INVESTIGATING THE PRODUCTION OF AUTO – ANTIBODIES AT DIFFERENT STAGES OF B CELL DEVELOPMENT

By

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DECLARATION

I hereby declare that this submission is my own work towards the M.Sc. and that to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the university, except where due acknowledgement has been made in the text.

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DEDICATION

I dedicate this work to my loving husband, Mr. Samuel Ato Andam-Akorful for his love , advice, encouragement, prayers and support. I also dedicate it to my daughter Ewurasi Andam-Akorful. I love you so much.

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that is associated with the production of anti -nuclear autoantibodies. The production of class switched IgG autoantibodies is important in the pathology of the disease. Understanding autoantibody production and the mechanism by which these B cells escape from tolerance is crucial in studying SLE. Recently, a study showed that similar to mature B-cells, immature B-cell also undergo class switch recombination(CSR) and express the enzyme activation-induced cytidine deaminase (AID) which is required for CSR. Based on this result, it could be suggested that anti-nuclear IgG autoantibodies are produced at early B-cell development. To test this hypothesis, an auto-immune mouse model, 564Igi-AIDtg is used to determine the developmental stage at which class switched autoantibodies are produced. The 564Igi mouse has an anti-RNA antibody gene knocked-in the immunoglobulin (Ig) heavy and light gene loci. These mice are on an AID-deficient background and contain an inactive GFP-AID transgene where AID is only functional once GFP gene is excised by cre recombinase. Since CD21 B-cell surface marker is expressed on mature B-cells, while CD19 is expressed throughout B-cell development, by crossing the 564Igi-AIDtg with a cre+ mouse where the cre recombinase is under the CD21 or CD19 promoter, AID can be expressed in different B-cell stages. My results show that most of the 564Igi CD21cre unlike the 564Igi CD19cre mice do not have RNA binding antibodies and do not make antibody staining nucleoli. This observation suggests that immature B cells make class switched anti-RNA and idiotype positive IgG2a and IgG2b self -reactive antibodies.

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LIST OF ABBREVIATIONS.

AID	– Activation Induced Deaminase
ANA	– Anti-Nuclear Autoantibody
Cre	– Cyclization Recombination
CSR	– Class Switch Recombination
EBV	- Epstein-barr Virus
ELISA	– Enzyme Linked Immunosorbent Assay
HEp	– Human Epithelial Cells
IC	– Immune Complexes
Id	– Idiotype
Ig	– Immunoglobulin
NZB	– New Zeeland Black inbred mice
NZW	– New Zeeland White inbred mice
RAG	- Recombinase-Activating Gene
SLE	- Systemic Lupus erythematosus
snRNP	- Small Nuclear Ribonucleoprotein
TLR	– Toll-Like Receptor
V(D)J	– Variable (Diversity) Joining

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that is best characterized with the production of mainly IgG anti-nuclear autoantibodies. Lupus can be fatal and it has no cure. It commonly affects organs such as kidney, joints, heart and lungs (Croker, *et al*, 2005).The symptoms are non-specific hence diagnosis is based on certain criteria which includes malar rash, renal disorder, discoid rash, photosensitivity, oral ulcers, arthritis, anti- double stranded DNA, anti- Smith and anti- nuclear antibodies (Heinlen ,et al.,2010).

In SLE, there is deposition of circulating immune complexes (IC) consisting of antigen-antibody complex which are produced due to defective clearance of autoantibodies. These IC eventually get deposited in affected vital organs and tissues such as the kidneys, heart and lungs causing end organ inflammation. The deposition of these immune complexes can lead to production of cytokines and chemokines . Cytokines augment the humoral immune response leading to further autoantibody production. Inflammation in an organ like the kidney may lead to severe glomerulonephritis and eventually organ failure (Reviewed in Merrill, et al 2004).

The incidence of SLE in Africa including Ghana appears to be rare (Vasudevan, A et al, 2010). This is in contrast to the high prevalence in people in developed world with African ancestry. This may be because not many studies have been done in this disease area in Africa (Vasudevan, A et al, 2010). Again, due to the non- specific symptoms of SLE many people who have the disease go unnoticed and eventually die from the various end organ damages which is associated with SLE.

Environmental factors and the activities of some microorganisms such as viral infections have been shown to have an effect in the appearance and outcome of lupus due to their ability to lead to interferon production (Vasudevan, A et al, 2010).

1.2 Problem Statement

Autoimmune diseases, SLE inclusive, have been studied extensively over the years and there still remain many unanswered questions. SLE is known to be fatal and there is still no cure. The use of drugs such as corticosteroids, azathioprine and methotrexate are used in SLE management and they all have various levels of toxicity. It is therefore important to fully understand the disease so safer drugs can be developed and eventually find a cure. Class switch IgG autoantibodies is known to be responsible for disease pathogenesis in lupus and these IgG autoantibodies persist in the body even after tolerance mechanism. The mechanism by which there is a break in tolerance is not yet known though this is evident in SLE.

