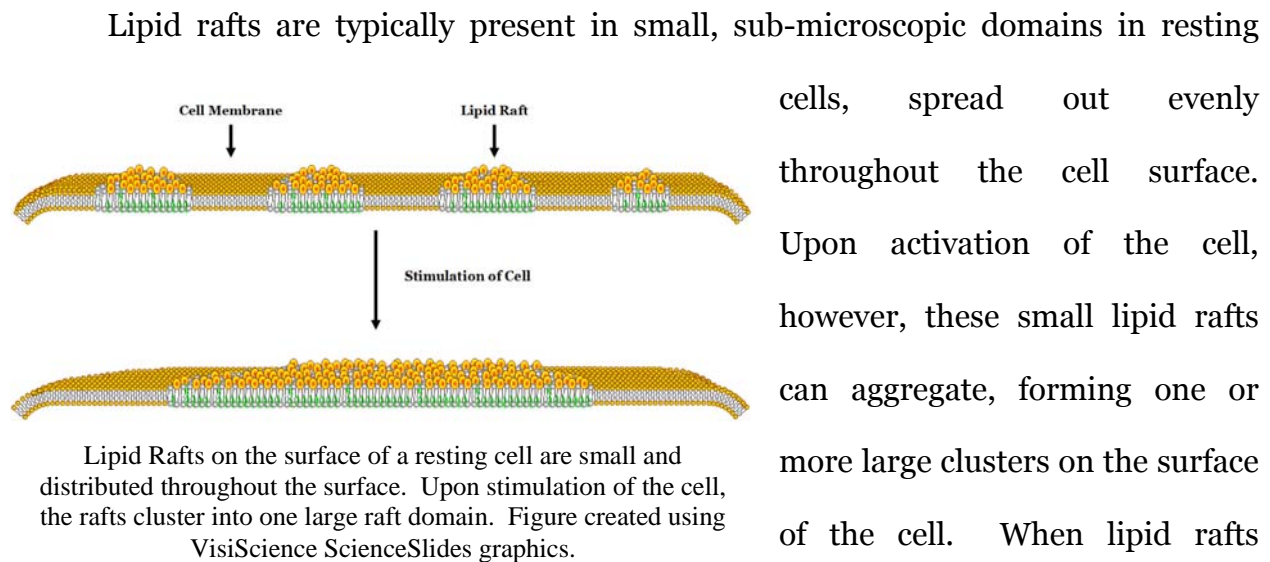
viral gene therapy can use the same tumor markers to direct genes that inhibit growth or angiogenesis toward only the tumor cells. This method typically involves replacing mutated genes or integrating new genes to introduce a new method of controlling the cells.³⁰

Both autoimmunity and cancer are serious problems which directly or indirectly affect a large percentage of the population. Because of this, much time and funding has gone toward finding effective therapies for both disorders. Although a singular cure for either cancer or autoimmune disorders is likely impossible, methods of treating specific types of disease will improve as time goes on and society's knowledge of science increases. Through studying different types of cancer or autoimmunity, and through studying how cells are regulated, the scientific community comes closer to understanding how these problems can best be treated.

³⁰ Janeway, 631-642.

Section IX: Lipid Rafts

In contrast to what is told to every Introductory Biology student, the lipids in a cell membrane are not as uniformly distributed as the “fluid mosaic” model indicates. In fact, different types of lipids form membrane microdomains, portions of the cell membrane composed of a large percentage of a particular type of lipids and their associated proteins. The most well-characterized membrane microdomain is the lipid raft, which is also called a glycolipid-enriched microdomain (GEM) or detergent-insoluble glycolipid-rich domain (DIG). Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains which also contain many proteins that are either constitutively associated with the lipid raft or recruited to the raft under certain conditions. Many of the raft-associated proteins are kinases, which are associated with the lipid rafts in order to aid in activating cell signaling cascades. (1)



cluster, they bring together many proteins and receptors which are associated with them and exclude others. While unclustered rafts are not easy to visualize, when rafts cluster they appear to represent as much as 40% of the cell membrane. As of yet, it remains unclear whether lipid raft clustering is the first event following cell activation, or if raft

clustering is a result of other receptors or proteins being induced to aggregate. Regardless of which event occurs first, evidence indicates that some receptors are nearly always associated with lipid rafts, while others are only associated under certain conditions. (2)

When lipid rafts cluster, they are extremely useful as signaling platforms for the cell, and are also thought to be a critical part of the formation of the immunological synapse. Because of raft-associated kinases and other proteins that are extremely useful in signaling cascades, when receptors are recruited to lipid rafts they are usually activated and signaling begins. Cross-linking of the BCR and the subsequent association of the BCR with lipid rafts has been extensively studied, and it appears that BCR signaling only begins in significant amounts following the association of the BCR with lipid rafts. It has also been suggested that lipid rafts may play an important role in the BCR-mediated endocytosis of pathogens. Along the same line, lipid rafts have also been implicated in some viral infections of cells, as they tend to cluster molecules necessary for access into the cells. Viruses that utilize lipid rafts to enter cells include HIV, Ebola, and the Epstein-Barr and Marburg viruses. (2, 3)

While the association of a receptor with a lipid raft is typically thought to increase the amount or effectiveness of cell signaling, many researchers have suggested that raft association may also decrease the effectiveness of certain signaling pathways. A model proposed by Kwang-Il Lim and John Yin at the University of Wisconsin, Madison, uses biophysical concepts to create an electrical circuit analogy and statistical equation for ligand binding to clustered receptors. The model proposes that raft-mediated receptor clustering can limit ligand binding because the ligand must first reach the cell surface, then find the clustered lipid raft area, then find its appropriate receptor. This model can

be used to explain instances in which lipid raft association does not aid the signaling of a particular molecule or receptor. (35)

Because of the many important functions that lipid rafts seem to have, many methods have developed for studying lipid raft formation and raft association of proteins. In order to understand what functions depend on lipid rafts, researchers frequently use a technique called cholesterol depletion, which employs a substance (often cyclodextrin or glucocorticoids) that removes cholesterol from the cell membrane. Various processes in the cell can then be studied to determine if they take place as normal when lipid rafts cannot form. Through this method, it has been shown that the localization of many receptors depends on lipid rafts. In addition to cholesterol depletion, the association of molecules with lipid rafts has also been studied through gel electrophoresis. As the name implies, as detergent-resistant microdomains, lipid rafts can easily be isolated from other cell components by lysing the cell with detergent and removing all soluble molecules. The cell components remaining after treatment with detergent are the lipid rafts and their associated receptors, which can then be analyzed with SDS-PAGE or 2D gel electrophoresis techniques. (2, 4)

While the technologies of cholesterol depletion and detergent treatment have been extremely useful in studying lipid rafts, they do have their limitations. Detergent treatment of cells lyses the cells, making studying their signaling processes in addition to the presence of the signaling molecules impossible; additionally, gel electrophoresis can be ineffective in detecting small amounts of a protein, meaning that it is difficult to study a receptor that is present in small amounts on the cell surface. Cholesterol depletion is highly controversial as a technique for studying living cells, as it can heavily disrupt many processes in the cell, making it difficult for the cell to recover. Because of

these limitations, less invasive methods of studying lipid rafts have developed. Visualization using lipid raft markers provides a non-invasive alternative to studying cell membrane dynamics and signaling processes. The most common marker for lipid rafts is the ganglioside GM1, which can be visualized by staining with the B subunit of the cholera toxin molecule, conjugated to a fluorescent molecule such as GFP or FITC. GM1 has been shown, through many different experiments, to associate with lipid rafts in mature B cells, an extremely important fact to remember when reading the end of this thesis. (4, 5)

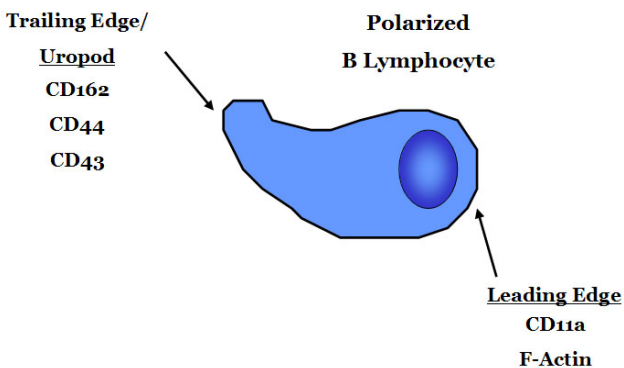
Section X: Cell Polarization and Signaling

