ABSTRACT

Title of Thesis: B CELL MEMORY, CD23, AND LIPID METABOLISM: A PRELIMINARY STUDY

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Each of us receives multiple vaccinations since our birth, which protect us from many infectious pathogens. These vaccines establish long-term protection through generating a memory subset of lymphocytes. This study explores whether CD23, an Fcε receptor, and a high fat diet have any role in regulating the generation of memory B cells. The role of CD23 in B cells was examined using CD23 transgenic mice. My data show that both mouse and human memory B cells express lower levels of CD23 than follicular B cells. In response to antigenic stimulation, CD23 co-aggregates with the B cell receptor. However, the percentages of isotype switched B cells as well as other major peripheral B cell subsets in the spleen are not altered in unimmunized CD23 transgenic mice, where B cells express 1.5 fold of the normal level of CD23, in comparison to those

of wild type mice. This implicates that CD23 does not have any significant role in the generation of memory B cells.

The impact of diet on B cells was examined using mice fed with a high fat and/or high cholesterol diet. The mice used possessed either a knockout of the gene *NPC1L1*, an intestinal cholesterol transporter or the wild type gene as an additional level of control. In comparison with mice fed a normal Chow diet and NPC1L1 knockout mice, neither a high fat diet nor a high fat/high cholesterol diet caused any significant changes in the percentages of isotype switched B cells in the spleen. However, both the high fat diet with and without high cholesterol led to increases in the percentages of mature follicular B cells and decreases in the percentage of transitional B cells in a NPC1L1 independent manner. Furthermore, the percentage of marginal zone B cells is significantly increased in the spleens of mice fed with high fat and high cholesterol diet. This result suggests a possible role of high fat and high cholesterol diet in regulating the peripheral development of B cells.

B CELL MEMORY, CD23, AND LIPID METABOLISM: A PRELIMINARY STUDY

by

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Thesis submitted to the Faculty of the Graduate School of the
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DEDICATION

To the only true super hero I have ever met and the greatest guarding angel a son could ever have… Janice D. Montgomery.
Rest in peace.

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ABBREVIATIONS

ABC – ATP Binding Cassette Transporter Protein

AF488 – Alex Fluor 488

Ag – Antigen

APC – Allophycocyanin

APC – Antigen Presenting Cell

Balb/c – An inbred strain of albino laboratory mice originally founded in1913 by Halsey J. Bagg

BCR – B Cell Receptor

BSA – Bovine Serum Albumin

Btk – Bruton's Tyrosine Kinase

C57BL6 – An inbred strain of black laboratory mice created in 1921 by C. C. Little

CD21 – Cluster of Differentiation 21

CD23 – Cluster of Differentiation 23

CD27 – Cluster of Differentiation 27

CD4 – Cluster of Differentiation 4

CD8 – Cluster of Differentiation 8

DAG – Diacylgycerol

DME-BSA – Dulbecco's Modified Eagle Medium supplemented w/ Bovine Serum Albumin

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic Acid

ELISA – Enzyme-linked Immunosorbent Assay

Fab – Fragment Antigen Binding

FACS – Fluorescence-Activated Cell Sorter

Fc – Fragment Crystallizable Region

FcR – Fc Receptor

FcγRIIb – Fc Gamma Receptor IIb

FcεR – Fc Epsilon Receptor

FDC – Follicular Dendritic Cells

FITC – Fluoresin Isothiocyanate

FO – Follicular Cell

FSC – Forward Scatter

HFD – High Fat Diet

hPBMC – Human Peripheral Blood Mononuclear Cells

Ig – Immunoglobulin

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM – Immunoglobulin M

Igα – Immunoglobulin alpha

Igβ – Immunoglobulin beta

IP3 – Inositol 1,4,5-trisphosphate

ITAM – Immunoreceptor Tyrosine-based Activation Motif

ITIM – Immunoreceptor Tyrosine-based Inhibition Motif

KO – Knock Out

L1 – Abbreviation for the NPC1L1 Transmembrane Protein

LPS – Lipopolysaccharide

Lyn – Yamaguchi Sarcoma Viral (v-yes-1) Oncogene

MFI – Mean Fluorescence Intensity

MHC I – Class I Major Histocompatibility Complex

MHC II – Class II Major Histocompatibility Complex

mIg – Membrane Immunoglobulin

mIgM – Membrane Immunoglobulin M

mRNA – Messenger Ribonucleic Acid

MZ – Marginal Zone

NF-κB – Nuclear Factor Kappa-Light Chain Enhacer of Activated B cells

NFAT – Nuclear Factor of Activaed T cells

NP – A hapten also known as 4-Hydroxy-3-nitrophenylacetyl

NPC1L1 – Neimann-Pick C1-Like 1 Transmembrane Protein

PAMP – Pathogen-Associated Molecular Pattern

PBS – Phosphate-Buffered Saline

TIRFM – Total Internal Reflection Fluorescence Microscopy

PFA – Paraformaldehyde

PI3K – Phosphoinositide 3-kinase

PIP – Phosphatidylinositol 3-phosphate

PIP2 – Phosphatidylinositol (3,4)-bisphosphate

PKC – Protein Kinase C

PLCγ – Phospholipase C Gamma

RBC – Red Blood Cells

RNA – Ribonucleic Acid

SH2 – Src Homology 2 Domain

SHIP – SH2 Domain-Containing Inositol 5'-phosphatase

SHP-1 – SH2-Containing Tyrosine Phosphatase 1

SSC – Side Scatter

Syk – Spleen Tyrosine Kinase

TCR – T Cell Receptor

TCR – T Cell Receptor

Tg – Transgenic

WBC – White Blood Cell

Wt – Wild type

INTRODUCTION

Though the specific chemical and biological components of vaccines used today may differ considerably, they all essentially seek a similar result: the successful priming of the immune system such that a strong secondary response will be generated upon subsequent exposure to the same pathogenic antigen of choice. The effective nature of this secondary response is known as immunological memory. Immunological memory is established through the production and proliferation of memory cells. B cells and T cells are the only well established immune cell types to produce memory subsets [1]. The primary focus of my thesis is the memory B cell, whether the generation of these memory B cells is affected by the expression of the cell surface protein CD23 and/or a high fat diet. Preliminary data has shown that memory B cells generally have lower CD23 expression when compared to naïve non-memory cells [2]. Previous studies have shown that an over-expression of CD23 (via the use of transgenic mice) reduces the production of IgE and IgG antibodies upon secondary exposure [3]. This suggests a role for CD23 in down-regulating memory B cell responses. Data generated from my work not only corroborates previous data showing low CD23 expression on the memory B cell population, but also shows a decrease in the percentage of memory B cells present in the spleen of CD23 transgenic mice. The central hypothesis of this project is that the CD23 molecule plays a negative regulatory role in the generation of memory B cell responses by either inhibiting the generation of memory B cells or the activation and differentiation

of memory B cells into plasma cells. A mechanistic understanding of CD23's role in memory B cell activation can help to broaden our knowledge of memory B cell activation and the secondary immune response.

The Immune System

The immune system has two major branches: the innate and the adaptive immune systems. Working with physical barriers like skin and mucosal membranes, the innate immune system acts as a rapid second line of defense against potentially harmful pathogens. Neutrophils, macrophages, natural killer cells, and the complement proteins are all players of the innate immune system. These cell types and proteins can act in an antigen-nonspecific manner in that they do not recognize any specific antigen expressed by pathogens. Instead, they recognize the more general pathogen-associated molecular patterns (PAMPs) [4]. Some of the more common PAMPs include bacterial and fungal cell wall components, flagellin, LPS, double stranded RNA, and unmethylated DNA. In addition to rapidly responding to pathogens, the innate immune response can also play an important role in alerting and directing the adaptive immune system to the danger through antigen presentation and cytokine production [1]. In this way, both the innate and adaptive immune systems can work together to clear infections. The cells of the immune system, both innate and adaptive, communicate through the use of specialized chemical signals (in the form of secreted cytokines) and cell surface receptors [5]. These secreted cytokines can act as signals to other immune cells by binding to specific receptors on the surfaces of the cells. This binding can initiate a cytoplasmic signaling cascade that stimulates the cell into performing some specified action [5]. The general characteristics of the innate immune system is that it is very quick, but nonspecific, and it

responds similarly each time it's activated. The adaptive immune system, however, has the ability to adjust the level and specificity of its response depending on the type of pathogen as well as how often a specific pathogen has been encountered.

The development of immunological memory is the hallmark of the adaptive immune system [1]. Lymphocytes are the major players of adaptive immunity. Cells of the adaptive immune system express antigen receptors on their surfaces. These antigen receptors are members of the immunoglobulin family that can bind to specific epitopes present within a particular antigen [6, 7]. The cells of the adaptive immune system use these antigen receptors to constantly monitor their surroundings for the presence of pathogenic epitopes. The two key players of this adaptive immune system are T cells and B cells.

T cells are considered to be somewhat like the directors of the adaptive system. This cell type has the ability to utilize its antigen receptor (also known as the T cell receptor or TCR) to scan other cells for the presence of foreign antigens [1]. All nucleated mammalian cells express a group of specialized proteins known as the major histocompatibiltiy complexes (MHCs) on their surfaces [8]. There are three different classes of MHC molecules present on mammalian cells, but current knowledge suggests that only class I MHC (MHC I) and class II MHC (MHC II) are important players in the adaptive immune system [1]. The MHC I molecules are responsible for presenting protein antigen from the cytoplasm. The fragmented peptides of the antigens are bound to MHC I and then displayed on the cell's surface. MHC II molecules, on the other hand, are responsible for presenting protein antigen that has been collected extracellularly via endocytosis. Expressed primarily by cells classified as professional antigen presenting

cells (APCs), including dendritic cells, macrophages, and B cells, MHC II complexes bind fragments of endocytosed extracellular proteins in endosomes and display them at the surface of professional APCs. T cells can use their TCRs to scan antigen presented by MHCs [4, 9]. The two major T cell subsets responsible for monitoring cells expressing MHCs are the CD4 positive helper T cell and the CD8 positive cytotoxic T cell [10]. The CD8 positive T cells are called 'cytotoxic' since they have the ability to directly kill their targeted cell [10]. Under normal conditions, CD8 positive T cells bind specific antigen-loaded MHC I molecules which induces signaling cascades in T cells and triggers their killing mechanisms which lead to apoptosis or necrosis of the target cell [6]. The CD4 positive T cells are helper cells that play major roles in monitoring and regulating the responses of other immune cells. CD4 positive T cells scan the MHC II molecules on the surfaces of professional APCs using their TCRs. The binding of TCRs to antigen-loaded MHC II will activate T cells to secrete cytokines that in turn activate antigen presenting cells [4, 9, 11].

One of the major contributions of B cells to the adaptive immune system is to mediate antigen-specific antibody responses. B cells scan for antigen using B cell receptors (BCRs). Different from TCRs on T cells, BCRs can directly bind to specific antigens without the presentation by MHC molecules [4, 9]. The specificity of BCRs to antigen allows them to effectively capture, internalize, process, and eventually present the antigen on the B cell surface. Through presenting antigen bound to MHC II, B cells can activate CD4 positive T helper cells, and activated T cells in turn provide essential help for B cell activation [11]. Once activated, B cells proliferate and differentiate into memory B cells and antibody producing plasma cells [5, 12]. Once differentiated, these

plasma cells will continuously secrete antibody specific for their cognate antigen. The additional signals generated from T helper cells to B cells through antigen presentation is essential for generating memory B cells and secondary antibody responses.

The binding of antigen or antigen-MHC complexes to TCRs or BCRs causes these surface receptors to oligomerize in lipid rafts [6, 13], which helps to recruit many cytoplasmic signaling molecules to the receptors and induces their activation. The activation of these molecules induces signaling cascades which can lead to cell proliferation and expansion of lymphocyte clones that are specific for the threat. For T cells, this proliferation leads to the generation of effector, memory, and regulatory T cells [14]. For B cells, activation of signaling cascades can result in differentiation of B cells into antibody-producing plasma cells [9]. This initial antibody response mainly consists of relative low affinity IgM isotype antibodies named as the primary immune response [Fig. 1]. These IgM antibodies have the ability to bind antigen with specificity and to opsonize their targets. The resulting effect of the antibody opsonization is the neutralization of their secreted toxins [15] and 'flagging' of the pathogens for complement and immune cell-mediated killing [15]. Antibody opsonized pathogens are more efficiently phagocytosed and killed by phagocytotic cells than pathogens that have not encountered antibody [16]. In addition, IgM-immobilized pathogens are very effective in activating the complement and the innate immune system [15].

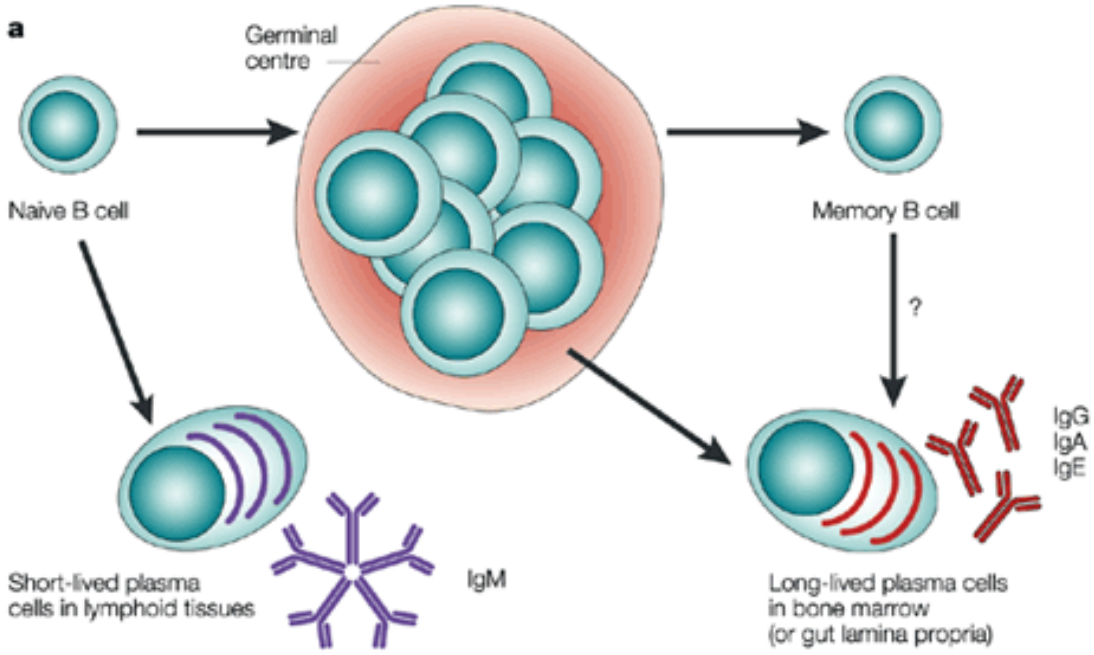

Figure 1: Simplified model of B cell activation [17].