When lymphocytes are activated, they often assume a polarized morphology. This occurs as part of cell migration and very often as a part of intercellular signaling. Polarized lymphocytes have two very different ends: a leading edge, at the front of the cell, and a trailing edge, or uropod, at the rear of the cell. Each end may have associated lipid rafts, a specific protein composition, and many discrete functions. The leading edge of a polarized lymphocyte is thought to be composed of receptors that recognize chemokines, chemical signals which tell the cell to move in a certain direction. It also contains many substrate-adhesion molecules, which help the cell stop migrating and enter an infected area or special compartment of the body. The trailing edge, or uropod, contains molecules associated with endocytic trafficking in the cell, as well as those necessary for intercellular adhesion. The molecules associated with the separate ends of the cell are thought to be related to the functions of each end, and in many cases this line of thinking has been backed up by experimental results. (6)

When a lymphocyte becomes polarized, the cell membrane typically reorganizes, with some receptors forming a “cap” at one end of the cell. This reorganization is generally thought to be mediated by changes in the cytoskeleton and by lipid raft clustering. In accordance with this idea, some of the markers of the trailing edge of the cell have been shown to be physically associated with cytoskeletal elements such as actin and tubulin. (6, 7) In some studies, the formation of a receptor cap in the uropod has been found to be blocked by cytochalasins, which block actin polymerization. This data indicates that actin has a large role in cell polarization and receptor localization in polarized cells. Interestingly enough, colchicines, which block microtubule polymerization, have been found to not interfere with the formation of a receptor cap at

the uropod, indicating that while some receptors may be associated with tubulin, microtubules likely do not play a role in uropod formation. (7) Other studies have shown that when cells become polarized, molecules such as syndecan-1 and syndecan-4 may be involved in the sequestering of receptors in lipid rafts, particularly in the uropod of the cell. (9)

Many studies have examined the role of cell polarization in lymphocytes. Typically, this involves either determining the effect of cytoskeletal rearrangement on



A polarized B cell. Leading edge and trailing edge markers are shown.

cell surface molecules or examining receptor localization in polarized cells by visualization of the receptor of interest. While the first technique is relatively simple and usually involves disruption of the cytoskeleton, the second technique can be slightly more complicated. First, a reliable marker of

cell polarization must be found. This involves testing various cell surface molecules to determine which molecules segregate reliably to either the leading edge or the uropod of the cell. Following the discovery of a good marker, a co-staining procedure must be performed to determine whether the molecule of interest co-localizes with the marker. Many markers of cell polarization have been found in T cells, as many polarization experiments have been conducted in T cells. The ganglioside GM1, a marker of lipid rafts, has been found to segregate to the leading edge of polarized lymphocytes, as has CD11a (also known as LFA-1) and F-actin. Characterized uropod markers include CD44, CD43, ICAM-1, and PSGL-1 (CD162), all cellular adhesion molecules. (8, 9) Antibodies

and fluorescently-labeled antibodies to all of these molecules have been created and are widely commercially available. As with lipid rafts, the study of receptor localization through visualization helps the researcher examine cell processes in intact, healthy cells, making it much more likely that the results being found are representative of what occurs *in vivo*.

Section XI: The BLYS/APRIL System – Novel TNF Family Members

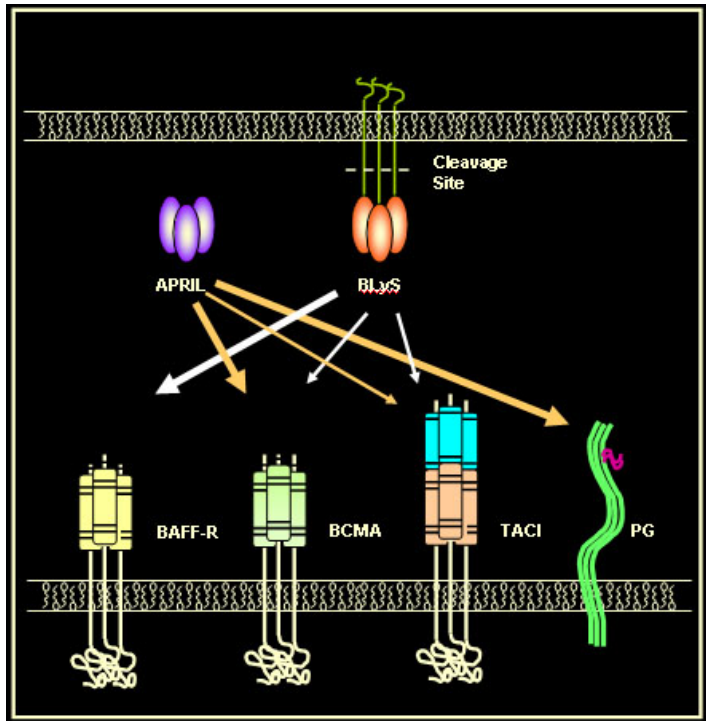
One major family of cytokines that has been extensively characterized is the tumor necrosis factor, or TNF, family. The TNF family is a group of proteins with homology to the original tumor necrosis factor protein. All of these proteins form trimers, and many TNF family members are membrane-bound. The TNF family of proteins and TNF receptors has many functions in innate and adaptive immunity, possessing stimulatory and anti-stimulatory functions. Examples of widely known TNF family members include CD40 ligand (CD40L), which is expressed by CD4⁺ T cells, and Fas ligand (FasL), which is expressed by CD8⁺ T cells. While CD40L commonly stimulates B cells to proliferate, FasL directs many cell types to undergo apoptosis. Defects in the FasL apoptotic pathway result in lymphoproliferative diseases and autoimmunity, a rare instance when autoimmunity is caused by a defect in a single gene. While many TNF receptors, like Fas, have “death domains” in their cytoplasmic tails, some, such as CD40, lack this domain and act through TNF-receptor associated factors, or TRAFs. (1)

Two novel ligands in B cells have recently been identified as a part of the TNF family. B cell lymphocyte stimulator, BLYS (also known as BAFF, zTNF4, THANK, TALL-1, and TNFSF-13b), and a proliferation inducing ligand, APRIL (also known as TALL-2, TRDL-1, and TNFSF-13a), are some of the most recent additions to the tumor necrosis factor family. These ligands, as well as their three known receptors and one additional binding partner, have been found to have a role in cell proliferation and apoptosis. Because of their function in B lymphocytes, this ligand/receptor family has been thought of as a potential target for treatments against autoimmunity and B cell

malignancies, and many experiments have sought to determine the role of BLYS and APRIL in various types of autoimmune disease and cancer. (10)

B lymphocyte stimulator, or BLYS, is a TNF family member that is commonly thought of as an enhancer of B cell survival. The BLYS locus is on human chromosome 13q34. BLYS is expressed on myeloid

lineage cells and is upregulated by inflammatory cytokines such as IFN- γ and IL-10. It has been shown to attenuate apoptosis, heighten the humoral immune response, and co-stimulate a CD40L response, perhaps by increasing the ability of the B cell receptor to phosphorylate the coreceptor CD19. Because it is a critical survival signal, newly formed and mature B cells compete for BLYS to extend cell survival. Mice that are



The BLYS and APRIL system of ligands and receptors. BLYS binds to BAFF-R, BCMA, and TACI, while APRIL binds to BCMA, TACI, and proteoglycans. Figure courtesy of Jaime Darce.

deficient in BLYS have a reduction in peripheral B cells after the T₁, or first transitional, stage of development, with the exception of B₁ cells, which are thought to go through a different developmental pathway. These mice also have a reduced T cell independent and T cell dependent humoral response. (11) When BLYS acts through its primary receptor, BAFF-R, it acts as a survival factor, allowing B cells to avoid apoptosis. Through both its soluble form and its membrane-bound form, it also acts as a B cell maturation signal, mediating class switching in maturing B cells. (10, 12, 14)

Normal B cells maintain homeostasis through a balance of production, survival, proliferation, and apoptosis. Abnormal production of BlyS can disrupt this balance and disturb tolerance, resulting in cancer and autoimmunity. This is evidenced by the fact that mice that are transgenic for BlyS overexpression develop autoimmune symptoms and B cell lymphomas, and also by the fact that serum levels of BlyS are elevated in human patients with autoimmune diseases and B-lymphoid malignancies. (12)

A proliferation-inducing ligand, or APRIL, is a cytokine which was first characterized by enhancing proliferation in B cells when it interacts with some of its receptors, but it can also repress proliferation through its interaction with the receptor TACI. In addition, APRIL can promote T cell independent type 2 (TI-II) responses and has been shown to promote IgM secretion *in vitro*. The gene for APRIL is found on human chromosome 17p13.1, and it shares significant sequence homology with BlyS, making the two molecules much more similar to each other than to any other TNF family members. APRIL is mainly secreted as a soluble molecule and is expressed by monocytes, dendritic cells, and T cells. In addition to its functions in B cells, it is thought to be important for T cell activation, as anti-APRIL antibodies interfere with T cell stimulation *in vitro*. While APRIL is thought to enhance proliferation, mice with an APRIL deficiency exhibit normal immune development, indicating that some of the function of APRIL may be redundant. APRIL expression is higher in tumor cells than in normal cells, however, suggesting that APRIL may have some effect on tumor cell proliferation. (12, 13, 14)

To date, there are four characterized binding partners for BlyS and APRIL. BlyS binds to its own receptor, BAFF-R (or BR3), as well as B cell maturation antigen (BCMA), and transmembrane activator and CAML interactor (TACI). APRIL, like BlyS,

binds to BCMA and TACI, but also binds to heparin sulfate proteoglycans (HSPG) on the surface of cells. Interestingly, the surface APRIL uses to bind to BCMA and TACI is different than the surface it uses to bind to HSPG, suggesting that it may be able to bind to both HSPG and another receptor at once. (10, 15)

Much information about the receptors for BLYS and APRIL can be gleaned from animals whose cells lack these receptors. BAFF-R knockout mice display a severe decrease in mature B cells beyond the T1 stage, similar to the phenotype of BAFF-deficient mice. This suggests that BAFF-R is responsible for BLYS-mediated B cell survival. Interestingly, it has also been discovered that the immunodeficient model A/WySnJ mouse strain has a natural mutation in the third exon of the BAFF-R gene. In contrast to BAFF-R knockouts, which display a severe phenotype, BCMA knockout mice have no drastic B cell deficiency and only appear to have a slight reduction of plasma cells, indicating that the majority of important BLYS and APRIL signaling takes place through their other receptors and BCMA may act as a backup system for these receptors. Mice deficient in TACI display an increase in peripheral B cells, yet a decrease in T cell independent (TI) responses, particularly TI-II, and a defect in IgA production. This surprising phenotype suggests that TACI may be a negative regulator of B cell survival, but may also positively regulate TI humoral responses. In addition, these mice also display autoimmune disorders similar to systemic lupus (SLE) as well as B cell lymphomas, most likely due to the increase in peripheral B cells. Much more information on TACI will be discussed in the next section. (11, 12)

Because of their regulatory roles on lymphocyte survival and proliferation, the ligands and receptors of the BLYS/APRIL system have been linked to many different autoimmune diseases and cancers. In the absence of tumor necrosis factor,

overexpression of BLyS leads to a high incidence of B cell lymphoma, and BLyS has been found to bind to malignant B cells from patients with B cell chronic lymphocytic leukemia (B-CLL), non-Hodgkins lymphoma (NHL), and multiple myeloma (MM). APRIL has also been shown to bind to B-CLL cells. Malignant lymphocytes have higher reactivity with BLyS and APRIL than normal human plasma cells, suggesting that BLyS and APRIL binding might provide an alternative survival pathway for tumor cells. In accordance with this idea, MM cells have been shown to survive for longer with access to BLyS than without it, and the bone marrow of patients with MM expresses much more BLyS mRNA than that of normal patients. MM cells and B-CLL cells also express a high level of TACI and BAFF-R, indicating that their ligands may be responsible for at least some of their survival. (10, 12, 17) In addition to their functions in cancer, BLyS and APRIL have also been connected to autoimmune disorders. They appear to have both pro- and anti-inflammatory functions in rheumatoid arthritis (RA), and levels of BLyS are elevated in the serum of patients with SLE and Sjögren's Syndrome. (10, 16, 18)

Because BLyS, APRIL, and their receptors are intrinsically linked with autoimmunity and B cell malignancies, this system makes a logical target for treatment for these diseases. In order to discover new ways to treat RA, SLE, MM, B-CLL, and NHL, many researchers are striving to better understand the ligands and their receptors in order to design strategies to prevent or encourage BLyS and APRIL binding to their receptors. Treatments based on this ligand-receptor system could provide new hope for patients who have not yet found an effective treatment for their cancer or autoimmune disorder.

Section XII: TACI

One of the important receptors in the BLyS/APRIL system is transmembrane activator and CAML interactor, or TACI, which binds to both BLyS and APRIL. TACI was discovered by Götz-Ulrich von Bülow and Richard J. Bram, who were then at the University of Tennessee in Memphis. Von Bülow and Bram cloned and characterized the gene for TACI, which is located on human chromosome 17p11.2, and created a strain of TACI knockout mice. Since the first discovery of TACI, von Bülow and Bram, now at Mayo Clinic, as well as other researchers, have done much work to further elucidate the structure and function of the TACI protein in both normal and disease systems. (14, 19)

TACI is a type III transmembrane protein with an extracellular amino terminus and two repeated cysteine-rich motifs, which is characteristic of the TNF receptor



superfamily. Unlike many members of the TNFR family, however, TACI lacks a death domain in its cytoplasmic tail and must act via other signaling elements in the cytoplasm. TACI is expressed in subsets of B cells, as well as activated CD4⁺ T cells, and its mRNA can be found in spleen, small intestine, thymus, and peripheral lymphocytes. TACI was first characterized by its interaction with the protein CAML, or calcium-modulator and cyclophilin ligand, and its subsequent activation of the protein NF-AT, or nuclear factor of activated T cells transcription factor. NF-AT activation is extremely important to lymphocyte action, and the CAML protein is a co-inducer of NF-AT activation, a process which is dependent on calcineurin. The cross-linking of TACI appears to have an effect on AP-1 and NF- κ B activation as well as NF-AT activation. (19)

TACI.
Courtesy of
Jaime Darce.

The function of TACI has been determined through the creation of both knockout and transgenic mice. TACI knockout mice have enlarged spleens with normal architecture, indicative of the increased number of mature circulating and splenic B cells, almost double the number than that of wild type mice. They also have a marked decrease in IgM and IgA, indicating defects in B cell class switching. These knockout mice have a normal T cell dependent response, but impaired TI responses, particularly to TI-II antigens. This phenotype resembles that of B cell lymphomas, and also resembles autoimmune disorders like SLE. In contrast to the autoimmune phenotype of knockout mice, the administration of soluble TACI protein into mice inhibits antibody production and the production of germinal centers in the spleen, and the injection of soluble TACI into spontaneously autoimmune mice suppresses kidney damage and increases survival in those mice. Based on this data, it appears that TACI is a positive regulator of B cell activation in TI-II responses, as well as a negative regulator of B cell proliferation. (20)

In a very recent discovery, another ligand besides BlyS and APRIL has been found for TACI. Syndecan-2, a transmembrane heparan sulfate proteoglycan, is a ligand that is specific for TACI and does not bind to BCMA or BAFF-R. This discovery was made using recombinant TACI-Fc fusion protein as a probe in a cDNA library screen. The screen was performed using 293T cells, which do not express detectable levels of mRNA for BlyS or APRIL. While it is known that BlyS binding to TACI typically downregulates cell proliferation, and APRIL binding mediates TI-II responses and B1 B cell function, the function of syndecan-2 in binding to TACI is still unknown. It is, however, known that syndecan-2 can mediate TACI signaling, and as syndecans typically mediate cell adhesion, motility, proliferation, and differentiation, the binding

of syndecan-2 to TACI may mediate any of these functions. This new development will certainly prove interesting in future TACI research. (21)

Part III: TACI Localization in B Cells

Section XIII: Experimental Logic

As a summer student at the Mayo Clinic, I was assigned to a lab based on my interests, but as I had little knowledge of immunology, I was unsure of exactly what my interests were. I entered the lab of Diane Jelinek, unaware of what project I would work on and how it would turn out. The Jelinek lab primarily focuses on molecular and cellular immunology and tumor biology, with a special concentration on normal and malignant B lymphocytes, B cell chronic lymphocytic leukemia, and multiple myeloma. Because I was entering into the summer with little knowledge in the field of immunology, the lab designed a project for me based on a side project of Jaime Darce, a graduate student in the lab.

Prior to the summer, Jaime and a postdoctoral fellow in the lab, Xiaosheng Wu, had made some very interesting observations during the course of their research. In conducting a thymidine proliferation assay to determine the effect of APRIL binding on stimulated B cells, they noticed an interesting discrepancy. When primary human peripheral blood B cells were stimulated with cytokines (IL-2, IL-4, IL-10, IL-12) and CD40L, a T cell dependent stimulus, they were induced to proliferate. The proliferative effect of CD40L on the B cells was stopped by the addition of APRIL at the same time as the CD40L, indicating that APRIL binding to TACI had a negative effect on CD40L-mediated proliferation of B cells. While this was what Jaime and Xiaosheng expected, they found that they got entirely different results when they changed the stimulus. CpG, a T cell independent stimulus, also induced the B cells to proliferate when added with cytokines (IL-15). When APRIL was added at the same time as the CpG, however, although the APRIL bound to the B cells, it did not have any anti-proliferative effect.