The B Lymphocyte

Antibodies are a product of the adaptive immune system. They are synthesized and secreted by plasma cells, the effector subset of B lymphocytes or 'B cells' [18]. During development in the bone marrow, B cells undergo multiple steps of gene-rearrangement to express a functional BCR [19]. The BCR is a cell surface molecule that allows the B cell to bind and recognize its cognate antigen [19-21]. B cells expressing BCRs that bind to self antigen in the bone marrow are negatively screened and prevented from maturing under normal circumstances [19].

Once the immature non self-reactive B cells finally migrate away from the bone marrow to the secondary lymphoid tissues, they become known as transitional B cells [22]. Transitional B cells receive surviving signals in the secondary lymphoid tissue and become naïve mature B cells [22]. Mature B cells that reside in the spleen or lymph nodes can be divided into two main populations: follicular B cells (FO) and marginal

zone B cells (MZ). FO B cells are the major subset of B cells in the secondary lymphoid tissue and reside within the lymphoid follicles. FO B cells express low levels of IgM, high levels of IgD and CD23, but not CD5 and have the ability to recirculate. In contrast, the MZ B cells usually remain located along the marginal edge of the lymphoid follicle and the red pulp of the spleen. The marginal zone is an area where macrophages and dendritic cells also reside. MZ B cells are non-recirculating, but can be rapidly recruited into the early adaptive immune response with the help of T cells [23]. MZ B cells usually express high levels of IgM and CD21, but low levels of IgD, and CD23. Recent studies show that MZ B cells play an essential role in transporting antigen into B cell follicles [23].

B Lymphocyte Activation

The B cell receptor (BCR) is the central player in B cell activation [19]. The BCR consists of two major parts: the antigen-binding and the signaling subunits. The antigen-binding subunit consists of membrane immunoglobulins (mIgs), providing two antigen-binding sites in each mIg. The signaling subunit is comprised of the Igα and Igβ heterodimer [Fig. 2]. While all five isotypes of Igs have membrane form, naïve B cells express mIgM and mIgD-based BCR. The Igα and Igβ heterodimer has distinctly longer cytoplasmic tails than mIgM [21]. Each of their cytoplasmic tails contains an immunoreceptor tyrosine-based activation motif (ITAM). Upon binding of antigen to the BCR, the tyrosine residues of the ITAMs are phosphorylated by Src family kinases [20]. Phosphorylation of the ITAMs allows for the docking of spleen tyrosine kinase (Syk), a Src homology 2 (SH2) domain-containing kinase [21, 24]. Antigen binding also induces the phosphorylation of the ITAMs on the BCR co-receptor CD19 [19], which recruits

another SH2 domain-containing kinase, phosphatidylinostitol 3-kinase (PI3K). Though recruitment of PI3K via ITAMs on CD19 is considered to be the major mechanism by which PI3K locates to the plasma membrane, it has also been suggested to be recruited by other mechanisms [19]. Once bound to phosphorylated ITAMs, Syk can associate with and phosphorylate adapter proteins such as B-linker protein (BLNK). The activated BLNK protein can then recruit phospholipase Cγ (PLCγ) to the complex. The phosphorylation of PLCγ by Syk partially activates it. Meanwhile, the activated PI3K molecule phosphorylates and converts phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) [19]. This conversion of PIP2 to PIP3 allows for the recruitment of the Bruton's tyrosine kinase (Btk) protein to the membrane through direct interaction with PIP3. Once associated with the membrane and in proximity of the other signaling molecules, Btk can phosphorylate PLCγ, which in combination with the phosphorylation by Syk, activates the PLCγ molecule [19]. Activated PLCγ can convert PIP2 into diacylglycerol (DAG) and soluble inositol-1,4,5-trisphosphate (IP3). IP3 activates calcium flux. Calcium and DAG activate protein kinase C (PKC) that, in turn, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is critical for B cell development, maturation, and proliferation [25]. Calcium can bind to the serine/threonine phosphatase calcineurin. Calcineurin can then dephosphorylate the protein nuclear factor of activated T-cells (NFAT), which causes it to translocate into the nucleus where it participates in transcriptional activation of genes associated with proliferation [19]. While the signaling transduction of the BCR in naïve mature B cells has been extensively studied, the mechanisms underlying memory B cell activation remain to be defined.

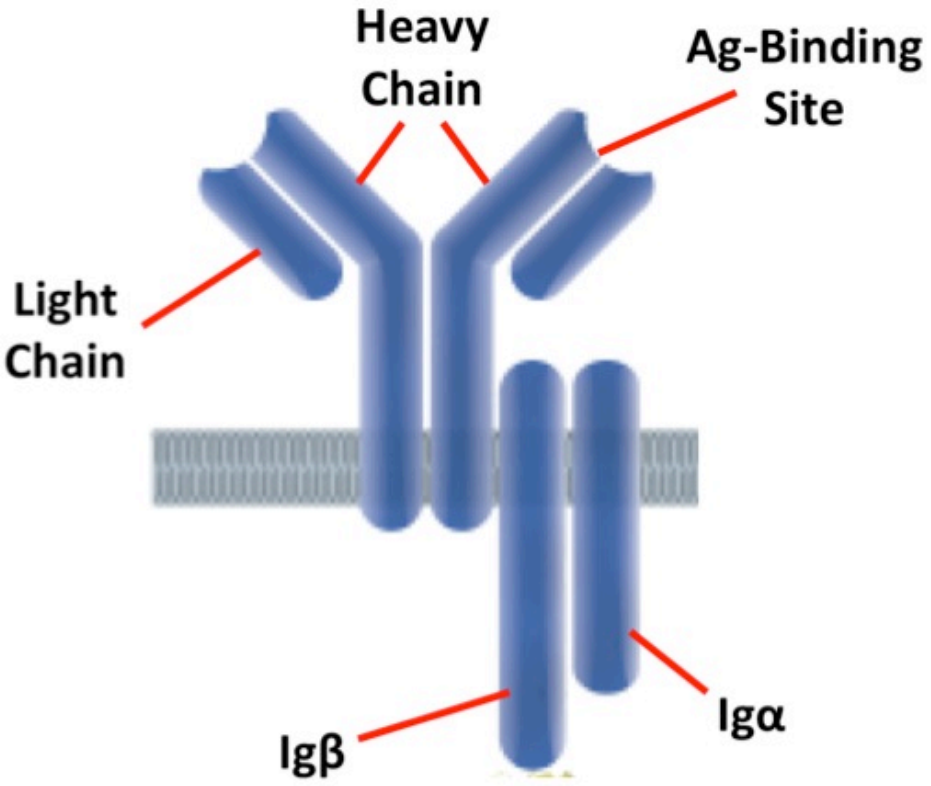

Figure 2: Schematic of the B cell receptor complex [21].

The BCR activation process is balanced by down regulation to control the level and duration of B cell activation. Malfunctions in these down regulatory mechanisms are associated with autoimmune diseases. A major negative regulatory co-receptor in B cells is Fcγ receptor IIB (FcγRIIB). Upon binding to antibody-antigen immune complexes, FcγRIIB can inhibit B cell activation through its downstream signaling molecule SH2-containing tyrosine phosphatase 1 (SHP-1). The cytoplasmic tail of FcγRIIB contains the inhibitory immunoreceptor tyrosine-based inhibition motifs (ITIMs). FcγRIIB binds to the Fc domain of an IgG-antigen immuno-complex that colligates FcγRIIB with the BCR that binds to antigen. The colliagtion brings the cytoplasmic tails of the Fc receptor and the BCR into close proximity causing the ITIMs on the Fc receptor to become phosphorylated by Lyn kinase [26]. Once phosphorylated, the ITIMs can recruit

inhibitory signaling molecules like SHIP-1 as well as tyrosine phosphatases SHP-1 and SHP-2. SHP-1 and SHP-2 dephosphorylate many of the signaling molecules associated with BCR signaling [27]. SHIP converts PIP3 to PIP2, blocking the recruitment of Btk to the plasma membrane [28]. CD23 is another Fc binding coreceptor expressed on FO B cells, but its role in B cell signaling and activation remain unclear.

Memory B Cells

In the presence of cognate antigen and T cell help, B cells undergo rapid proliferation, resulting in the formation of very large clusters of B cells known as germinal centers [7]. Within the germinal centers, B cells undergo isotype switching (typically to the IgG isotype) which determines the immunological function of the antibodies their descendant plasma cells will be able to generate [Fig.1]. In the presence of T cell help via antigen presentation by B cells and follicular dendritic cells, B cells go through somatic hypermutation and affinity maturation, where the variable regions of their mIg genes become highly mutated and B cells expressing high affinity antibody are selected for survival and differentiation into memory B cells. Therefore memory B cells express isotype switched receptors that have a greater affinity for their cognate antigen than naïve B cells [8].

The memory B cell is a key component of the adaptive immune system and is considered to be the central player in the development of long-term humoral immunity. They are long-lived and differentiate into long-lived plasma cells that secrete high affinity antibodies upon stimulation [29, 30]. Upon reencountering an antigen, memory B cells that were generated from the first antigen encounter mount a rapid and high affinity secondary antibody response. Therefore, a major characteristic that separates

memory B cells from their naïve parent cells is their significantly lower threshold for activation and rapidly robust proliferative activity [29]. Evidence of these differences can be observed during the secondary immune response where the activation and proliferation of memory B cells results in the rapid production of antibody-secreting plasma cells [29] and differences in serum antibody titers after primary and secondary antigen exposure [Fig. 3].

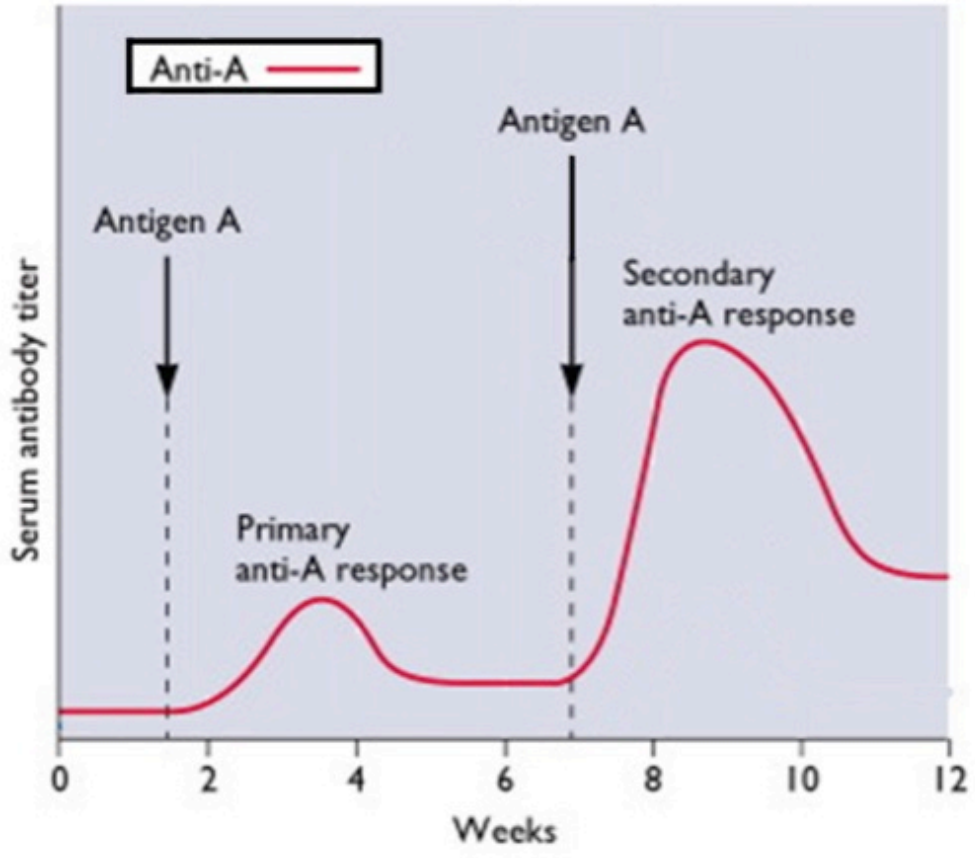

Figure 3: Diagram displaying serum antibody titer after primary and secondary immunization with generic 'Antigen A' [31].

The ability to produce memory cells that can rapidly respond to and clear specific pathogenic antigens before they are able to do much damage is a very powerful attribute of the immune system. Vaccines developed today attempt to take advantage of this process in order to protect us from dangerous pathogens. The most effective vaccines are

those that can induce the development of large numbers of memory cells that can react quickly to their target pathogen without causing any adverse effects to the host organism or patient. From a scientific standpoint, it is somewhat surprising how little we know conclusively about how memory B cells are generated and activated. Although several studies have identified multiple strategies for stimulating stronger memory B cell responses, the underlying molecular mechanisms driving this process still remain unclear [32].

The CD23/FcεRII Molecule

The CD23 molecule was originally discovered as an Fc receptor of IgE (FcεRII). It is a 45kD type II membrane protein and is expressed by several different cell types, most notably activated macrophages, follicular dendritic cells, and B cells [33]. There are currently two known isoforms of the molecule expressed in humans: CD23a and CD23b. The two CD23 isoforms differ only by the first 6-7 residues of their cytoplasmic N-terminal regions [34]. Current studies show the expression of the CD23a isoform to be B cell exclusive [33], however expression of the CD23b isoform can be induced in several different cell types including B cells, T cells, monocytes, and dendritic cells [35]. There is currently only one known isoform of CD23 present in mice, which most closely resembles the CD23a human isoform [33]. The CD23 molecule is initially classified as a low affinity receptor for IgE, but this only appears to be the case when the molecule exists as a monomer [36]. When CD23 becomes oligomerized, its affinity for IgE becomes comparable to that of the high affinity IgE receptor, FcεRI [37]. CD23 has been suggested to act as a negative regulator of both IgE and IgG responses [33, 34]. Though the exact mechanism of this action is not completely understood, it has been shown that

binding of IgE to CD23 leads to an inhibitory signaling cascade that is associated with down-regulation of IgE responses [36]. Fujiwara et al. [38] found that both the antigen-specific and overall levels of IgE and IgG were greater during secondary exposure to antigen in CD23 deficient mice when compared to wild type mice. Payet et al. [34], using a CD23 transgenic mouse model, found that both the primary and secondary IgE and IgG responses were significantly decreased in the mice that over-expressed CD23 when compared to wild type mice. Their results further suggested that expression of CD23 on FDCs play a key role in down-regulating the IgE response through interactions with CD21 on IgE positive B cells [34]. A more recent study shows that CD23 positive B cells could bind and effectively transport IgE-bound antigen to FDCs where it is then processed and presented to T helper cells [39]. These data clearly support the notion that CD23 plays a regulatory role in both IgE and IgG antibody responses through multiple mechanisms. The findings that CD23 expression not only affected the primary response but also the secondary immune response imply that this molecule may play a role in the activity of memory B cells. What remains to be addressed is whether CD23 regulates the development of memory B cells or differentiation of memory B cells into plasma cells. It is possible that cross-linking of CD23 by immunological complexes containing IgE induces inhibitory signals in B cells. This would help to explain the observation of lower antibody titers in mice over-expressing CD23. This thesis aims to explore these essential questions with the hope of shedding light on some of the mechanisms by which humoral memory responses are regulated.

Lipid Metabolism and Immunity

Recent studies have shown that inflammatory immune responses may play a significant role in diseases caused by lipid metabolism disorders like obesity and type II diabetes [40-43]. Multiple studies have demonstrated that development of these metabolic diseases can be exacerbated by the disruption of the reverse cholesterol transporter (RCT) pathway [42]. The RCT pathway is responsible for reducing blood plasma cholesterol levels via a high-density lipoprotein (HDL) mechanism. Upon entering a blood vessel with a high concentration of cholesterol, macrophages can take up large amounts of the sterol and become immobilized which keeps them in a sustained inflammatory state and can lead to lesions associated with vascular plaque buildup [41]. In addition to inflammation, recent studies have demonstrated a direct link between the development of insulin resistance and B cell-mediated autoimmunity in obese mice [42]. B cells were found to be responsible for high fat diet-induced insulin resistance, the production of pathogenic IgG autoantibodies, and activation of pro-inflammatory T cells and macrophages in adipose tissues. This combination subsequently led to insulin resistance and glucose intolerance in obese mice.

The Neimann-Pick C1-Like 1 protein (NPC1L1) is a transmenbrane protein associated with lipid metabolism and more specifically with cholesterol transport and absorption into the liver and small intestine [44]. In humans, the NPC1L1 protein (L1) is primarily expressed in the liver with slightly less expression in the small intestine. In mice, the L1 protein is almost exclusively expressed in the small intestine [44, 45]. In studies involving a L1 KO mouse model, it was found that *NPC1L1* gene knock out mice developed a high level of resistance to hypercholesterolemia after being placed on a high

cholesterol diet when compared to wild type [44, 46]. A somewhat similar phenomenon is observed in human patients suffering from high cholesterol who are administered ezetimibe (a drug designed to target and inhibit the L1 protein). Multiple studies with human patients treated with ezetimibe demonstrate the drug's effectiveness in lowering blood cholesterol levels [47, 48].

Studies conducted by Winer et al. [42] suggest that high fat diet-induced obese mice developed visceral fat tissue inflammation and symptoms of type II diabetes due, in large part, to the activity of B cells. Their data shows that although pro-inflammatory macrophages, T cells, and B cells are all recruited to the fat tissue in obese mice that develop disease, B cell deficient obese mice showed no development of disease and reductions in proinflammatory cytokines produced by T cells [42]. When serum IgG antibodies from symptomatic mice are transferred into B cell deficient obese mice, they quickly develop both insulin resistance and glucose intolerance [42]. The development of the disease in obese mice could be attenuated through treatments designed to deplete B cells [42]. Considering their data, Winer et al. concluded that B cells contribute to the development of insulin resistance and glucose intolerance through producing autoantibodies and activating T cells in adipose tissues. However, how disorders of lipid metabolism impact on B cell activation and self-tolerance is largely unknown.

The goal of this research is to explore the role of CD23 in and the impact of high fat diet on the generation of memory B cells. Using CD23 transgenic mice, I examined the effect of over expression of CD23 on peripheral B cell subsets. Using mice fed with a high fat diet, I examined whether the high fat diet has any impact on peripheral B cell subsets.

SIGNIFICANCE

The B cell-mediated antibody response is an essential component of adaptive immunity. Memory B cells are responsible for humoral memory responses. Immunological memory is the basis of vaccination. Because of their longevity, the regulation of memory B cell generation and activation is critical for the control of the immune response. Malfunctions in this regulatory mechanism could lead to deficiencies in humoral protection against pathogens or the long-term production of autoantibodies that are associated with autoimmune diseases and allergic reactions [49, 50]. This could lead to the overreaction of the host's immune system to otherwise harmless antigens in the form of chronic allergies or diseases like asthma [30]. Memory B cells that are reactive to self antigens could lead to the development of life-threatening auto-immunity where the immune system becomes a deadly enemy to the host organism itself [16, 51]. As was described in the introduction, the activity of B cells and B cell-generated autoantibodies has been directly linked to obesity-associated insulin resistance [42]. Obesity induced diabetes has become epidemic in the United States and many European countries and effective intervention is urgently needed. This is where a more thorough understanding of the regulatory mechanisms of memory B cells becomes most valuable.

My Master thesis research has generated preliminary data for future examination of several major questions: does CD23 affect memory B cell development and activation, does CD23 impact BCR signaling, and does the proper function of cholesterol transporters linked to obesity affect the B cell mediated immune response? In addressing the major questions of this project, we will increase our understanding of the underlying mechanisms for the regulation of long-term B cell-mediated immunity. A more complete

understanding of these mechanisms could lead to more effective vaccine development strategies and advancements in the treatment of B cell-associated autoimmune diseases.

METHODS

Isolation of B cells

The human B cells were acquired from hPBMCs isolated from leukopak blood donations using Ficoll gradient centrifugation and the Miltenyi Biotec™ B cell isolation kit along with AutoMACS™. After successful isolation, the cells were counted, treated with a cryopreservation solution containing DMSO, aliquoted into separate vials containing approximately $4.0x10^6$ cells/mL, and then stored in liquid nitrogen.

The mouse B cells were harvested and then isolated from ground mouse spleens using Ficoll gradient centrifugation followed by treatment with T cell lysis buffer (Thy1.1 antibody and complement proteins). After a period of panning (37°C; 5.0% CO_2), the cells were counted and then diluted to an approximate concentration of $2.0x10^6$ cells/mL. The cells were then stored at 4°C before use.

Mouse Models

To address the role of CD23 in B cell activation, I used both CD23 transgenic and knockout mice that were kindly provided by Dr. Daniel H. Conrad from the Department of Microbiology and Immunology at the Virginia Commonwealth University in Richmond, Virginia. Wild type Balb/cJ mice (Jackson Laboratory) were used as controls for these experiments. The CD23 transgenic mice used in this study were established through use of a vector containing a major histocompatibility heavy chain (IgH) enhancer. Use of this enhancer helps to direct expression of the vector to lymphocytes and yields a significant increase in CD23 expression in these target cells [34]. The CD23 knock out (KO) mice were established through use of a vector designed to replace exons

3 and 4 of the *Fcer2a* gene (CD23) with a neomycin resistance cassette. All mice homozygous for the mutant allele effectively silence CD23 expression [60].

To examine the relationship between lipid metabolism and B cell development, I used a NPC1L1 knock out (L1 KO) strain of C57BL/6 mice that were graciously provided by Dr. Liqing Yu at the Department of Animal and Avian Sciences (ANSC) at the University of Maryland at College Park. Wild type C57BL/6 mice were used as controls for these experiments. The L1 KO mice used for this experiment were established through use of a vector designed to disrupt the *NPC1L1* gene at the unique restriction enzyme site AfeI in exon 2 through use of a neomycin resistance cassette [44].

In vitro Activation of B cells

B cell activation was carried out by stimulating the cells *in vitro* with fluorescently-labeled $F(ab')_2$ anti-human or anti-mouse IgG+M antibody (15μg/mL). The antibody was added to the cell solution at a 1:200 dilution and allowed to incubate in a 37°C chamber for 5, 15, and 20min time points. A 0min time point was used as an unstimulated control. This method of stimulation simulates the interaction between B cells and soluble antigen [28].

Immunofluorescence Microscopy Analysis

For the microscopy studies, human B cells were thawed and then allowed to adhere to poly lysine-coated microscope slides. Once the cells had adhered to the slides, they were then stimulated utilizing the method described previously while still on the slides. After stimulation at various predetermined time points, the slides with the human B cells were removed from the 37°C incubation chamber and treated with FcγR blocker at 4°C before being subsequently stained with CD19, CD27, and CD23 antibodies. After

several washing steps with media (DME-BSA), the cells were fixed with a 4% solution of paraformaldehyde (PFA) at 4°C. Phosphate-buffer saline (PBS) was used to wash away the residual PFA and then all excess liquid was removed from the slides. Mounting media (ProLong® Gold Antifade Reagent) was then applied and coverslips were carefully placed onto the slides. The slides were sealed using clear nail polish and stored in low light conditions at 4-8°C for at least 12 hours before analysis using either the Zeiss 710 or Leica SP5X confocal microscopes.

Flow Cytometry Analysis

In order to address the question of whether or not memory B cells expressed CD23, I compared the CD23 surface expression levels between memory B cells and naïve B cells using flow cytometry. The B cells isolated from hPBMCs were initially thawed in a 37°C water bath and then treated with FcγR blocker before being stained for CD19 (B cell marker), CD27 (human memory B cell marker), and CD23 at 4°C. Unstained cell samples as well as samples of cells stained with only a single color were used as controls for all experiments involving flow cytometry. After staining was completed, the cells were then washed, fixed, and analyzed using the BD FACSCanto flow cytometer. CD19 and CD27 double positive cells were gated as memory B cells and the cells that stained CD19 positive, but CD27 negative were considered naïve B cells. The mean fluorescence intensity (MFI) of CD23 of these two populations was then determined.

In order to further address the question of CD23 expression in isotype switched vs naïve B cells in mice, cells were isolated as previously described from the spleens of CD23 transgenic and wild type Balb/c mice. The B cells were treated with FcR blocker

and then stained for B220 (B cell marker), IgM, IgD, CD23, and CD138 (plasma cell marker) at 4°C. Since there is no known memory B cell marker for mice, both IgM and IgD stains were included in order to more clearly visualize the naïve and isotype switched populations. After staining, the cells were washed, fixed, and then analyzed using flow cytometry. Any cells staining positive for CD138 were considered to be of the plasma cell subset and were excluded from any further analysis. Those cells staining positive for B220, but negative for both IgM and IgD were considered to be of the isotype switched B cell population. The B cells staining positive for either IgM or IgD or both were counted as part of the naïve B cell population. Each of these populations was then analyzed for CD23 expression by determining the MFI.

To address the question of whether CD23 expression could effect the development of different B cell subsets, I compared the relative percentages of the subsets present in the spleens of CD23 transgenic, CD23 knock out, and wildtype mice. The B cells were isolated from the spleen as was previously described and then treated with FcγR blocker before staining for B220, IgM, IgD, CD23, CD21, and CD138. Again, those cells that exhibited positive staining for CD138 were not analyzed. B cells that stained negative for both IgM and IgD were counted as isotype switched cells. The follicular subset of B cells (FO) were identified by positive IgM, IgD, and CD23 staining coupled with low staining of CD21. Marginal zone B cells (MZ) were gated via positive staining of IgM and CD21, low staining of CD23, and negative staining for IgD. The T1 subset of B cells were identified through positive IgM, IgD, and CD23 staining, but low CD21 staining. And the T2 subset of B cells was gated through positive staining of IgM and CD23, low staining of CD21, and negative staining for IgD.

In order to address the questions raised in the lipid metabolism study, I compared the relative percentages of different B cell subsets present in the spleens of L1 KO and wild type mice. The B cells were isolated from the spleen as was previously described and then treated with FcγR blocker before staining for B220, IgM, IgD, CD23, CD21, and CD138. Again, any cells staining positive for CD138 were not considered for analysis. Cells that stained negative for both IgM and IgD were counted as isotype switched B cells. Positive IgM, IgD, and CD23 staining coupled with low staining of CD21 identified the FO subset of B cells. MZ B cells were gated via positive staining of IgM and CD21, low staining of CD23, and negative staining for IgD. The T1 subset of B cells were identified through positive IgM, IgD, and CD23 staining, but low CD21 staining. And finally, the T2 subset of B cells was gated through positive staining of IgM and CD23, low staining of CD21, and negative staining for IgD.

Mouse Diet Treatment

For the lipid metabolism study, both the L1 KO and wild type mice were placed on either a normal chow (Prolab RMH 3000) or synthetic high fat diet (Diet Core of Wake Forest University School of Medicine) containing a low (<0.02%) or high (~0.16%) cholesterol supplement at 6 weeks of age. The mice were maintained on their respective diets for a total of 18 weeks while their weights were monitored on a weekly basis [46]. After 16 weeks of the diet treatment, the food intake of the mice was monitored daily for the final 2 weeks of the treatment [46].

RESULTS

Do memory B cells alter their levels of CD23 expression?

In order to address the question of whether or not memory B cells expressed CD23, I compared the CD23 surface expression levels between human memory B cells (CD19 and CD27 double positive) and human non-memory B cells (CD19 positive only) using flow cytometry [Figs. 4a-d]. As was previously described, the human B cells were isolated from leukopaks of healthy blood donors. The mean fluorescence intensity (MFI) of CD23 of these two populations was determined. My results show that the MFI of CD23 in the memory B cells was slightly higher than what was seen in the non-memory B cells [Fig. 4d] though this difference was not statistically significant (P=0.412).

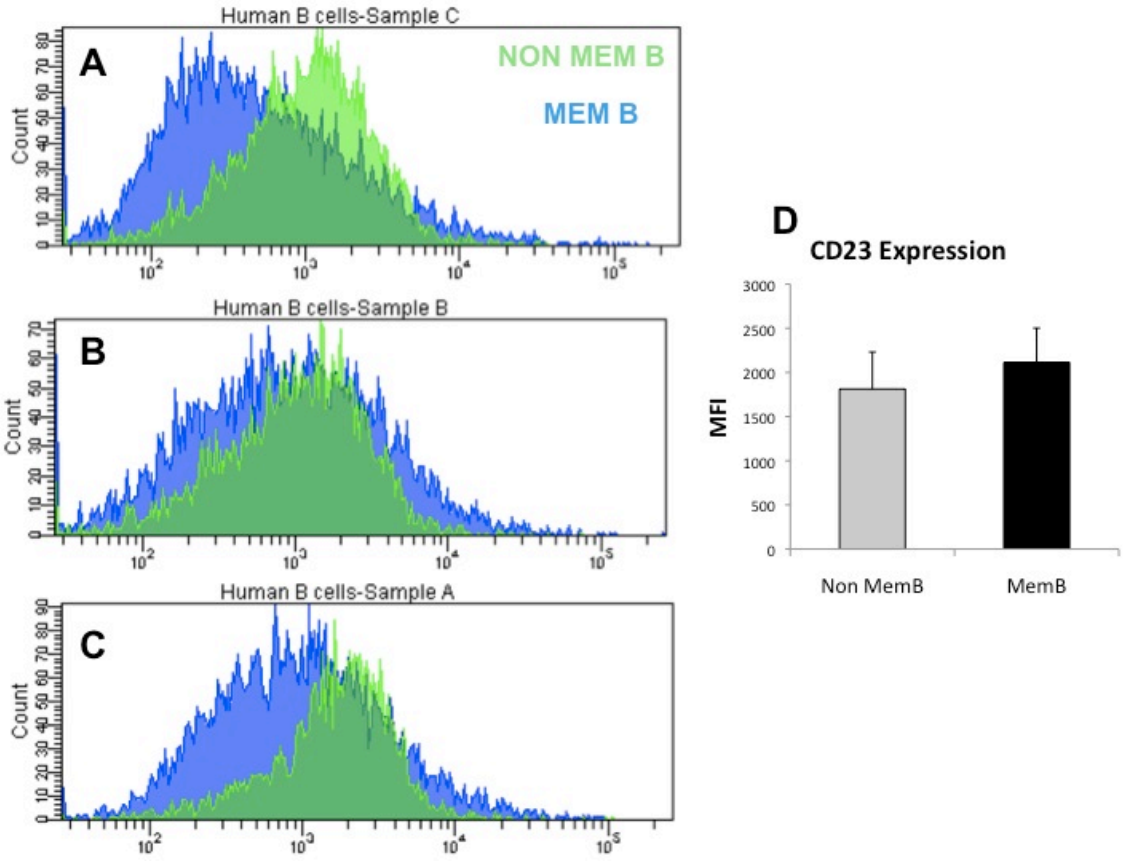

Figure 4: CD23 expression in human memory B cells. (A-C) Representative histograms of five independent experiments displaying mean fluorescence intensity of CD23 expression on human memory B cells (Blue) and human non-memory B cells (Light Green). Human peripheral blood B cells were stained with anti-CD19 (B cell marker), anti-CD27 (memory B cell marker), and anti-CD23 before analysis using flow cytometry. CD19+ CD27+ cells were gated as memory B cells (Black Bar) and CD19+ CD27- cells as non-memory B cells (Grey Bar). (D) Shown are the average MFI values (+SD) from five independent experiments (P=0.412).