This was a curious difference, as in each case APRIL was binding to TACI, but it was having different effects on the cells.

Intrigued by the differential effect of APRIL on the stimulated B cells, Jaime and Xiaosheng set out to determine what might cause APRIL to signal differently through TACI in each case. One of the things they examined was the localization of TACI on the surface of these cells. Using fluorescent labeling and confocal microscopy, they made a very interesting observation: TACI was capping at one end in a small subset of the CpG-stimulated cells, creating a polarized morphology in these cells. Through the lower-power confocal microscope in the lab, they saw that the receptor was localizing on one end of the cell, but only in the CpG-stimulated cells. From this observation, my Summer Undergraduate Research Fellowship project was born.

My task, for my summer project, was to characterize the localization of TACI on the surface of normal human B cells under various stimuli. For several different stimuli, I examined the localization of TACI using fluorescent labeling and confocal microscopy. Following the general characterization of TACI localization, my next job was to examine colocalization with markers of polarized cells and with lipid raft markers. For someone unfamiliar with immunology and many of the necessary lab techniques, this was a tall order, but with hard work and a little luck, I was able to finish all of the goals of my project.

In the next few sections of this thesis, techniques, biological molecules, and concepts will be briefly described in a glossary format. The aim of these sections is to provide a general background on the techniques used in my summer research and the reagents and equipment used in these techniques. This background should be sufficient for understanding this thesis, but for an in-depth background on any of these

techniques, it would be best to search scientific papers, manufacturers' websites, or the internet for more information.

Section XIV: Glossary of Methods and Molecules of Interest

The following is an overview of the techniques and molecules used in my project, listed in the order of their use in the project. This glossary is intended to provide a very general background of the techniques. For a more detailed background to any of the techniques or molecules listed in this glossary, please consult a textbook or scientific papers.

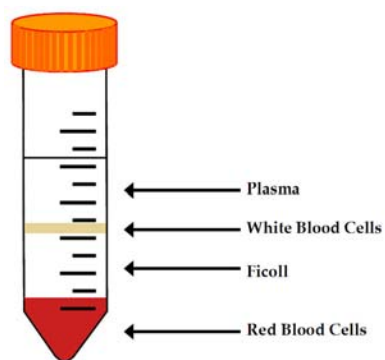
Methods

Buffy Coat

The layer of white/yellow plasma from which the red blood cells have settled out; primarily contains white blood cells. (22) When blood centers get donations, they must filter out the white blood cells and platelets before blood can be used for a transfusion. The buffy coat is used for research or other medical purposes.

Cell Isolation – Density Centrifugation

Isolating a particular group of cells from a larger group of cells or a solution (i.e. from a buffy coat). Cell isolation can be done by density gradient centrifugation, which uses a density gradient (such as that established by the sugar Ficoll) to separate cells by density. Ficoll solution can be added by underlaying beneath the cells (which are suspended in a solution of saline). The cells are then spun in a centrifuge, which forces the more dense matter to the bottom of the tube. To isolate white blood cells from a buffy coat, the middle layer that floats between the clear and red layers is removed for further purification.



Washing Cells

Cells may be “washed” for purification by adding saline and spinning the cells in a centrifuge.

Automated Magnetic Cell Sorting (AutoMACS)

The process of magnetically labeling and sorting cells. Cells can be labeled with an antibody attached to a magnetic bead and sorted based on the marker selected for. The cells are sorted by running them through a magnetic column, which sorts the cells based on being positive or negative for the magnetic



Image courtesy of Miltenyi Biotec, Inc.
www.miltenyibiotec.com

bead. Positive, double positive, and negative selection can be performed. The AutoMACS machine used in this experiment was made by Miltenyi Biotec, Inc. (23)

Cell Culture & Stimulation

Cells can be cultured by placing them in a media and incubating them, in order to allow for studying the cells at a later time. The media depends on the type of cell and length of the culture period, and may have additional nutrients or antibiotics added. Primary human B cells may last up to 5 days without a stimulus, and longer if stimulated. In cell culture, any stimulus can be added to the cells at any time point to study the effect of the stimulus, either short term or long term. In the case of lymphocytes, these stimuli are typically coupled with cytokines to mimic an *in vivo* immune response. The cells can be removed from culture and isolated from the media in order to be studied further. (24)

Labeling Cells with Antibodies

Antibodies can be made to a biological molecule through many different methods. These antibodies will label the location of a particular molecule. If the antibodies are attached to a fluorescent molecule, such as GFP or FITC, the molecule that the antibodies identify will be clearly visible under a microscope or selectable in flow cytometry.

The typical process of labeling cells involves fixing the cells (preserving them in the state they are in and permeabilizing the membrane) and adding fluorescently labeled antibodies. In addition to antibodies, ligands can also be added to identify a particular receptor or molecule.

Confocal Laser Scanning Microscopy

Confocal microscopy is a method of microscopy used to obtain high-resolution and 3D images. The microscope has a pinhole sitting conjugal to the plane of light



(confocal), which keeps light from other planes from hitting the detector. This method allows for blur-free samples of thick specimens. The laser scanning microscope scans the specimen point by point and line by line, and reassembles the image on a computer screen. Multiple scans of the specimen using this method aids in enhancing image resolution and eliminating background, making confocal a very powerful imaging technique. Moving the focus plane during imaging allows for “optical slices” of the

specimen to be taken, making it possible to create and analyze a three dimensional stack of the slices after imaging. Confocal microscopy is an incredibly diverse and useful technology for biomedical research.

While confocal microscopy can be used for bright field images, the ability to do fluorescent microscopy has greatly enhanced the technique. Fluorescent microscopy exploits the excitation and emission spectra of fluorescently labeled antibodies and other molecules in order to capture the location of certain molecules in an image. Multiple wavelengths of light can be used to image molecules with labels of different colors, and most confocal microscopes have two or more wavelength channels for this purpose. Following the same idea, confocal technology can be used for more advanced purposes, such as FRET, FRAP, or FLIP, which further exploit the excitation wavelengths of fluorescently labeled molecules to make more concrete observations about the molecules of interest. While these technologies are beyond the scope of this thesis, many very interesting reviews can be found online which thoroughly summarize the use of each. (25)

Reagents

Saline

A salt solution. Frequently used for diluting biological materials such as blood for research purposes.

Ficoll

Also called Isolymp, Ficoll is a sugar solution used to set up a density gradient in density gradient centrifugation. Ficoll is useful in separating a certain cell type from a solution based on density of the cells.

MACS Selection Cocktail

MACS selection cocktail is a solution of biotinylated antibody (or antibodies) used to select for a particular molecule on the surface of a group of cells. The antibody is biotinylated, making it possible to magnetically label the antibody with magnetic colloid. The combination of selection cocktail and magnetic colloid is used for selection in AutoMACS cell sorting. In the experiment described in this thesis, the selection cocktail used was for CD19, a cell surface marker of B-lineage cells.

Magnetic Colloid

More simply termed magnetic beads, magnetic colloid is a solution added to cells that have been labeled with biotinylated antibodies. The magnetic beads bind to the biotinylated antibodies, labeling the cells for magnetic cell sorting using AutoMACS.

RPMI

A medium used for the culturing of human leukocytes. The medium is sterile and contains nutrients to support the growth of human cells. Additional nutrients or antibiotics may be added to the medium to ensure the proper growth of cells. (26)

Molecules

Cell Sorting:

CD19

A cell surface molecule that makes up part of the B cell co-receptor. CD19 is often used as a marker of B-lineage cells for flow cytometry and cell sorting. In this experiment, CD19 was used for AutoMACS cell sorting. (1)

Stimuli:

CD40L

Also known as CD154, CD40L is a molecule on T cells that interacts with the molecule CD40 on B cells during a T cell dependent immune response. CD40L drives B cells into the cell cycle, causing them to proliferate both *in vivo* and *in vitro*. It is essential for TD responses. In the experiment described in this thesis, CD40L was used with **IL-2**, **IL-4**, **IL-10**, and **IL-12** to stimulate the B cells. (1)

CpG

Short, single-stranded, unmethylated bacterial DNA. CpG binds to TLR9 on B cells, stimulating them for T cell independent (type 1) responses. It is a potent stimulator of B cells *in vitro*, and has been found to be possibly helpful in lentiviral-based gene therapy. In this experiment, CpG was used along with **IL-15** to stimulate the cells. (1, 28)

LPS

LPS, or lipopolysaccharide, is a component of the cell wall of gram-negative bacteria. It is an inducer of T cell independent type 1 responses and has adjuvant effects. LPS acts

through binding to LPS-binding protein and TLR4 on B cells. In this experiment, LPS was used in the context of **IL-4** to stimulate the B cells. (1)