In order to further investigate these findings, a similar experiment was conducted using Balb/c wild type mice [Figs. 5a-c]. Again, as was previously described, mouse B cells were isolated from the spleen. Since there is currently no known specific surface marker for mouse memory B cells, I focused on the subset of isotype switched B cells (negative for both IgM and IgD). Since the development of memory in B cells is generally accepted as occurring after isotype switching, choosing this subset seemed logical. The difference in MFI observed in the mice was much more pronounced [Figs. 5a-b], but its significance was merely borderline-at best (P=0.050). Also, in contrast to

the human B cell data, the isotype switched mouse B cells appear to express less CD23 than the naïve mouse B cells [Fig. 5c].

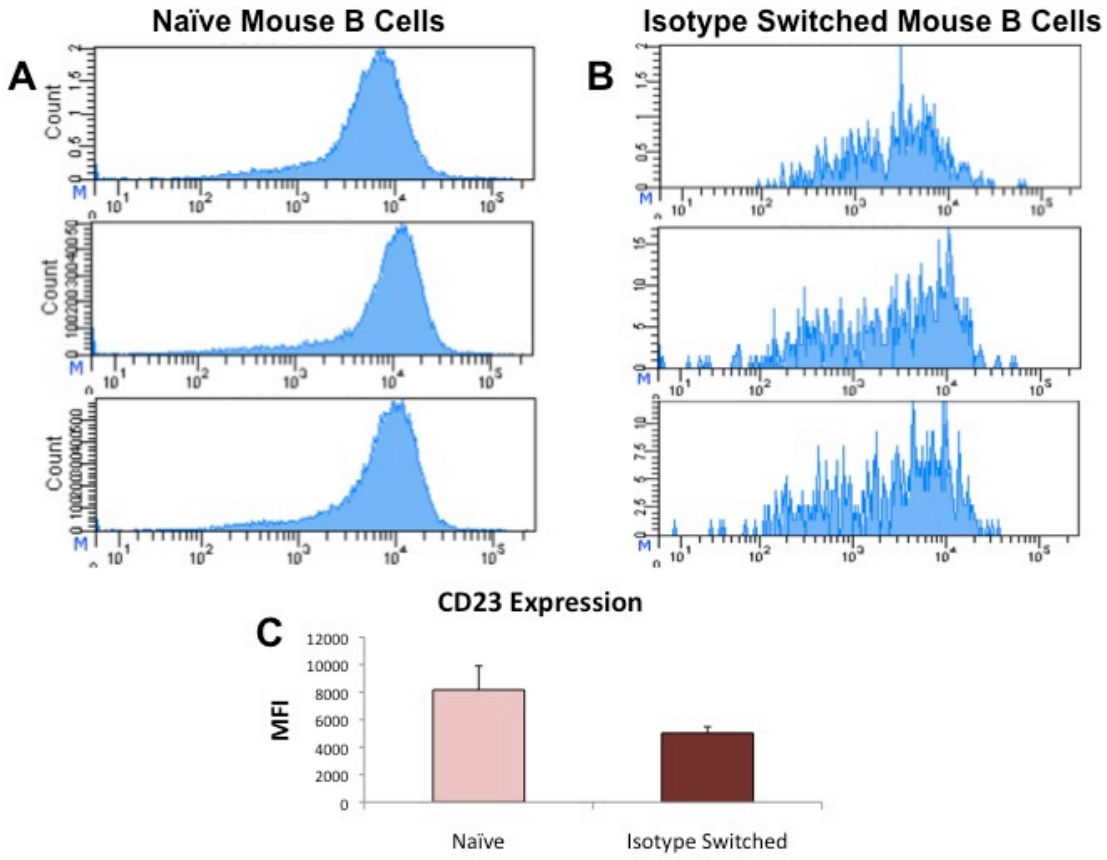

Figure 5: CD23 expression in mouse B cells. Representative histograms of two independent experiments, displaying mean fluorescence intensity of CD23 expression on (A) mouse naïve B cells (left row of histograms) and (B) mouse isotype switched B cells (right row of histograms). Splenic mouse B cells were stained with anti-B220 (B cell marker), anti-IgM, anti-IgD, anti-CD138 (plasma cell marker), and anti-CD23 before analysis with flow cytometry. B220+ IgM+ and/or IgD+ cells were gated as naïve B cells and B220+ IgM- IgD- cells were gated as isotype switched B cells. (C) Shown are the average MFI values (+SD) from two independent experiments using three mice per experiment (P=0.050).

How much do the B cells in CD23 transgenic mice over-express CD23?

To address the role of CD23 in B cell activation, I used CD23 transgenic mice that were kindly provided by Dr. Daniel H. Conrad at Virginia Commonwealth University, Richmond, Virginia. This strain of mice constitutively over-expresses CD23 [34]. To compare the expression level of CD23 in the transgenic mice with wild type mice, splenocytes were harvested from age-matched CD23 transgenic, CD23 KO, and

wild type mice and stained for B220 (B cell marker), CD138 (plasma cell marker), IgM, IgD, and CD23 before analysis using flow cytometry. Any cells staining positive for CD138 were determined to be of the plasma cell subset and were excluded from further analysis. B220 positive cells were gated as B cells and all events within the forward scatter and side scatter range for live cells were considered 'live cells'. Analysis of the data shows that there was an increase in CD23 expression in the total live cells harvested from CD23 transgenic mice when compared to their wild type counterparts [Fig. 6a], but that this difference held no statistical significance (P=0.282). The analysis also showed a predictably sharp decrease in CD23 expression (P=0.013) in the CD23 KO mice when compared with wild type [Fig. 6a]. And then, somewhat surprisingly, though the CD23 transgenic mice showed higher expression of this surface protein over their wild type counterparts [Fig. 6a], the difference was not statistically significant (P=0.077). In B cells harvested from the CD23 transgenic mice, there was also an increase in CD23 expression when compared to their wild type counterparts [Fig. 6b]. Similar to the results observed for the total live cells, however, the difference of expression between the transgenic mouse B cells and the wild type mouse B cells held no statistical significance (P=0.250). Again, similar to the results of the total live cell analysis, there was a sharp decrease in CD23 expression (P=0.013) in the CD23 KO mice when compared with wild type [Fig. 6b]. Finally, almost identical to the total live cell data, the CD23 transgenic mice showed higher expression of this surface protein over their wild type counterparts [Fig. 6b], but the difference was not statistically significant (P=0.062).

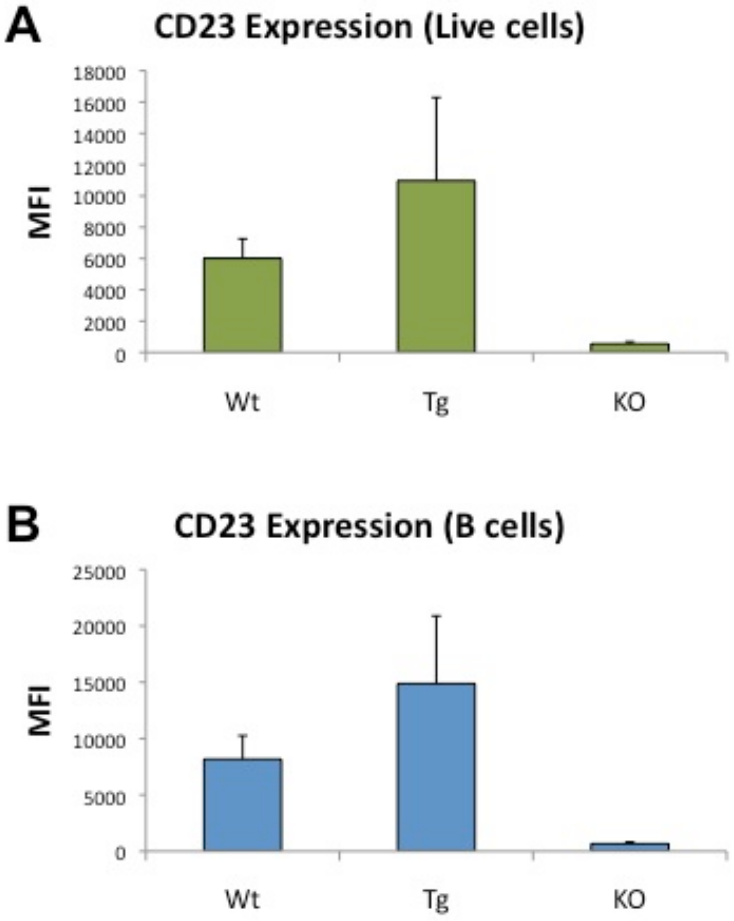

Figure 6: Comparison of CD23 expression in total (A) live cells and (B) B cells only. Mouse splenocytes were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), and anti-CD23 before analysis using flow cytometry. B220+ CD138- cells were gated as B cells and live cells are gated based on the forward scatter (FSC) and side scatter (SSC). Shown are the average MFI values (+SD) from two independent experiments using three mice per expression strain (Wt, Tg, and KO). 6A P-Values (Wt v Tg/Wt v KO/Tg v KO): 0.282/0.013/0.077. 6B P-Values (Wt v Tg/Wt v KO/ Tg v KO): 0.250/0.013/0.062. Wt: wild type mice; Tg: CD23 transgenic mice; KO: CD23 knock out mice.

Does CD23 over-expression in CD23 transgenic mice affect B cell subsets in the spleen?

The over-expression of CD23 has been shown to influence the humoral immune response in mice [34]. Here, I examined the percentages of B cell subsets present in the spleens of CD23 transgenic mice and compared them to the same B cell subsets in the spleens of wild type mice. The splenocytes were stained for B220, CD138, IgM, IgD, CD24, CD21, and the CD23 molecule and analyzed using flow cytometry. The five

different B cell subsets that were observed and for which data was analyzed are as follows: follicular B cells (FO), marginal zone B cells (MZ), transitional 1 B cells (T1), transitional 2 B cells (T2), and isotype switched B cells. All cells staining positive for B220 were considered to be B cells. Any cells showing positive staining for CD138 were determined to be plasma cells and were excluded from any subsequent analysis. The FO B cell subset was defined as double positive for IgM and IgD, CD23 positive, CD24 low, and CD21 low. The MZ B cell subset was defined as double positive for IgM and CD21, CD23 low, CD24 positive, and IgD negative. The T1 B cell subset was defined as double positive for IgM and IgD, CD23 negative, CD21 low, and CD24 positive. The T2 B cell subset was defined as double positive for IgM and CD23, CD24 low, CD21 low, and IgD negative. The isotype switched B cell subset was defined as CD21 positive, CD23 low, CD24 low, and double negative for IgM and IgD. The resulting data showed a slight increase ($P=0.043$) in the percentage of isotype switched B cells in CD23 transgenic mice when compared to the wild type mice [Fig. 8]. There was also a significant increase in the percentage of MZ [Fig. 7b] and T1 [Fig. 7c] B cells present in the spleens of CD23 KO mice when compared to the CD23 transgenic mice ($P=0.033$ and 0.011, respectively). After analyzing all the data, there were no differences in population observed for either the FO or the T2 B cell subsets when comparing between the CD23 transgenic, CD23 KO, and wild type mice [Figs. 7a, d].

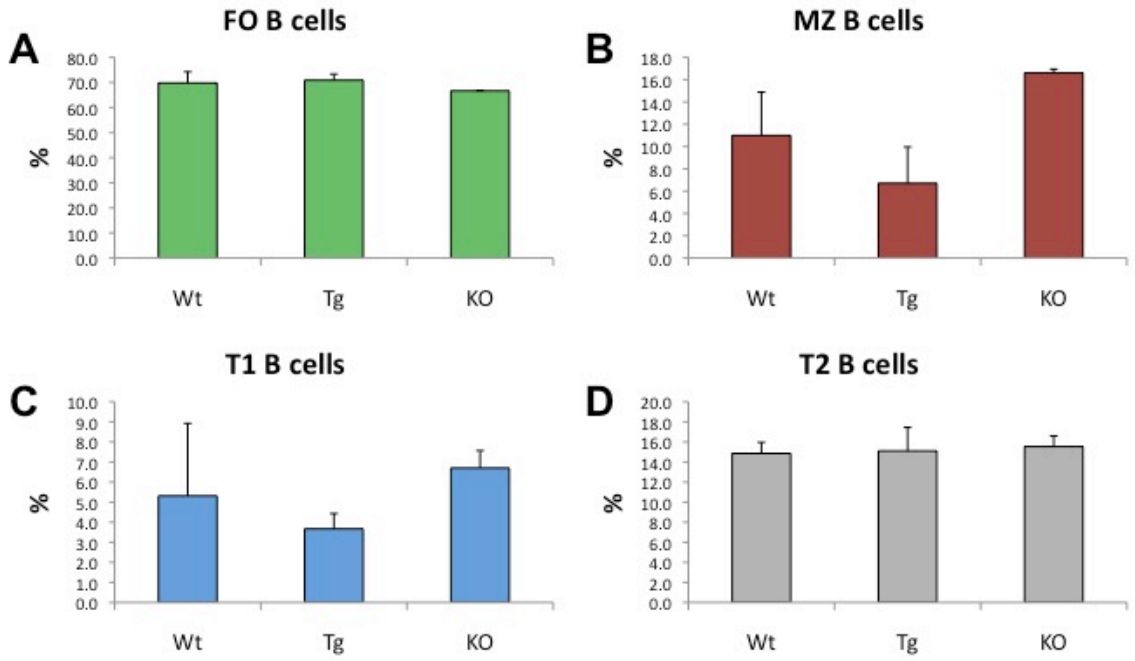

Figure 7: Effect of CD23 over-expression on B cell subsets in the spleen. Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. (A) The follicular B cell subset (FO) was gated as B220+ CD138-IgMlo IgD+ CD23+ CD21Int (Green Bars), (B) the marginal zone B cell subset (MZ) was gated as B220+ CD138- IgMhi IgD- CD23lo CD21hi (Maroon Bars), (C) the transitional 1 B cell subset was gated as B220+ CD138- IgM+ IgD+ CD21lo CD23+ (Blue Bars), and (D) the transitional 2 B cell subset was gated as B220+ CD138- IgM+ IgD- CD21lo CD23+ (Grey Bars). Shown are the average percentages (+SD) from two independent experiments using three mice per expression strain (Wt, Tg, and KO). 7A P-Values (Wt v Tg/Wt v KO/Tg v KO): 0.755/0.179/0.096. 7B P-Values (Wt v Tg/Wt v KO/Tg v KO): 0.371/0.090/0.033. 7C P-Values (Wt v Tg/Wt v KO/Tg v KO): 0.460/0.645/0.011. 7D P-Values (Wt v Tg/Wt v KO/Tg v KO): 0.496/0.145/0.791.

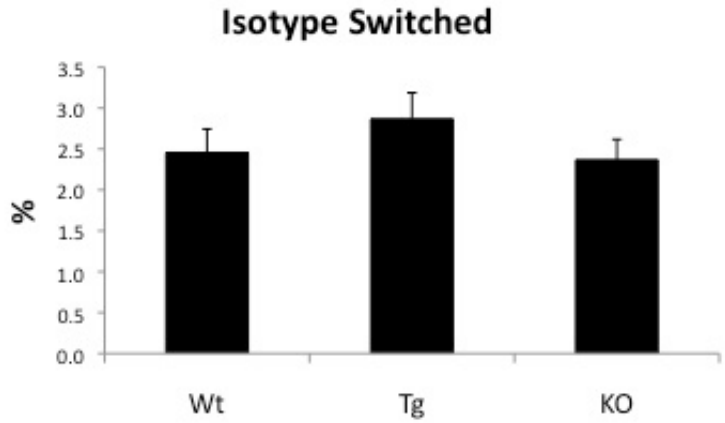

Figure 8: Effect of CD23 over-expression on the isotype switched B cell subset in the spleen. Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. The Isotype switched B cell subset was gated as B220+ CD138- IgM- IgD- CD21+ CD23lo. Shown are the average percentages

(+SD) from two independent experiments using three mice per expression strain (Wt, Tg, and KO). P values (Wt v Tg/Wt v KO/Tg v KO): 0.043/0.352/0.243.

Does CD23 co-cluster with B cell receptor in response to antigen stimulation?

BCR self-aggregation and cap formation has been shown to play crucial roles in B cell activation [28]. To explore whether the CD23 molecule plays a role in BCR activation, I analyzed the cellular distribution of CD23 in relation to the distribution of BCR after stimulation with soluble antigen. Human B cells were incubated in a 37°C chamber with fluorescently-labeled $F(ab')_2$ fragment of anti-IgG+M antibody for 0, 5, 15, and 20min time points. After the stimulation period, the B cells were stained with fluorescently-labeled antibodies against CD19, CD27 and CD23 at 4°C. After fixation, the cells were mounted to poly-lysine slides and analyzed using confocal microscopy. Analysis of these confocal images suggests that surface CD23 can co-cluster with the BCR on stimulated cells [Fig. 9]. In un-stimulated cells (0min time point), neither the BCR nor the CD23 molecule clustered on the cell surface [Fig. 9]. This apparent co-clustering could either be coincidental or it could imply a role for CD23 in antigen-induced BCR aggregation and/or signaling. Though an exact cause for this co-clustering has not yet been elucidated, its pattern seems similar to the inhibitory mechanism that FcγRIIb utilizes to suppress B cell activation [26, 52, 53]. Since CD23 (FcεRII) also belongs to the class of surface molecules known as Fc receptors, and has been previously implicated to play an important part in down-regulating the IgE response [3, 37, 54-56], it would not be too far a stretch to think that this co-clustering is indicative of a role for this molecule in B cell activation.

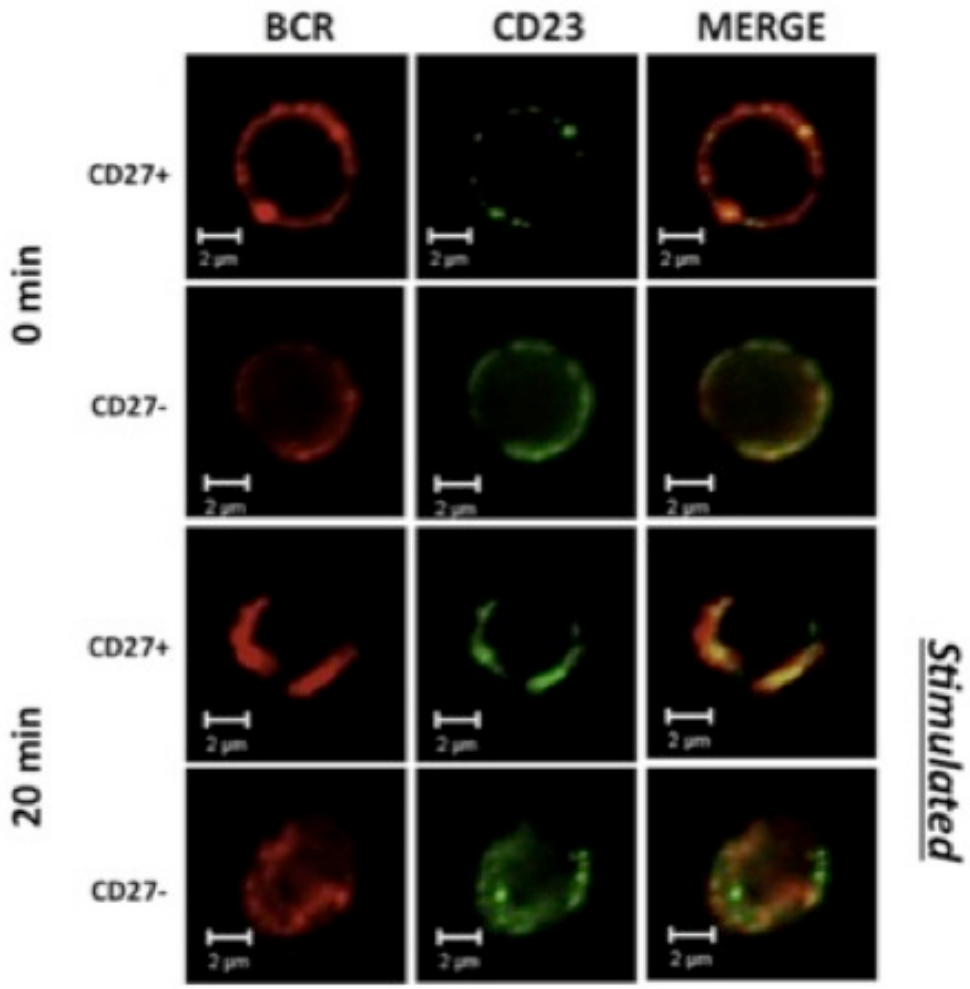

Figure 9: The surface distribution of CD23 in stimulated and un-stimulated human B cells. B cells were treated with fluorescently-labeled anti-IgG+M F(ab')$_2$ antibodies to simulate interactions with soluble antigen for 5, 10, 15, and 20 minutes in a 37°C incubation chamber. Cells incubated for 0 minutes were used as un-stimulated control samples. After stimulation, the cells were immediately transferred to 4°C conditions and stained with fluorescently-labeled anti-CD19, anti-CD27, and anti-CD23 antibodies. Cells were fixed with 4% paraformaldehyde (PFA) solution mixed in non-sterile phosphate buffered saline (PBS). B cell receptor is represented in red and CD23 is represented in green. Shown are representative images from five experiments.

Does high fat diet affect the development of peripheral B cells?

To examine the affect of diet-induced obesity and cholesterol on the development of B cells, I isolated B cells from the spleens of cholesterol transporter Neimann-Pick C1-Like 1 protein (NPC1L1) deficient and wild type mice. The mice had been placed on either normal chow (Prolab RMH 3000) or high fat diet (Diet Core of Wake Forest University School of Medicine) with a relatively high (~0.16%) or low level of

cholesterol (<0.02%) for 18 weeks [46]. The isolated B cells were then stained for the surface markers for different subsets of peripheral B cells, including B220, IgM, IgD, CD23, CD24, CD21, and CD138 before analysis using flow cytometry. The 5 different B cell subsets that were observed and for which data was analyzed are as follows: FO B cells, MZ B cells, T1 B cells, T2 B cells, and isotype switched B cells. All cells staining positive for B220 were considered to be B cells. Similar to the previous CD23 study, any cells showing positive staining for CD138 were determined to be plasma cells and were excluded from any subsequent analysis. The FO B cell subset was defined as double positive for IgM and IgD, CD23 positive, and CD21 low. The MZ B cell subset was defined as double positive for IgM and CD21, CD23 low, CD24 positive, and IgD negative. The T1 B cell subset was defined as double positive for IgM and IgD, CD23 positive, CD21 low, and CD24 positive. The T2 B cell subset was defined as double positive for IgM and CD23, CD24 low, CD21 low, and IgD negative. The isotype switched B cell subset was defined as CD21 positive, CD23 low, CD24 low, and double negative for IgM and IgD.

The resulting data showed a significant increase (P=0.001) in the percentage of FO B cells present in the spleens of wild type mice fed the high fat diet with low cholesterol compared to that in wild type mice fed the chow diet [Fig. 10a]. There appeared to be no difference (P=0.076) in the percentage of FO B cells in the spleens of wild type mice fed with the high fat diet with low cholesterol when compared to those fed with the high cholesterol diet [Fig. 10a]. In L1 KO mice, there was also a significant increase (P=0.016) in the percentage of FO B cells present in the spleens of mice fed with the high fat/low cholesterol diet when compared to those fed the normal chow diet [Fig.

10b]. Although there appeared to be a slight increase in the percentage of FO B cells present in the spleens of L1 KO mice fed the high cholesterol diet over the low cholesterol diet [Fig. 10b], the data reflected no significance (P=0.058). When directly comparing the percentages of FO B cells present in the spleens of L1 KO mice against those present in wild type mice fed the same diet regimen, there were no significant differences [Fig. 10c].

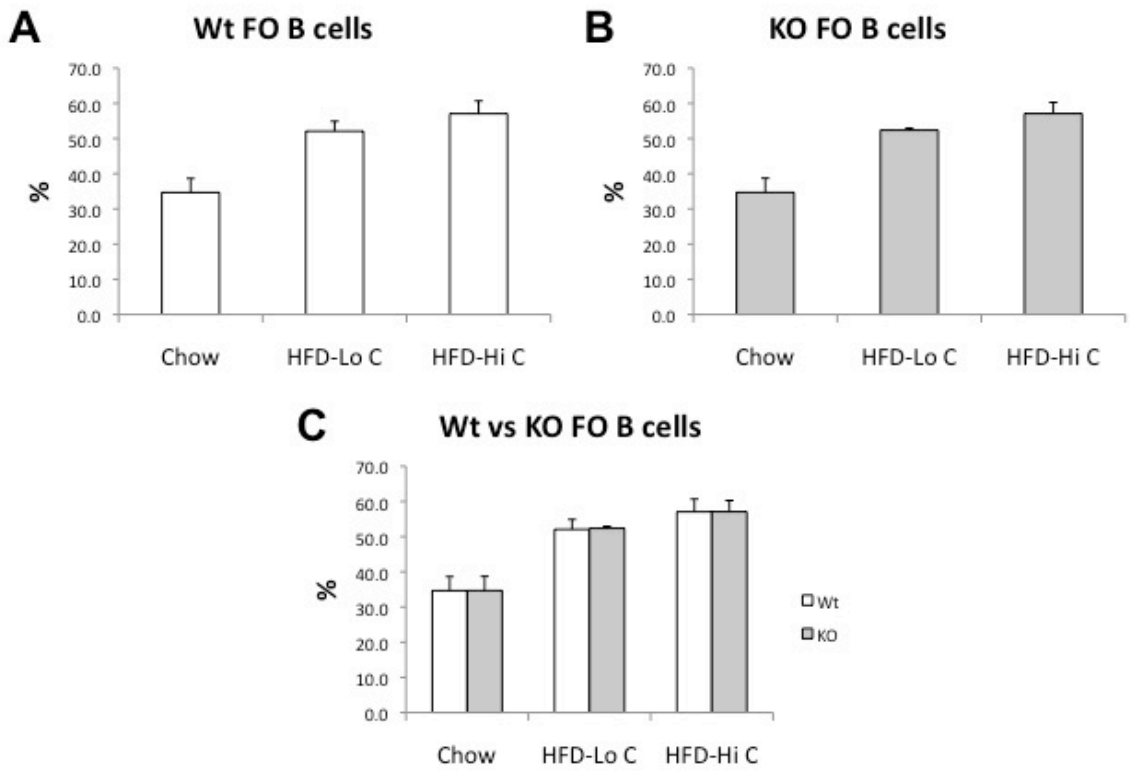

Figure 10: Effects of high fat diet on splenic FO B cells of wild type (A) and L1 KO mice (B) after high fat diet. Mice were fed either a normal chow diet, a high fat diet with low cholesterol (<0.02% cholesterol)), or a high fat diet with high cholesterol (0.16% cholesterol). Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. The FO B cell subset was gated as B220+ CD138- IgMlo IgD+ CD21Int CD23+. Shown are the average percentages (+SD) from two independent experiments using four mice per expression strain (Wt and KO). 10A P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.001/0.076. 10B P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.016/0.058. 10C P-Values (Chow Wt v Chow KO/HFD Lo C Wt v HFD Lo C KO/HFD Hi C Wt v HFD Hi C KO): 0.996/0.847/0.992.

When looking at the relative percentages of MZ B cells present in the spleens of mice fed the high fat/low cholesterol diet with those fed the normal chow diet [Fig. 11a],

there appeared to be no difference (P=0.198). The difference between those wild type mice fed the low cholesterol diet versus those fed the high cholesterol diet [Fig. 11a], however, was rather apparent (P=0.002). Interestingly, the L1 KO mice fed the high fat/low cholesterol diet showed a significant increase (P=0.010) in the percentage of MZ B cells present in the spleens over those fed the chow diet [Fig. 11b]. In the L1 KO mice fed either the high fat diet with low cholesterol or high cholesterol [Fig. 11b], there appeared to be no difference in the percentage of MZ B cells present in the spleens (P=0.487).

When directly comparing the MZ B cell percentages in the spleens of L1 KO mice against those of wild type mice fed the same diet, there were significant differences observed [Fig. 11c]. Interestingly, when fed the normal chow diet, the L1 KO mice appeared to have significantly less MZ B cells present in the spleen when compared to wild type (P=0.003). Similarly, the L1 KO mice fed the high fat/high cholesterol diet also showed a significantly smaller (P=0.015) percentage of spleen MZ B cells when compared to their wild type counterparts who were fed the same diet [Fig. 11c]. By contrast, when both strains of mice were fed the high fat/low cholesterol diet [Fig. 11c], the L1 KO mice had a greater percentage of splenic MZ B cells (P=0.034).