Anti-Ig (α -Ig)

An antibody to the B cell receptor (BCR) that causes the BCR to cross-link, activating the B cell. Anti-Ig can be directed against Ig molecules of any class, and is typically mitogenic for the B cells. It is used to study T cell independent type 2 responses. In this experiment, anti-IgA and anti-IgG were used in the context of **IL-2**, **IL-4**, **IL-10**, and **IL-12** to stimulate the cells. (1)

IL-2

Interleukin-2, or IL-2, is the cytokine that is most central to an adaptive immune response. IL-2 is secreted by naïve T cells, T_{H1} cells, and some CD8⁺ T cells, and drives T cell development. In B cells, IL-2 stimulates cell growth and J-chain synthesis. (1)

IL-4

Interleukin-4, or IL-4, is a cytokine secreted by T_{H2} T cells. In a T cell-B cell interaction, it is focused at the site of contact between the T cell and the B cell. IL-4 activates B cells, induces growth and class switch (predominantly to IgG1 and IgE), and increases expression of MHC Class II molecules. (1)

IL-10

Interleukin-10, or IL-10, is a cytokine secreted by T_{H2} cells. It increases MHC Class II expression and aids in activating B cells for a T cell dependent response. (1)

IL-12

Interleukin-12, or IL-12, is a cytokine that is naturally produced by macrophages in response to antigenic stimulation. IL-12 typically stimulates T_{H0} differentiation to T_{H1} cells, as well as NK cell development. It has also been shown to have a role in autoimmunity and allergic disease. (1)

IL-15

Interleukin-15, or IL-15, is a cytokine that is similar in properties to IL-2, but utilizes a different receptor complex on the cell surface. IL-15 induces the proliferation of T cells as well as normal and malignant B cells. It has been connected to multiple myeloma. (1, 27)

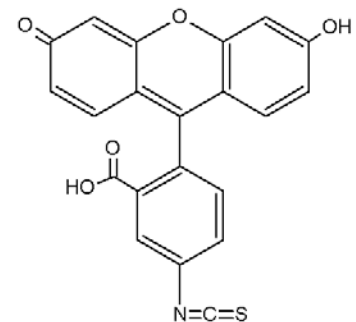
Fluorescently Labeling Cells:

Streptavidin

Streptavidin is a tetrameric protein that binds very tightly to biotin. It is frequently used to tag biotinylated antibodies using a conjugated fluorescent or enzymatic protein. (34)

FITC

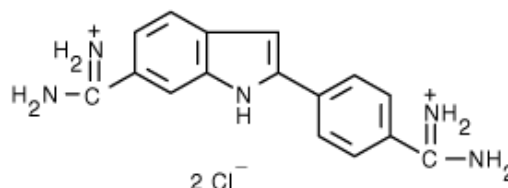
Fluorescein Isothiocyanate, or FITC, is a small, organic, fluorescent molecule that is the most commonly used dye for FACS and confocal microscopy. The optimal excitation wavelength for FITC is 495 nm, and it emits light at 519 nm, in the green light range. Interestingly, fluorescein is also the dye



used commonly in eye exams, and is also used to dye the Chicago River green every St. Patrick's Day. (1, 29, 30)

DAPI

4', 6-diamidino-2-phenylindole, or DAPI, is a fluorescent stain that binds to DNA. DAPI easily passes through the cell membrane, making it effective for staining the nucleus of



live and fixed cells. The optimal excitation wavelength for DAPI bound to DNA is 358 nm, and its emission wavelength is 461 nm, which creates a bright blue color. (29, 31,32)

PE

Phycoerythrin, or PE, is a large, fluorescent protein that is commonly used for FACS and confocal microscopy. Its excitation wavelength is 480 nm and its emission wavelength is 578 nm, creating an extremely bright orange-red color. (1, 29).

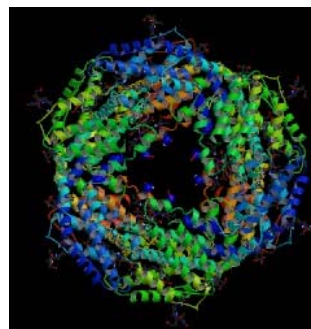


Image courtesy of Protein Data Bank
<http://www.rcsb.org/pdb/>

Cell Surface Markers:

GM1

GM1 is a ganglioside that is highly enriched in lipid raft portions of the cell membrane. It is typically used to mark lipid rafts in lymphocytes, using the β subunit of cholera toxin, which binds tightly to GM1. (5,8)

CD162

P-selectin glycoprotein ligand-1 (PSGL-1), or CD 162, is a cell surface molecule that is expressed constitutively on leukocytes. It is commonly used as a marker of the trailing edge, or uropod, of the cell, as it migrates to the trailing edge in a polarized lymphocyte. (7, 33)

LFA-1/CD11a

Leukocyte Function Antigen-1 (LFA-1), or CD11a/CD18, is a β_2 integrin, or cell adhesion molecule, found on the surface of leukocytes. LFA-1 interacts with ICAM-1 and ICAM-3 to mediate interactions between T cells and B cells. Due to its cell adhesion functions, LFA-1 is commonly used as a marker of the leading edge in polarized lymphocytes. (1, 7)

Section XV: Experimental Materials and Methods

Isolation of B Cells

Normal human peripheral blood mononuclear cells (PBMC) were isolated from a buffy coat by density gradient centrifugation using Isolymph. Cells were washed with saline and viable cells were counted using a Beckman-Coulter cell counter. These cells were sorted using MACS, using CD19⁺ selection for B cells. The cells were then washed using RPMI complete media and resuspended at a concentration of 1×10^6 cells/100 μ L in RPMI.

Cell Culture and Stimulation

Previously sorted CD19⁺ B cells were cultured in RPMI complete media in a 48 well plate. The cells were either left unstimulated or stimulated with CD40L + IL-2, IL-4, IL-10, and IL-12; CpG + IL-15; LPS + IL-4; or anti-IgA and IgG + IL-2, IL-4, IL-10, and IL-12. The cells were incubated for several days at 37°C and removed at various time periods. Cells were examined when freshly isolated, and on days 1, 3, and 5 following isolation.

Immunofluorescent Staining

Once removed from the 48 well plate, the cells were washed and resuspended in FACS buffer. The cells were then stained using monoclonal antibodies (mAb) or ligands to one or more of: TACI, CD162, CD11a, and GM-1. Secondary antibodies, conjugated to FITC or PE, were added when necessary. Control antibodies were used that were appropriate for the molecule being stained for. Cells were incubated with the antibodies in a dark room at 4°C and washed in between incubations with FACS buffer. Following

fluorescent staining and washing, cells were resuspended in 1% paraformaldehyde (PFA) to fix the cells for microscopy.

Please Note: Throughout the course of the experiment, many other molecules were tested briefly as markers of polarization for human B cells. The molecules listed above were found to be the most effective markers, and therefore, other molecules used and the results associated with those markers will not be discussed in this thesis.

Confocal Microscopy

Once stained for fluorescent microscopy, the cells were mounted on a 10-well microscope slide. Vectashield DAPI stain was added to the cells and the slide was covered with a cover slip. The cells were examined by confocal microscopy using a Zeiss LSM 510 inverted confocal microscope. All images were taken on one or more color channel at a resolution of 300X.

Section XVI: Experimental Results

TACI is Differentially Localized in CpG-Stimulated B Cells

Following the stimulation of CD19⁺ PBMC and incubation for several days, the cells were stained and analyzed to determine the localization of TACI on the cell surface.

It was initially found that while TACI is uniformly distributed on the surface of B cells that are untreated or are stimulated with CD40L, a subset of CpG-stimulated B cells exhibits a capping of TACI at one end of the cell (**Fig. 1**). The capping effect was evident by day 3 of the

experiment and grew more pronounced by the fifth day following stimulation. This effect occurs in approximately 10% of TACI-positive cells under CpG stimulation, while it was observed in only 1% of TACI-positive CD40L-stimulated B cells.