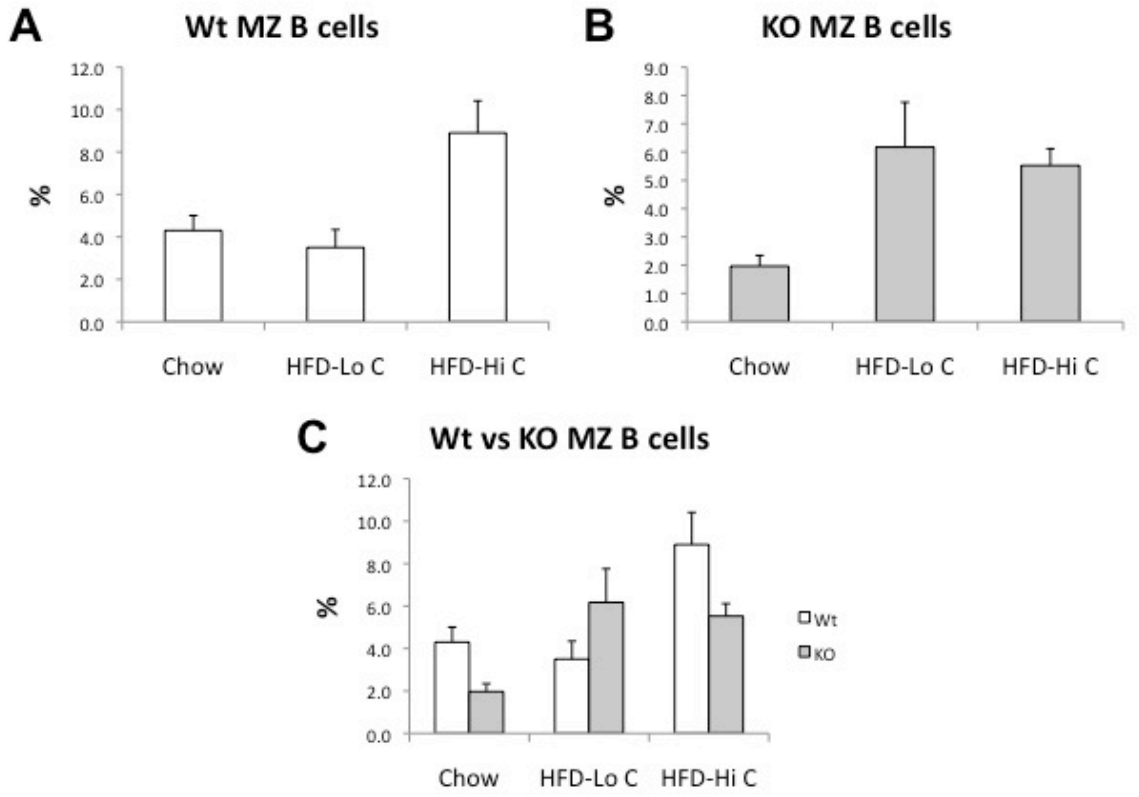

Figure 11: Effects of high fat diet on splenic MZ B cells of wild type (A) and L1 KO mice (B) after high fat diet. Mice were fed either a normal chow diet, a high fat diet with low cholesterol (<0.02% cholesterol)), or a high fat diet with high cholesterol (0.16% cholesterol) Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. The MZ B cell subset was gated as B220+ CD138- IgM+ IgD- CD21+ CD23lo. Shown are the average percentages (+SD) from two independent experiments using four mice per expression strain (Wt and KO). 11A P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.198/0.002. 11B P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.010/0.487. 11C P-Values (Chow Wt v Chow KO/HFD Lo C Wt v HFD Lo C KO/HFD Hi C Wt v HFD Hi C KO): 0.003/0.034/0.015.

Looking at the transitional B cells, we see no significant difference (P=0.205) in the percentage of spleen T1 B cells in wild type mice fed the high fat/low cholesterol diet over those fed the normal chow diet [Fig. 12a]. Interestingly, wild type mice fed the high fat/high cholesterol diet showed a dramatically smaller percentage (P<0.001) of T1 B cells present in the spleen when compared to mice fed the high fat/low cholesterol diet [Fig. 12a]. The L1 KO mice fed the high fat/low cholesterol diet showed a pronounced decrease (P<0.001) in spleen T1 B cells when compared to those fed the normal chow diet [Fig. 12b]. However, when analyzing the percentages of T1 B cells present in the L1

KO mice fed either the high fat/high cholesterol or low cholesterol diet [Fig. 12b], no significant difference was observed (P=0.886).

When directly comparing the T1 B cell percentages in the spleens of L1 KO mice against those of wild type mice fed the same diet, there were significant differences observed [Fig. 12c]. The L1 KO mice fed the normal chow diet appeared to have a larger percentage (P=0.005) of T1 B cells present in their spleens when compared to wild type fed the same diet [Fig. 12c]. The L1 KO mice fed the high fat/high cholesterol diet also showed a higher percentage (P=0.004) of T1 B cells present in the spleen over wild type mice fed the same diet [Fig. 12c]. In contrast, wild type mice fed the high fat/low cholesterol diet appeared to have a larger percentage (P=0.006) of splenic T1 B cells when compared to L1 KO mice fed the same diet [Fig. 12c].

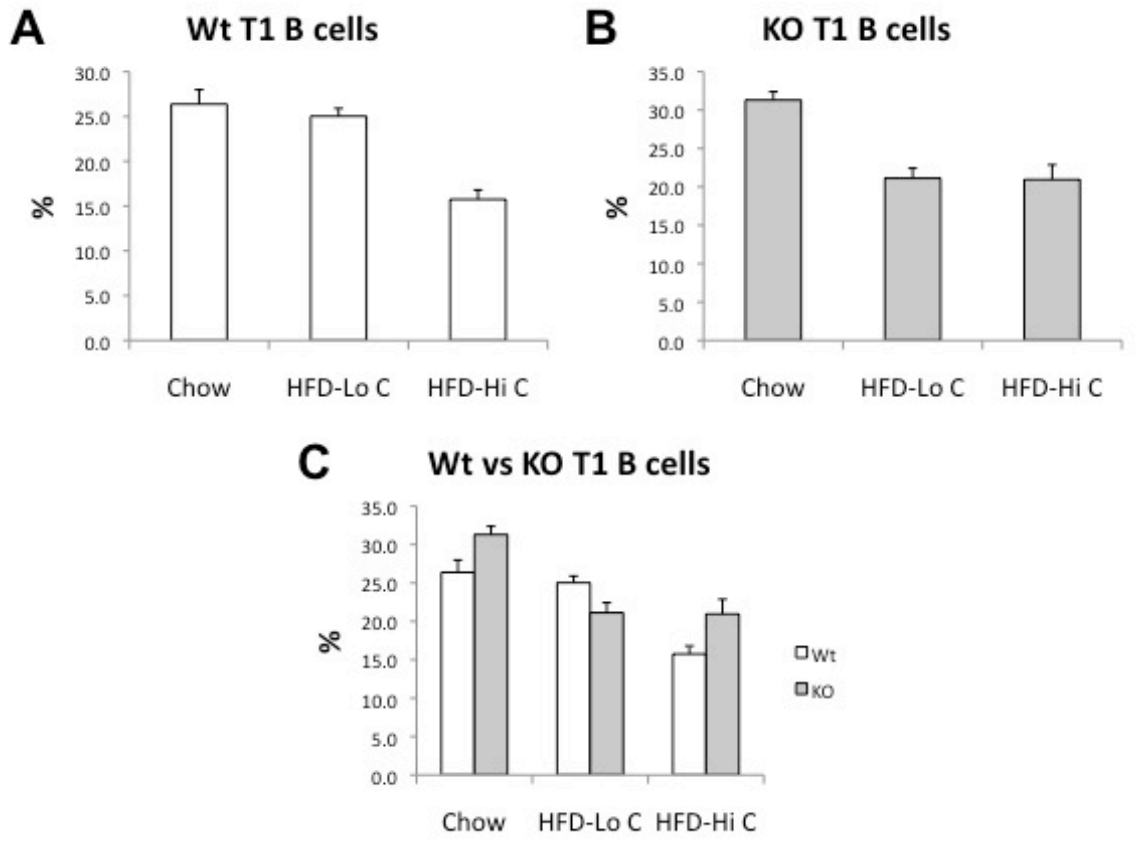

Figure 12: Effects of high fat diet on splenic T1 B cells of wild type (A) and L1 KO mice (B) after high fat diet. Mice were fed either a normal chow diet, a high fat diet with low cholesterol (<0.02% cholesterol)), or a high fat diet with high cholesterol (0.16% cholesterol) Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. The T1 B cell subset was gated as B220+ CD138- IgM+ IgD+ CD21lo CD23+. Shown are the average percentages (+SD) from two independent experiments using four mice per expression strain (Wt and KO). 12A P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.205/<0.001. 12B P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): <0.001/0.886. 12C P-Values (Chow Wt v Chow KO/HFD Lo C Wt v HFD Lo C KO/HFD Hi C Wt v HFD Hi C KO): 0.005/0.004/0.006.

Continuing with the transitional B cells, we can see a clear decrease (P=0.008) in the percentage of T2 B cells present in the spleens of wild type mice fed the high fat/low cholesterol diet when compared to wild type mice fed the normal chow diet [Fig. 13a]. A difference in the percentages of T2 B cells could not be observed (P=0.098), however, in the spleens of wild type mice fed either the high fat/high cholesterol diet when compared to those fed the high fat/low cholesterol diet [Fig. 13a]. L1 KO mice fed either the high fat/low cholesterol diet or the normal chow diet showed no difference (P=0.199) in the

percentage of T2 B cells present in the spleen [Fig. 13b]. Similarly, the L1 KO mice fed either the high fat/high cholesterol or high fat/low cholesterol diet showed no difference (P=0.566) in spleen T2 B cell percentages [Fig. 13b].

When directly comparing the percentage of T2 B cells in the spleens of L1 KO mice fed the normal chow diet against those of wild type mice fed the same diet, there was a significant decrease observed (P=0.013) for the L1 KO mice [Fig. 13c]. There was also a decrease in the percentage (P=0.026) of T2 B cells observed in the spleens of L1 KO mice fed the high fat/high cholesterol diet when compared to their wild type counterparts that were fed the same diet [Fig. 13c]. When comparing the percentages of T2 B cells in the spleens of L1 KO mice fed the high fat/low cholesterol diet with wild type mice fed the same diet [Fig. 13c], there were no differences observed (P=0.405).

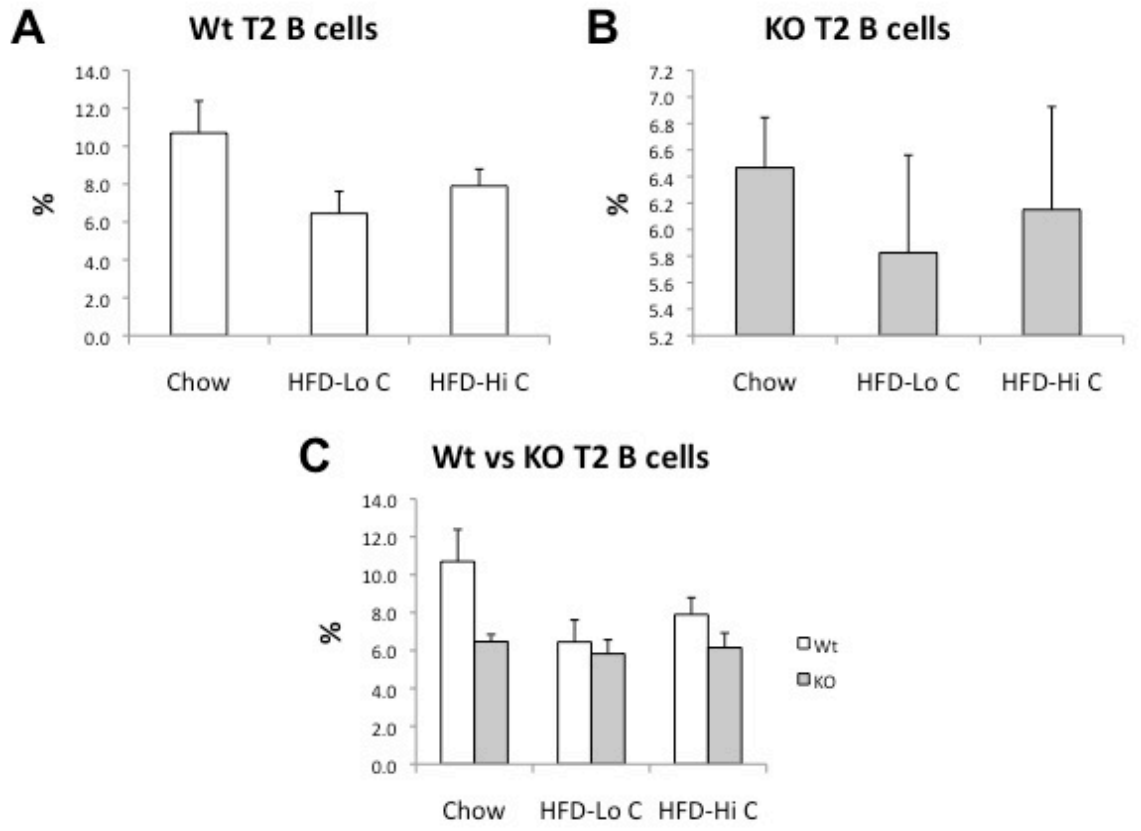

Figure 13: Effects of high fat diet on splenic T2 B cells of wild type (A) and L1 KO mice (B) after high fat diet. Mice were fed either a normal chow diet, a high fat diet with low cholesterol (<0.02% cholesterol)), or a high fat diet with high cholesterol (0.16% cholesterol) Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. The T2 B cell subset was gated as B220+ CD138- IgM+ IgD-CD21lo CD23+ CD24lo. Shown are the average percentages (+SD) from two independent experiments using four mice per expression strain (Wt and KO). 13A P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.008/0.098. 13B P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.199/0.566. 13C P-Values (Chow Wt v Chow KO/HFD Lo C Wt v HFD Lo C KO/HFD Hi C Wt v HFD Hi C KO): 0.013/0.405/0.026.

After analyzing the percentage of isotype switched B cells present in the spleen, there was no significant difference observed (P=0.208) when comparing wild type mice fed either the high fat/low cholesterol or normal chow diet [Fig. 14a]. There was, however, an increase in the percentage (P=0.006) of splenic isotype switched B cells observed in wild type mice fed the high fat/high cholesterol diet over the wild type mice fed the high fat/low cholesterol diet [Fig. 14a]. For the L1 KO mice, there was no difference observed (P=0.696) in the percentage of isotype switched B cells present in the

spleen of mice fed the high fat/low cholesterol diet when compared to L1 KO mice that were fed the normal chow diet [Fig. 14b]. Interestingly, the L1 KO mice that were fed the high fat/high cholesterol diet showed a marked decrease (P=0.003) in the percentage of splenic isotype switched B cells when compared to L1 KO mice that were fed the high fat/low cholesterol diet [Fig. 14b].

When directly comparing the isotype switched B cell percentages in the spleens of L1 KO mice fed the normal chow diet against those of wildtype mice fed the same diet [Fig. 14c], there were no significant differences observed (P=0.950). There was a significant decrease (P=0.011) in the percentage of isotype switched B cells observed in the spleens of the L1 KO mice fed the high fat/high cholesterol diet when compared to wild type mice fed the same diet [Fig. 14c]. Interestingly, when comparing the percentages of isotype switched B cells present in the spleens of mice fed the same high fat/low cholesterol diet, it was found that the L1 KO mice had a higher percentage (P=0.002) of this B cell subset than their wild type counterparts [Fig. 14c].