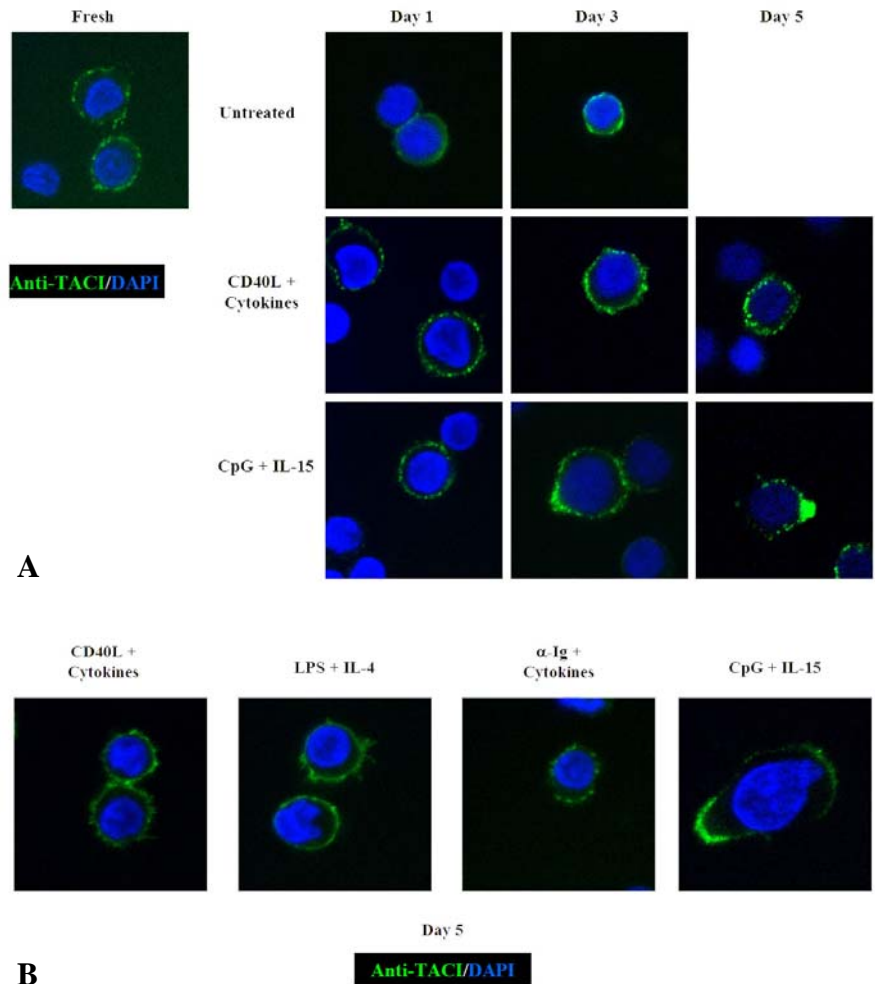


Fig. 1 TACI is differentially localized in CpG-stimulated B cells. (A) In B cells stimulated with CpG, but not with CD40L, TACI localizes at one end of the cell. The capping phenotype appears around day 3 and becomes more drastic by day 5 following stimulation. (B) Cells stimulated with LPS and anti-Ig Ab do not exhibit TACI capping.

Following the initial observations that TACI is differentially localized on CpG-stimulated B cells but not on CD40L-stimulated cells, the research was expanded to include additional stimuli. To determine if the capping phenomenon was common to all TI stimuli, LPS was added as a TI-I stimulus, and α -Ig was added as a TI-II stimulus. The capping phenomenon was not observed with either LPS or α -Ig, and with both stimuli TACI remained uniformly distributed around the cell surface. These results suggest that TACI capping is not common to all TI responses, but do not necessarily indicate that CpG is the only stimulus that causes this phenomenon.

TACI Colocalizes with Lipid Rafts Following CpG Stimulation

To further characterize the localization of TACI, a colocalization experiment was carried out to determine if TACI was associating with lipid rafts under any of the stimuli studied. Colocalization with lipid rafts was examined using the marker GM1, which is known to be constitutively raft-associated (6). In unstimulated B cells, TACI and GM1 are both uniformly distributed throughout the cell surface, showing little to no colocalization. This same pattern is repeated for CD40L, LPS, and α -Ig. Under CpG stimulation, however, both TACI and GM1 exhibit capping on one end of the cell, and a significant amount of colocalization is observed (**Fig. 2**). These results indicate that upon CpG stimulation, TACI localizes to the lipid raft portion of the cell surface.

TACI Does Not Colocalize with Trailing Edge Marker CD162 Following CpG Stimulation

As the cells that exhibited TACI capping had a polarized appearance, the localization of TACI was compared to the localization of known leading edge and trailing

edge markers for polarized lymphocytes. CD162 (also known as PSGL-1) is a known marker of the trailing edge in both B cells and T cells, and therefore TACI was first compared to CD162 under both CD40L and CpG stimulation (7, 9). LPS and α -Ig were omitted from this experiment, as they failed to cause the polarized phenotype and seemed to have no significant effect on TACI localization.

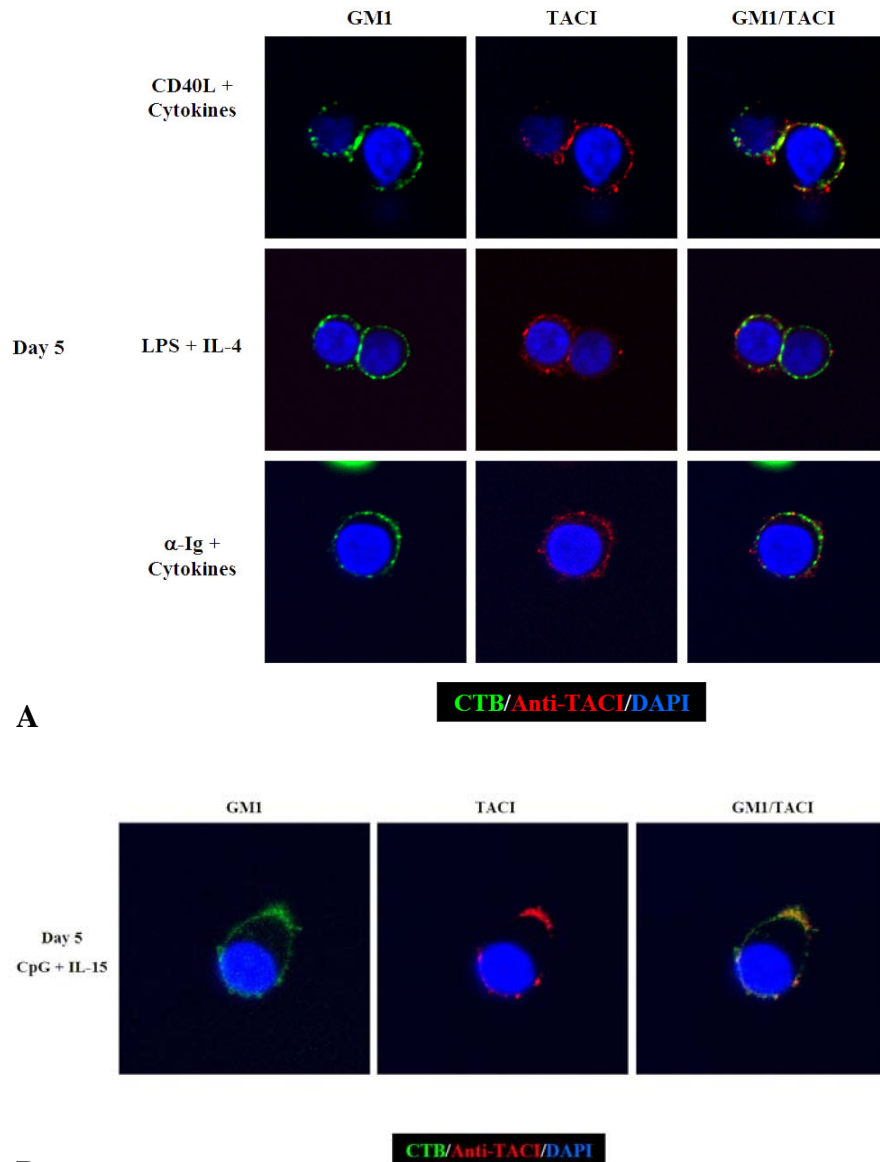


Fig. 2 TACI colocalizes with lipid rafts following CpG stimulation. (A) In B cells stimulated with CD40L, LPS, and anti-Ig Ab, TACI does not colocalize with lipid raft marker GM1. (B) In cells stimulated with CpG, TACI colocalizes with GM1 and the cells exhibit a polarized morphology.

In untreated cells, as well as those stimulated with CD40L, both TACI and CD162 were evenly distributed throughout the cell surface, exhibiting little to no colocalization. In contrast, following CpG stimulation, TACI and CD162 both exhibited differential

localization in a subset of cells, with each molecule moving to an opposite side of the cell (**Fig. 3**). The anti-colocalization of CD162 and TACI suggest that TACI is not migrating to the trailing edge of the polarized, CpG-stimulated B cells. These results were echoed by staining with trailing edge markers CD43 and CD44 (33; preliminary results not shown).

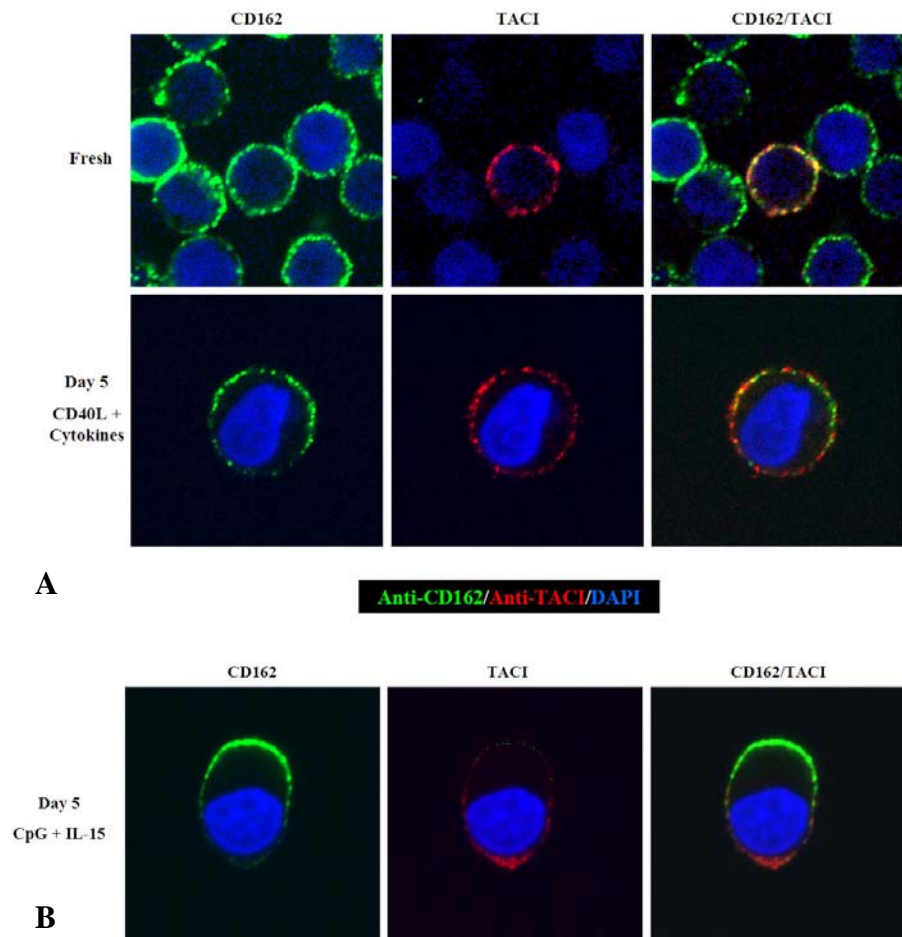


Fig. 3 TACI does not colocalize with uropod marker CD162 following CpG stimulation. (A) In fresh, unstimulated B cells and cells stimulated with CD40L, TACI does not colocalize with CD162, and both molecules are evenly distributed around the cell surface. (B) In cells stimulated with CpG, TACI and CD162 exhibit opposite polarized localization.

TACI Colocalizes with Leading Edge Marker CD11a in CpG-Stimulated B Cells

In order to confirm the results of the CD162 colocalization staining, TACI localization was compared to the localization of CD11a (also known as LFA-1), a known marker of the leading edge in polarized lymphocytes (7). In a similar

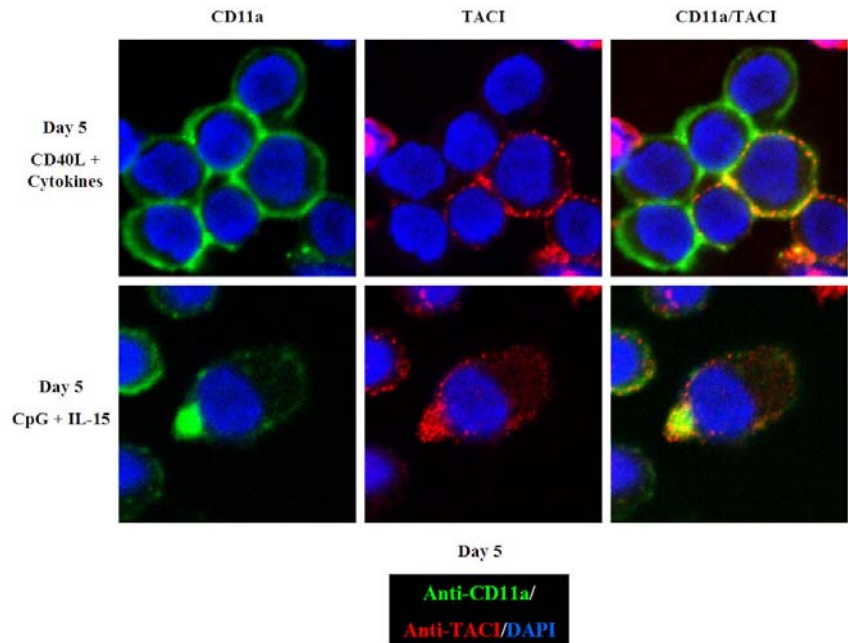


Fig. 4 TACI colocalizes with leading edge marker CD11a following CpG stimulation. In cells stimulated with CD40L, TACI and CD11a are evenly distributed throughout the cell surface on day 5 following stimulation. In cells stimulated with CpG, TACI and CD11a both localize to the same end of the cell.

manner to the other colocalization assays, in unstimulated and CD40L-stimulated B cells, TACI and CD11a showed no significant colocalization, with both molecules being uniformly distributed around the cell. In CpG-stimulated cells, however, TACI and CD11a both localized to the same compartment of the cell surface, exhibiting significant amounts of colocalization (**Fig. 4**). These results, along with the results of staining for CD162 and TACI, suggest that upon CpG stimulation, TACI migrates to the leading edge of the polarized B cell.

Section XVII: Discussion and Conclusion

In preliminary data before the summer's experiments were carried out, it was noted that when APRIL is added to CpG-stimulated B cells, the APRIL does not have the same antiproliferative effect as it does on CD40L-stimulated cells. This data was the inspiration for the experiments described in this thesis, but these experiments are only the first step to discerning the possible mechanism of this difference in APRIL function.

The first task in this long process was the imaging of TACI localization under CD40L and CpG stimulation. It was determined that TACI "caps," or localizes at one end of the cell, upon day 3 in B cells under CpG stimulation but not under CD40L stimulation (10% under CpG stimulation vs. less than 1% under CD40L stimulation). Further detail was added to this intriguing data, including the discovery that TACI does not display capping in B cells following LPS or anti-Ig Ab stimulation. These results indicate that the capping phenomenon is not based upon whether the response is T cell dependent or T cell independent. Although no further stimuli were tested during the summer's experiments, preliminary data from the lab later in the year suggests that the capping phenomenon may not be exclusive to CpG stimulation. Perhaps the CpG stimulation pathway, acting through TLR9, is the key to TACI capping. In order to determine this, we must first determine why TACI may be capping, and how this relates to the lack of APRIL function in CpG-stimulated B cells.

Because lipid rafts are a newly discovered feature of the cell surface of lymphocytes, and lipid rafts have been shown to cluster upon cell stimulation, it was important to determine whether TACI capping upon CpG stimulation was related to lipid rafts in any way. Through the summer's experiments, it was shown that TACI colocalizes with lipid rafts as both cluster at one end of the cell following CpG

stimulation. As of yet, it is unclear whether TACI colocalizes with lipid raft markers immediately following lipid raft clustering or if TACI is recruited to the lipid raft following raft clustering. This would be an interesting topic for further experiments.

To further characterize the localization of TACI in CpG-stimulated B cells, TACI localization was compared to that of trailing edge marker CD162 and leading edge marker CD11a. Because of the polarized morphology of the CpG-stimulated B cells, and the localization of TACI to the smaller end of the cell, it was expected that TACI was migrating to the uropod, or trailing edge, of the cell following stimulation. Upon comparing TACI localization to that of CD162 and CD11a, however, it is evident that TACI is localizing to the leading edge of CpG-stimulated, polarized B cells.

This summer's experiments have shown that upon CpG stimulation, and not CD40L stimulation or stimulation with other TI stimuli, TACI clusters at one end of the cell surface. Furthermore, TACI colocalizes with lipid rafts in the leading edge compartment of the cell membrane. These findings present the intriguing questions of why this clustering occurs, and why TACI may be drawn to the lipid raft portion and leading edge of the cell. While much about receptor clustering, lipid rafts, and cell polarization remains unknown, a few ideas come to mind that may help explain the results of these experiments.