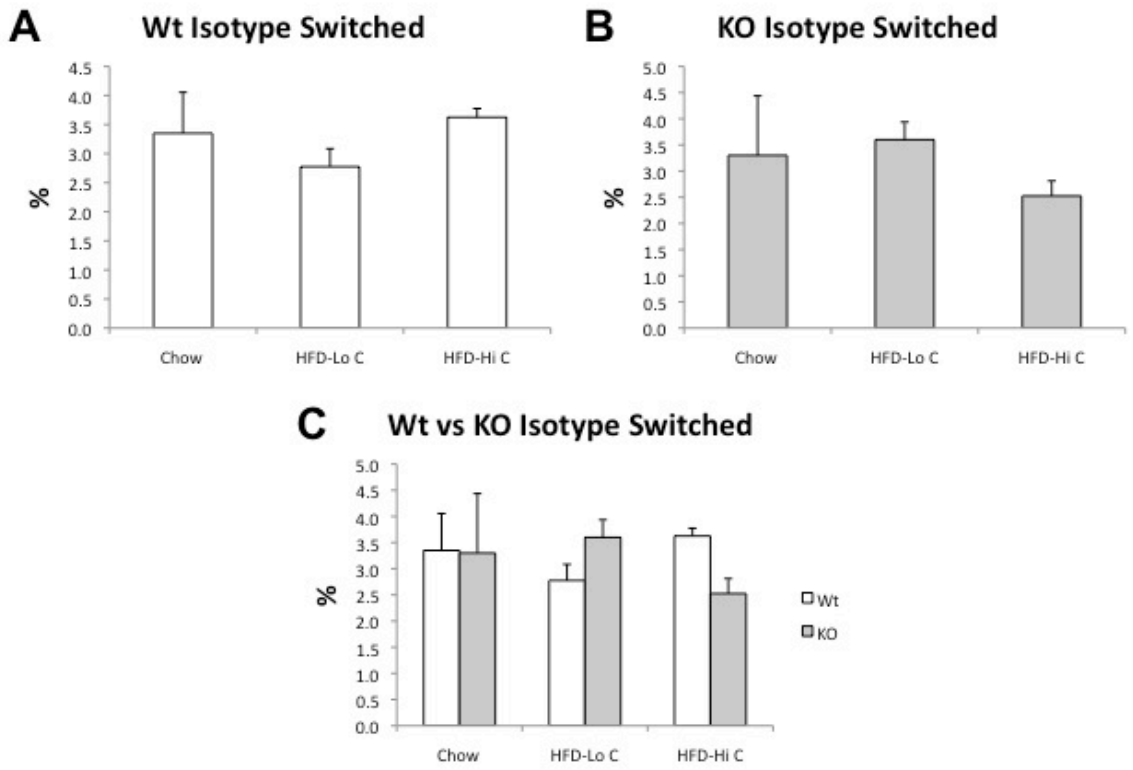

Figure 14: Effects of high fat diet on splenic FO B cells of wild type (A) and L1 KO mice (B) after high fat diet. Mice were fed either a normal chow diet, a high fat diet with low cholesterol (<0.02% cholesterol)), or a high fat diet with high cholesterol (0.16% cholesterol) Mouse B cells were stained with anti-B220 (B cell marker), anti-CD138 (plasma cell marker), anti-IgM, anti-IgD, anti-CD24, anti-CD21, and anti-CD23 before being analyzed by flow cytometry. The isotype switched B cell subset was gated as B220+ CD138- IgM- IgD- CD21+ CD23lo. Shown are the average percentages (+SD) from two independent experiments using four mice per expression strain (Wt and KO). 14A P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.208/0.006. 14B P-Values (Chow v HFD Lo C/HFD Lo C v HFD Hi C): 0.696/0.003. 14C P-Values (Chow Wt v Chow KO/HFD Lo C Wt v HFD Lo C KO/HFD Hi C Wt v HFD Hi C KO): 0.950/0.011/0.002.

	Normal Chow Diet	HFD+ low Cholesterol	HFD+ high Cholesterol
FO B cells	34.7% (±4.0)	52.1% (±2.8)	57.1% (±3.6)
MZ B cells	4.3% (±0.7)	3.5% (±0.8)	8.9% (±1.5)
T1 B cells	26.4% (±1.6)	25.0% (±0.9)	15.8% (±1.0)
T2 B cells	10.7% (±1.7)	6.5% (±1.2)	7.9% (±0.9)
Switched	3.4% (±0.7)	2.8% (±0.3)	3.6% (±0.2)

Table 1: Splenic B cell subset percentages (±SD) observed in wild type mice after various diets.

	Normal Chow Diet	HFD+ low Cholesterol	HFD+ high Cholesterol
FO B cells	34.7% (±4.0)	52.4% (±0.6)	57.1% (±3.2)
MZ B cells	1.9% (±0.4)	6.2% (±1.6)	5.5% (±0.6)
T1 B cells	31.3% (±1.1)	21.1% (±1.3)	20.9% (±1.9)
T2 B cells	6.5% (±0.4)	5.8% (±0.7)	6.2% (±0.8)
Switched	3.3% (±1.1)	3.6% (±0.3)	2.5% (±0.3)

Table 2: Splenic B cell subset percentages (±SD) observed in *NPC1L1* knock out mice after various diets.

SUMMARY OF PRELIMINARY DATA

In summary, the data generated suggests that isotype switched B cells down-regulate expression of CD23 when compared to naïve B cells [Fig. 5c]. The down-regulation of this inhibitory molecule potentially lowers the activation threshold of memory B cells. Utilizing the CD23 transgenic mouse model, it was found that over-expression of CD23 slightly increased the percentage of isotype switched B cells present in the spleen [Fig. 8], implying a possible role for CD23 expression in isotype switching. It is also worth noting that both the MZ and the T1 B cell populations were significantly increased in the CD23 KO mice [Figs. 7b, 7c]. Additionally, the human B cell data shows that CD23 clusters with BCR after *in vitro* stimulation with soluble antigen, suggesting the possible involvement by CD23 in BCR activation [Fig. 9].

In the study involving diet-induced obese mice, the data generated suggests that a diet high in fat has the ability to stimulate the development of follicular B cells in the spleen while somehow suppressing the development of B cells to the transitional stage [Figs. 10, 12, 13, Table 1, Table 2, and Table 3]. This transitional B cell decrease could be due to the increased maturation and subsequent migration into the splenic follicles induced by the high fat diet. It is also important to note that wild type mice fed a diet high in fat and cholesterol showed an increase in the amount of isotype switched B cells present in the spleen, which suggests that a high fat/high cholesterol diet can promote B cell isotype switching and possibly memory B cell development [Fig. 14, Table 1]. Interestingly, *NPC1L1* knock out mice fed the same diet showed a decrease in isotype switched B cells, which suggests that the cholesterol transporter may somehow play a role in B cell isotype switching [Fig. 14, Table 2].

Analysis of B cell development in mice fed with different diets suggests that the high fat diet somehow stimulates an increase in FO B cells in both the wild type and L1 KO mice regardless of the level of cholesterol [Figs. 10a, 10b]. This data appears to be consistent with recent studies suggesting that diets high in fat can stimulate immune responses [57-59]. The data also shows that the high fat diet stimulates an increase in MZ B cells when coupled with low cholesterol in wild type mice [Fig. 11a], but in the L1 KO mice an increase was seen in those fed the high fat diet regardless of the cholesterol content [Fig. 11b]. This data also seems to be consistent with generation of some form of low-level immune response, but interestingly, wild type mice fed a high fat/low cholesterol diet did not generate a change in splenic MZ B cells when compared to those fed the normal chow diet [Fig. 11a]. The amount of T1 B cells appears to decrease when wild type mice are fed the high fat/high cholesterol diet [Fig. 12a], but the percentage of this same B cell subset only decreases in L1 KO mice when fed the high fat/low cholesterol diet [Fig. 12b]. The percentage of T2 B cells also decreased after a high fat diet (regardless of cholesterol level), but only for the wild type mice [Fig. 13a]. This decrease in transitional B cell percentages could be indicative of this subset's migration into B cell follicles after an immune response. The percentage of isotype switched B cells increased in wild type mice after a high fat/high cholesterol diet [Fig. 14a], but this same diet in L1 KO mice caused a decrease in this B cell subset [14b]. This data suggests that the cholesterol transporter L1 may be essential for the generation of isotype switched B cells during the immune response.

INTERPRETATIONS/SUMMARY:

In conclusion, the work conducted in the CD23 study and the data generated therein suggest that B cells that have undergone isotype switching (which are most likely memory B cells) have down-regulated expression of CD23 when compared to naïve B cells [Fig. 5c]. Since over-expression of the CD23 molecule has been directly associated with inhibition of the humoral immune response (i.e. antibody production), one could hypothesize that the low expression of this molecule on the isotype switched B cell subset (memory B cells) may be necessary in order to lower the activation threshold of these cells. The study involving human B cells also showed that CD23 co-clustered with the BCR after *in vitro* stimulation, which implies a possible role in BCR-mediated B cell activation [Fig. 9]. It was also found that, in mice over-expressing CD23, there were slightly more isotype switched B cells in the spleen when compared to wild type mice [Fig. 8]. These observations might at first seem to contradict each other, until one considers that over-expression of CD23 could somehow drive isotype switching while also suppressing the activation of B cells.

Analysis of B cell development in mice fed with different diets suggests that the high fat diet somehow stimulates an increase in FO B cells in both the wild type and L1 KO mice regardless of the level of cholesterol [Figs. 10a, 10b]. This data appears to be consistent with recent studies suggesting that diets high in fat can stimulate immune responses [57-59]. The data also shows that the high fat diet stimulates an increase in MZ B cells when coupled with low cholesterol in wild type mice [Fig. 11a], but in the L1 KO mice an increase was seen in those fed the high fat diet regardless of the cholesterol content [Fig. 11b]. This data also seems to be consistent with generation of some form of

low-level immune response, but interestingly, wild type mice fed a high fat/low cholesterol diet did not generate a change in splenic MZ B cells when compared to those fed the normal chow diet [Fig. 11a]. The amount of T1 B cells appears to decrease when wild type mice are fed the high fat/high cholesterol diet [Fig. 12a], but the percentage of this same B cell subset only decreases in L1 KO mice when fed the high fat/low cholesterol diet [Fig. 12b]. The percentage of T2 B cells also decreased after a high fat diet (regardless of cholesterol level), but only for the wild type mice [Fig. 13a]. This decrease in transitional B cell percentages could be indicative of this subset's migration into B cell follicles after an immune response. The percentage of isotype switched B cells increased in wild type mice after a high fat/high cholesterol diet [Fig. 14a], but this same diet in L1 KO mice caused a decrease in this B cell subset [14b]. This data suggests that the cholesterol transporter L1 may be essential for the generation of isotype switched B cells during the immune response.

When directly analyzing the effect of knocking out the *NPC1L1* gene on the percentages of these B cell subsets, there appeared to be no difference in the amount of FO B cells present in the spleens of the L1 KO mice when compared to wild type regardless of the diet [Fig. 10c]. This suggests that the cholesterol transporter L1 does not directly impact B cell migration into follicles during the immune response. The L1 KO mice did, however, show an increase in the percentage of MZ B cells (6.2%) over wild type mice (3.5%) when fed the high fat/low cholesterol diet [Fig. 11c]. It is difficult to propose an explanation for this phenomenon since previous data/studies have shown that disrupting the expression of the L1 cholesterol transporter has a negative effect on the development of obesity-related metabolic disorders [45, 60, 61], which should

logically include indirect suppression of the inflammatory immune response. The L1 KO mice did, however, show a decrease in the percentage of the MZ B cell subset when compared to the levels observed in wild type mice after being fed either the normal chow (1.9% vs 4.3%) or the high fat/high cholesterol diet (5.5% vs 8.9%) [Fig. 11c]. The T1 B cell subset saw an increase in percentage in the L1 KO mice over the wild type mice when fed either the normal chow (31.3% vs 26.4%) or the high fat/high cholesterol diet (20.9% vs 15.8%) [Fig. 12c]. This data could be evidence that the cholesterol transporter L1 is at least partially associated with the development of B cells out of the T1 stage. The high fat/low cholesterol diet showed a decrease of the T1 B cell percentage in the L1 KO mice (21.1%) when compared to wild type mice (25.0%) fed the same diet [Fig. 12c]. Interestingly, the L1 KO mice also saw a decrease in the T2 B cell subset when compared to wild type after either the high fat/high cholesterol (6.2% vs 7.9%) or normal chow diet (6.5% vs 10.7%) was administered [Fig. 13c]. Again, this data seems to suggest that the L1 cholesterol transporter plays a role in the development of B cells out of the T1 stage. The isotype switched B cell subset had an increase in percentage in the L1 KO mice (3.6%) over wild type mice (2.8%) after being fed the high fat/low cholesterol diet [Fig. 14c], but this same subset showed a decrease in percentage for the L1 KO mice (2.5%) when compared to wild type mice (3.6%) after being fed the high fat/high cholesterol diet [Fig. 14c]. These final findings are somewhat difficult to explain as well. An increase in the percentage of isotype switched B cells goes hand-in-hand with an increase in B cell stimulation and activation. We also know that high cholesterol absorption can lead to activation of the inflammatory immune response. If knocking out the *NPC1L1* gene effectively blocks cholesterol absorption, then how might it be possible for L1 KO mice

fed a high fat/low cholesterol diet to have higher relative percentages of isotype switched B cells than wild type mice? We currently know that the L1 transporter protein plays a crucial role in cholesterol absorption through the intestine, but perhaps this transmembrane protein also plays at least a small role in the reverse cholesterol transport system (RCT). If this were the case (though admittedly, highly unlikely), it could be used to speculate that knocking out or interrupting the function of the *NPC1L1* gene could conceivably lead to a slight buildup of cholesterol—enough at least to stimulate isotype switching in B cells. A rather large problem with this theory is that other transporter proteins are still expressed in the L1 KO mice—namely a key player in the RCT system known as the ATP binding cassette (ABC) transporter. Existing actually as a heterodimer comprised of both ABCG5 and ABCG8 proteins [62, 63], one of the more well established roles of this ABC transporter complex in the intestines is the active secretion of cholesterol out into the intestinal lumen [62, 64]. Once in the intestinal lumen, the cholesterol can then be excreted from the body. In what way knocking out expression of the L1 transporter could affect the normal function of the ABC transporter complex or any of the RCT-associated proteins in this situation would need to be thoroughly explored before any preliminary conclusions could be drawn.

FUTURE DIRECTIONS

Future Project #1: Identify the steps in memory B cell responses where CD23 plays a role.