Typically, receptors cluster on the cell surface in response to a stimulus in order to mount an effective, or stronger, response to the specific stimulus. Additionally, lipid rafts are commonly thought of as signaling platforms for clustered receptors, as many of the molecules that cluster in lipid rafts are instrumental in activating signaling pathways. The B cell receptor is one example of a receptor which clusters in lipid rafts, as the BCR must crosslink in order for cell signaling and antigen uptake to occur. In this

model of receptor clustering, the idea is that receptors cluster and become associated with lipid rafts in order to increase the efficiency of cell signaling. This presents an interesting dilemma in the case of TACI, however. In the results that led to this summer's experiments, the CpG-stimulated cells, in which TACI forms clusters, were not as strongly affected (or affected at all) by APRIL binding to TACI on the cell surface. It is, perhaps, possible that APRIL has a different function in these cells, and that in this case TACI is carrying out some form of signaling but does not negatively regulate cell proliferation. An alternative to this explanation is the model proposed by Kwang-Il Lim and John Yin, who suggest that in some cases, receptor clustering and localization in lipid rafts can inhibit signal transduction rather than activating it. (35) By this model, TACI clustering upon CpG stimulation would be a way for the cell to stop TACI from negatively regulation proliferation. Only time will tell if either of these two explanations, or another explanation, can fully discern the reasons for TACI clustering upon CpG stimulation.

Further complicating the dilemma of TACI clustering is the data suggesting that TACI migrates to lipid rafts in the leading edge of the polarized, CpG-stimulated B cells. As much remains to be learned about cell polarization, no concrete answers can be found from this data, but patterns characteristic of the leading edge may provide a hint as to the reasons for TACI clustering in the leading edge of the cell. The leading edge of a polarized lymphocyte is commonly associated with the recognition of chemokines and other signaling molecules, as well as substrate adhesion and intercellular adhesion, particularly migration into tissues and B cell-T cell interactions. As with lipid rafts, it is interesting that TACI migrates to a compartment of the cell in which it is expected to have heightened signaling, while TACI signaling appears to be ineffective in instances in

which TACI clusters. It is also interesting that the leading edge is thought to be instrumental in B cell interactions with T cells, while TACI migrates to the leading edge in response to a T cell independent stimulus. The current profile of a polarized lymphocyte offers many possibilities for TACI function in the leading edge, and additionally it is possible that TACI migrates to the leading edge as a result of being excluded from the trailing edge of the cell. It is also important to remember that much of the research on cell polarization has been carried out on T cells, and therefore questions which are considered answered in T cells may in fact turn out differently in B cells. As more experimentation is carried out in B cells, and cell polarization and TACI localization are examined more carefully, some of these questions may eventually be answered.

While the summer's experiments brought about many answers, they also created many more questions. First among these is the discrepancy in markers for cell surface compartments. In B cells as well as T cells, ganglioside GM1 is used as a marker of lipid rafts on the cell surface. In T cells, it is commonly associated with uropod rafts, but no literature exists describing the localization of GM1 in polarized B cells. (36, 37) Interestingly, in the experiments described in this thesis, TACI has been found to colocalize with leading edge marker CD11a, as well as GM1, in polarized B cells. This data lends the suggestion that perhaps in B cells, GM1 is located in lipid rafts in the leading edge, not the uropod. More experimentation and analysis are needed in order to confirm this suggestion; however it is most definitely an interesting possibility.

Next among the questions raised is the question of the function of TACI clustering. No experiments were carried out to tie the clustering of TACI back to the initial observation that CpG-stimulated B cells do not respond to APRIL in the same way

as cells under other stimuli. In order for the data described in this thesis to be useful, this connection needs to be examined in order to determine the clinical relevance of these findings. A further experiment along this line would be a confocal assay for APRIL binding to the stimulated B cells. One very interesting possibility raised by the data in these experiments is that APRIL binding to TACI utilizes another APRIL binding partner, heparan sulfate proteoglycans, to strengthen the binding reaction with TACI; in this case, TACI clustering may isolate TACI from the heparan sulfate proteoglycans, making APRIL binding ineffective. Determining the location and extent of APRIL binding in the polarized B cells would help to bolster or eliminate this possibility and perhaps elucidate the role of TACI clustering in B cell response to APRIL.

Still other questions remain in regards to the TACI clustering dilemma. Although no clustering was found with CD40L, LPS, or anti-Ig Ab, one must wonder whether TACI clusters only in response to CpG, or if other stimuli may also have the same effect. If other stimuli also cause TACI to cluster, it becomes necessary to determine if the lack of response to APRIL is also coupled to these stimuli, or if TACI clustering may be unrelated to the APRIL response (or lack thereof). Additionally, commonalities may need to be determined between stimuli that cause TACI to cluster, in order to discover the mechanism or logic behind the clustering phenomenon.

All of the questions listed above, as well as many others, still remain to be explored. Because of the importance of APRIL and TACI to B cell research, these topics will certainly be more fully analyzed in the years to come.

Section XVIII: Further Reading

In the months following the experiments outlined in this thesis, much research has taken place to further elucidate the role of TACI in B cell function. Recent developments include the discovery of another binding partner for TACI, syndecan-2, and the connection of TACI to new disorders, including rheumatoid arthritis and Waldenström macroglobulinemia. While it is beyond the scope of this paper to describe each new discovery in detail, a list of the pertinent recent scientific literature follows.

Further Reading:

Bischof D, et al. 2006. Selective Activation of TACI by Syndecan-2. *Blood*. 107:3235-3242.

Describes the discovery of a new binding partner for TACI, syndecan-2, and the activation of TACI by syndecan-2.

Elsawa SF, et al. 2006. B-lymphocyte stimulator (BLyS) stimulates immunoglobulin production and malignant B-cell growth in Waldenström macroglobulinemia. *Blood*. 107:2882-2888.

Relates patterns of BLyS overexpression and expression of BCMA, BAFF-R, and TACI to patients with Waldenström macroglobulinemia.

Fu L, et al. 2006. Constitutive NF- κ B and NFAT Activation Leads to Stimulation of the BLyS Survival Pathway in Aggressive B Cell Lymphomas. *Blood*. First Edition Paper.

Describes the activation of the BLyS survival pathway in B cells in individuals with lymphomas.

Diaz-de-Durana Y, et al. 2006. TACI-BLyS signaling via B-cell-dendritic cell cooperation is required for naive CD8⁺ T-cell priming *in vivo*. *Blood*. 107:594-601.

Suggests the necessity of interactions between B cells and DCs via TACI and BLyS for the generation of adequate numbers of APCs to prime naïve CD8 T cells for CTL response.

Salzer U, Grimbacher B. 2005. TACItly changing tunes: farewell to a yin and yang of BAFF receptor and TACI in humoral immunity? New genetic defects in common variable immunodeficiency. *Curr Opin Allergy Clin Immunol*. 6:496-503.

Describes the discovery of genetic defects in TACI and BAFF-R in patients with common variable immunodeficiency (CVID); elaborates on possible roles for TACI in B cell biology.

Seyler TM, et al. 2005. BLyS and APRIL in rheumatoid arthritis. *J. Clin. Invest*. 115:3083-3092.

Relates TACI expression to a pattern of diffuse synovitis in patients with rheumatoid arthritis.

Zheng Y, et al. 2005. A role for B cell-activating factor of the TNF family in chemically induced autoimmunity. *J. Immunol*. 175:6163-6168.

Describes the phenomenon of Hg-induced autoimmune syndrome, as well as the role of BLyS in mediating this autoimmunity. TACI-Ig inhibits Hg-induced autoantibody production.

Closing Comments

Through all of the work I have done on my thesis this year, I have learned quite a bit. In addition to learning about the importance of having a work ethic and not leaving all of the work for a large project until the last minute, I have strengthened my basic knowledge of biology, as well as my background in immunology, immensely. I have built upon my understanding of the BLyS/APRIL system and TACI in B cells, and I have come to grasp the reasons and basic research behind my summer research project. I have also discovered much about the extensive complexity and interrelatedness of the field of biology.

Most importantly, writing a thesis has been a process of self-discovery. What I have written about in this paper is not just a summer research project anymore; it is a pathway to what I hope will be a long and satisfying career. Perhaps my interests will change slightly and I will move away from B cell biology or from the specific systems I have studied, but after spending a year intensively researching and learning about immunology, I know that it will be a lifelong passion. I have also discovered that I am capable of intensive, self-directed research, and that I will be able to handle the rigors of a graduate education in immunology.

As I finish this thesis, I finish my final year at Boston College, secure with the knowledge that regardless of what happens, something substantial has already come of my education here. I have certainly gained much during my four years here, and in particular I have developed the knowledge and discipline necessary to write something this long. It is my hope that as a reader, you have enjoyed this journey as much as I have.

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