Purpose/Rationale: The goal of this project is to investigate whether and where the CD23 molecule plays its role in the development of memory B cells and the humoral memory response. CD23 has been suggested to play a role in B cell activation, but the specifics regarding this role have not been elucidated [36]. Griffith et al. [39] found that cross-linking surface CD23 by antibodies can enhance B cell activation and even override BCR-mediated signaling. The findings that the over-expression and deficiency of CD23 suppress and enhance both IgE and IgG antibody responses suggest that CD23 acts as a down-regulator of B cell-mediated immune responses [3, 34, 37, 55, 56, 65]. It remains unknown whether CD23 has a role in the development and activation of memory B cells. My preliminary data show a decrease in CD23 expression in memory B cells, implicating a possible down-regulation of a negative regulator in memory B cells. In this future experiment, one would test the hypothesis that CD23 negatively regulates the generation of memory B cells and/or the activation and differentiation of memory B cells into plasma cells. In order to address this hypothesis, one can use the CD23 knockout and transgenic mouse models. First, it will be necessary to compare the relative percentages of different B cell subsets, particularly memory B cells in the spleens of wild type and CD23 knockout and transgenic mice, after immunization to identify which step of memory B cell development is regulated by CD23. Secondly, one will need to examine the effect of CD23 expression on the frequencies of memory B cells and humoral

memory responses. A third step would involve analyzing whether the expression level of CD23 has any impact on the differentiation of memory B cells into high-affinity plasma cells in vivo and in vitro. Lastly, it would be interesting to investigate whether the effect of CD23 on memory B cells is originated from the B cells themselves and not other cell types. This could be accomplished by adoptively transferring wild type, CD23 deficient or over-expressing B cells into B cell deficient mice and testing the generation of memory B cells and the differentiation of these B cells into antibody-secreting plasma cells. These experiments will determine whether CD23 plays a role in regulating the generation of memory B cells and the activation and differentiation of memory B cells into high affinity antibody-secreting cells.

Expected Results, Potential Pitfalls and Alternative Approaches: I would expect to see a decrease in the percentage of memory B cells in CD23 transgenic mice when compared with that in CD23 knockout and wild type mice. This could be associated with a lower frequency of high affinity antibody producing cells and low antibody titer in CD23 transgenic mice. CD23 may affect either the generation or activation of memory B cells or both. Because CD23 is expressed in other immune cells in addition to B cells, the defects found in CD23 knockout and transgenic mice may not solely originate from B cells. Therefore, adoptive transfer of B cells may lead to a partial defect. In addition to adoptively transferring B cells into B cell deficient mice, and alternative approach could be to transfer wild type T cells with mutated B cells into irradiated wild type mice.

Future Project #2: Determine how CD23 affects memory B cell activation.

Purpose/Rationale: This project investigates whether the expression, ligation, or colligation of the CD23 molecule can regulate BCR-mediated signaling in naïve and

memory B cells. The colligation of BCR with CD23 has been shown to down-regulate BCR activation and cause an apoptotic response in naïve B cells [36]. In contrast, cross-linking of CD23 with anti-CD23 antibodies has been found to increase naïve B cell activation [39]. How CD23 regulates BCR-mediated signal transduction and B cell activation remains to be explored. BCR clustering is known to be a key step in the initiation of naïve B cell activation, and the recruitment of signaling molecules to BCR clusters leads to the formation of signalosomes [21, 28]. While activation mechanisms of memory B cells have not been well studied, it is reasonable to assume that memory B cells also share this characteristic. The hypothesis is that CD23 can regulate signal transduction in B cells by influencing the kinetics and magnitude of surface BCR aggregation and by modifying signaling molecules recruited to BCR clusters. My preliminary experiments show that the surface CD23 molecules co-cluster with surface BCRs in response to BCR cross-linking supporting the hypothesis. To address the question of how CD23 regulates BCR-mediated signaling, one could use CD23 knockout and transgenic mouse models as well as human primary B cells. The investigator would need to determine the effect of CD23 expression, CD23 ligation, and CD23-BCR colligation on the activation of proximal signaling molecules, on the kinetics and magnitude of surface BCR clustering, and on the recruitment of signaling molecules to BCR clusters. These experiments would determine whether CD23 can regulate BCR signaling by modulating the formation of BCR signalosomes.

Expected Results, Potential Pitfalls and Alternative Approaches: I expect that CD23 cross-linking and CD23-BCR colligation will have an impact on signaling in B cells. Colligation of CD23 with BCR may increase the activation of molecules responsible for

the down-regulation of BCR signaling. If this is proven to be true, I also expect that colligation of CD23 to BCR will have a negative effect on BCR clustering as BCR clustering has been implicated as an important component of B cell signaling. If no change in the activation of proposed signaling proteins is observed, the investigator could expand the spectrum of signaling molecules. If one is unable to image CD23 and the BCR clusters using TIRF microscopy, they could instead try to colligate the BCR and CD23 with soluble antigen in vitro, stain the cells, fix the cells at various time points and then image them using confocal microscopy. Utilizing this strategy would allow the investigator to visualize the forming of the BCR cap and how CD23 may co-localize with the cap. One could also stain the cells for the activated signaling molecules by permeabilizing the cells and then treating with fluorescently tagged antibodies against the molecules of interest.

GLOSSARY

Adjuvant – An immunological agent that increases the antigenic response.

Amino Acid – An organic compound containing an amino group (NH_2), a carboxylic acid group (COOH), and any of various side groups, especially any of the 20 compounds that have the basic formula $NH_2CHRCOOH$, and that link together by peptide bonds to form proteins or that function as chemical messengers and as intermediates in metabolism.

Anergic – Diminution or lack of immunity to an antigen.

Antibody – Any of various proteins produced by B cells in response to the presence of an antigen. By becoming attached to antigens on infectious organisms antibodies can render them harmless or cause them to be destroyed.

Antigen – Any substance that stimulates an immune response in the body (especially the production of antibodies).

Atrophy – The partial or complete wasting away of a part of the body.

Autoanitbody – An antibody that reacts with the cells, tissues, or native proteins of the individual in which it is produced.

Autoimmunity – Of or relating to an immune response by the body against one of its own cells or tissues.

B Cell – Any of the lymphocytes that mature in the bone marrow and, when stimulated by a particular antigen, differentiate into plasma cells. Also called B lymphocyte.

Basophil – A leukocyte with basophilic granules easily stained by basic stains.

Calcinuerin – A phosphatase that activates the transcription factor NFAT via dephosphorylation.

Cancer – A class of disease in which a group of cells display uncontrolled growth through division beyond normal limits, invasion that intrudes upon and destroys adjacent tissues, and sometimes metastasis, in which cancer cells spread to other locations in the body via lymph or blood. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize.

Chemokine – Any of various cytokines produced in acute and chronic inflammation that mobilize and activate white blood cells.

Chemotaxis – The characteristic movement or orientation of an organism or cell along a chemical concentration gradient either toward or away from the chemical stimulus.

Cholesterol – An animal sterol that is normally synthesized by the liver; the most abundant steroid in animal tissues.

Cognate Antigen – The specific antigen that will bind to the BCRs of a given mature B cell.

Complement – The complement system consists of a number of small proteins found in the blood, generally synthesized by the liver, and normally circulating as inactive precursors (pro-proteins). When stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The end-result of this activation cascade is massive amplification of the response and activation of the cell-killing membrane attack complex. Over 25 proteins and protein fragments make up the complement system, including serum proteins, serosal proteins, and cell membrane receptors. They account for about 5% of the globulin fraction of blood serum.

Confocal Microscopy – An optical imaging technique used to increase optical resolution and contrast of a micrograph by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images.

Cytokine – Any of several regulatory proteins, such as the interleukins and lymphokines, that are released by cells of the immune system and act as intercellular mediators in the generation of an immune response.

Cytotoxic – Of, relating to, or producing a toxic effect on cells.

Dendritic Cell – An antigen-presenting leukocyte that is found in the skin, mucosa, and lymphoid tissues and that initiates a primary immune response by activating lymphocytes and secreting cytokines.

Differentiation – The process by which a less specialized cell becomes a more specialized cell type. Example: A naïve B cell differentiates into a memory B cell.

DNA – A nucleic acid that carries the genetic information in the cell and is capable of self-replication and synthesis of RNA.

ELISA – A diagnostic tool in medicine and plant pathology, as well as a quality-control check in various industries. This technique is used to detect (and quantify) the presence of an antigen in a sample.

ELISpot – The Enzyme-linked immunosorbent spot assay. Developed in 1983 by Cecil Czerkinsky and is considered a common method for monitoring immune responses in humans and animals.

Eosinophil – A type of white blood cell found in vertebrate blood, containing cytoplasmic granules that are easily stained by eosin or other acid dyes.

Flagellin – The principal substituent of bacterial flagellum, and is present in large amounts on nearly all flagellated bacteria.

Flagellum – A tail-like projection that protrudes from the cell body of certain prokaryotic and eukaryotic cells, and functions in locomotion.

Flow Cytometry – A laser based, biophysical technology employed in cell counting, sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second.

Fluorescence – The emission of electromagnetic radiation, especially of visible light, stimulated in a substance by the absorption of incident radiation and persisting only as long as the stimulating radiation is continued.

Germinal Center – A site within lymph nodes and lymph nodules (peripheral lymph tissues) where mature B lymphocytes rapidly proliferate, differentiate, undergo somatic hypermutation, and class switch their antibodies during a normal immune response to an infection.

Granulocyte – A category of white blood cells characterized by the presence of granules in their cytoplasm. They are also called polymorphonuclear leukocytes (PMN or PML) because of the varying shapes of the nucleus, which is usually lobed into three segments.

Immunization -- The process of inducing immunity to an infectious organism or agent in an individual or animal through inoculation with the organism or agent.

Immunoglobulin -- Any of a group of large glycoproteins that are secreted by plasma cells and that function as antibodies in the immune response by binding with specific antigens. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM.

Immunology -- The branch of biological science concerned with the study of immunity.

***in vitro* –** Made to occur outside the living organism in an artificial environment, such as a culture medium.

***in vivo* –** In the living organism.

Isotype – The genetic variations or differences in the constant region of the heavy chain of the Ig (immunoglobulins) classes and sub-classes.

Isotype Switching – A biological mechanism that changes a B cell's production of antibody from one class to another, for example, from an isotype called IgM to an isotype called IgG.

Kinase – Alternatively known as a phosphotransferase, it is a type of enzyme that transfers phosphate groups from high-energy donor molecules, such as ATP, to specific substrates in a process referred to as phosphorylation.

Knock Out – A genetic technique in which an organism is engineered to carry genes that have been made inoperative (have been "knocked out" of the organism).

Leukocyte – Any of various blood cells that have a nucleus and cytoplasm, separate into a thin white layer when whole blood is centrifuged, and help protect the body from infection and disease. Includes neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Also known as white blood cells (WBC).

Lipid – Any of a group of organic compounds, including the fats, oils, waxes, sterols, and triglycerides, that are insoluble in water but soluble in nonpolar organic solvents, are oily to the touch, and together with carbohydrates and proteins constitute the principal structural material of living cells.

Lymph – As the blood and the surrounding cells continually add and remove substances from the interstitial fluid, its composition continually changes and it changes into lymph fluid. It is then transported through lymph vessels to lymph nodes before emptying ultimately into the right or the left subclavian vein, where it mixes back with blood.

Lymph Node – A small ball-shaped organ of the immune system, distributed widely throughout the body including the armpit and stomach/gut and linked by lymphatic

vessels. Lymph nodes are garrisons of B, T, and other immune cells. Lymph nodes are found all through the body, and act as filters or traps for foreign particles. They are important in the proper functioning of the immune system.

Lymphocyte – A type of white blood cell in the vertebrate immune system, including NK cells, T cells, and B cells.

Macrophage – A type of phagocytic white blood cell produced by the differentiation of monocytes in tissues.

Mast Cell – A resident cell of several types of tissues containing many granules rich in histamine and heparin. Best known for its role in allergy and anaphylaxis.

Memory B Cell – A B cell sub-type that are formed following primary infection. Responsible for rapid secondary immune responses.

Memory T Cell – A specific type of infection-fighting T cell that can recognize foreign invaders such as bacteria or viruses, that were encountered during a prior infection or vaccination. At a second encounter with the invader, memory T cells can reproduce to mount a faster and stronger immune response than the first.

Metabolism – A set of chemical reactions that happen in living organisms to maintain life.

mRNA – Messenger RNA (mRNA) is a molecule of RNA encoding a chemical "blueprint" for a protein product. It is transcribed from a DNA template, and carries coding information to the sites of protein synthesis: the ribosomes.

Naïve B Cell – A fully matured B cell that has not yet encountered its cognate antigen.

Natural Killer Cell – A lymphocyte that is capable of binding to and killing virus-infected cells and some tumor cells by releasing cytotoxins. It is found in the bone marrow and spleen. Its cytotoxic activity is not antibody-dependent but is augmented by interferon.

Neutrophil – The most abundant type of granular white blood cell in mammals and forms an essential part of the innate immune system.

Opsonization – A process whereby opsonins (ex: antibodies) make an invading microorganism more susceptible to phagocytosis.

Phagocytosis – The cellular process of engulfing solid particles by the cell membrane to form an internal phagosome by phagocytes and protists. Phagocytosis is a specific form

of endocytosis involving the vesicular internalization of solid particles, such as bacteria, and is, therefore, distinct from other forms of endocytosis such as the vesicular internalization of various liquids.

Phosphatase – An enzyme that removes a phosphate group from its substrate via hydrolysis.

Plasma Cell – Also called plasma B cells, plasmocytes, and effector B cells, are white blood cells that produce large volumes of antibodies.

Plasma Membrane – A biological membrane consisting primarily of phospholipids that separates the interior of all cells from the outside environment.

Proliferation – Also known as 'cell growth'. When used in the context of cell division, it refers to growth of cell populations, where one cell (the 'mother cell') grows and divides to produce two 'daughter cells'.

Protein – Organic compound made of amino acids arranged in a linear chain and folded into a globular or fibrous form. The amino acids in a polymer are joined together by the peptide bonds between the carboxyl and amino groups of adjacent amino acid residues.

RNA – One of the three major macromolecules (along with DNA and proteins) that are essential for all known forms of life.

Serum – The clear portion of any liquid separated from its more solid elements.

Spleen – An organ in vertebrate animals that in humans is located on the left side of the abdomen near the stomach. It is mainly composed of lymph nodes and blood vessels. It filters the blood, stores erythrocytes, destroys old ones, and produces lymphocytes.

Splenocyte – Any one of the different white blood cell types as long as it is situated in the spleen or purified from splenic tissue.

Synergy – When any two or more agents work together to produce a result not obtainable by any of the agents independently.

T Cell – Any of the lymphocytes that mature in the thymus and have the ability to recognize specific peptide antigens through the receptors on their cell surface. Also called T lymphocyte.

Thymus -- a glandular organ of vertebrates, consisting in man of two lobes situated below the thyroid. In early life it produces lymphocytes and is thought to influence

certain immunological responses. It atrophies with age and is almost nonexistent in the adult.

Titer – A measure of concentration. Titer testing employs serial dilution to obtain approximate quantitative information from an analytical procedure that inherently only evaluates as positive or negative. The titer corresponds to the highest dilution factor that still yields a positive reading.

Transgene – An exogenous gene introduced into the genome of another organism

Wild Type – The typical form of a species of organism resulting from breeding under natural conditions.

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