In Ghana, there have been few studies on autoimmune diseases including lupus and hence there is not much data on lupus. A study conducted at Korle bu teaching hospital in 1983 showed that SLE and other autoimmune diseases is rare in Ghana (Affram ,et al.,1991). Currently, there is an on -going study in Ghana to determine the actual prevalence of autoimmune diseases in the country (unpublished). Many patients with lupus die unnoticed due to the non-specific symptoms and are usually diagnosed and treated for other diseases. Early detection and management of Lupus would lead to the reduction in fatality of lupus associated diseases as well as the ability to tell the exact number of people with lupus and other autoimmune diseases to be able to put in place proper interventions.

1.3 Justification

Understanding B cell development and their mechanism of tolerance is important in SLE since the disease results from the production and retention of auto-reactive B cells. B cell development occurs in distinct stages and it is a highly regulated processes. Self- reactive B cells are still retained despite tolerance mechanism of editing, anergy and deletion (Berland, *et al*, 2006). B-cells are marked by unique cellular markers they express at various stages of their development. The surfaces marker CD21 is expressed on mature B –cells while CD19 expression is first observed at the immature B-cell stage and continues to be expressed at the mature B-cell stage (Pablo, E *et al*., 2011).

IgG autoantibody production in SLE is important in disease pathology. In order to activate B-cells to switch from producing IgM antibodies to other isotypes, B-cells must undergo the process of CSR which requires AID. This recombination alters the antibody effector function without changing the antigen specificity (Stavnezer, 2011). Both mature and immature B-cells have been shown to undergo CSR (Han, *et al*., 2007).

To better understand lupus, a murine lupus model, 564Igi, has been generated. This model system was generated such that heavy and light chain genes encoding for 564 Ig have been targeted to the heavy and light chain loci of a non -autoimmune C57BL/6 mouse strain. This antibody (Ab) recognizes RNA, single stranded DNA and nucleosomes (Berland, *et al.* 2006).

For this particular study, the 564Igi mice on an AID- deficient background which contain an inactive green fluorescent protein (GFP)- AID transgene is used. This means that AID expression is blocked by the presence of the GFP. However, when

these mice are crossed with either CD21cre or CD19cre mice, the expression of cre recombinase causes the GFP gene to be excised, thus allowing AID to be expressed hence CSR and somatic hyper mutation can occur (Muto, *et al*., 2006). The cre recombinase under CD21 or CD19 promoter allows AID to be expressed at different B-cell stage.

1.4 Aims and Objective

The aim of this study was to use conditional AID expression in 564Igi CD19cre and 564Igi CD21cre mice to determine the stage in B cell development where class switched autoantibodies are made.

The objective of the study is to determine the B cells producing 564Igi Idiotype positive (Id+) class switched antibodies and anti RNA auto- antibodies.

CHAPTER TWO

2.0 Literature Review

2.1 SLE Overview

SLE is a chronic autoimmune disease characterized by anti-nuclear autoantibody production which is detectable years prior to disease onset (Fairhurst, *et al*, 2006). The production of these antibodies is against ubiquitous self antigens such as double stranded DNA, single stranded DNA and single stranded RNA. Cells which have undergone apoptosis and are not efficiently cleared could result in the generation of self- reactive B cells against nuclear antigens on the debris from these cells. These auto reactive B cells undergo affinity maturation and class switching. The antibodies produced binds to self –antigens and the antibody –antigen complex (IC) generated are eventually deposited in the affected tissues due to the body's inability to clear the high amount of the ICs being formed. In SLE, aberrant activation of compliment also occurs and this leads to inflammatory injury. (Toong, *et al*, 2011).

Lupus has a broad clinical and immunological manifestation and can affect any organ in the body such as the kidney, brain and skin. Some of the antigens of lupus includes ribonucleoprotein complex (Ro), La, an RNA binding protein and nuclear particles consisting of several different polypeptides (Sm) and nucleosomes. Ro, La and Sm are extractable nuclear antigens which are protein- RNA complexes. Sm can activate B cells through BCR and TLR. SLE is relatively rare and has a prevalence of 40 cases per 100,000 persons among Northern Europeans to more than 200 per 100,000 persons among black populations. It is noteworthy that the relatively low prevalence might be attributed to the difficult nature to produce precise estimates due to non-

specific diagnosis. Lupus is a potentially fatal disease easily confused with other disorders (Rahman, *et al*., 2008).

The disease mechanism involves both innate and adaptive immunity. The role of type 1 interferon (IFN) production in auto-reactive B cell stimulation has been implicated in lupus (Croker, 2005). In SLE patients, there is enhanced expression of genes that are IFN regulated. The role of microorganism in SLE has been speculated because viral nucleic acid and CpG DNA stimulate IFN. Nucleic acid containing ICs activate innate response via Toll-like receptors (TLRs) (Croker, 2005. These recognize molecular patterns on the surface of pathogens and thereby warn the immune system of an infection. TLR activation by host nucleic is prevented due to their location, however if endogenous DNA or RNA is brought into the same location, activation of TLRs occur. Studies have shown that TLR 7 which recognizes single –stranded RNA (ssRNA) is important in autoantibody production (Croker, 2005 and Berland, et al, 2006).

Lupus nephritis is a common complication of SLE which affects 14-55% of patients. This could be caused by anti- nucleosomes, anti- double stranded DNA, anti- Ro and anti Sm autoantibodies. Other complications of SLE are arthritis, anemia, rash, serositis, seitures, psychosis, rash, heart disease and CNS lupus (Rahman, et al, 2008). Anti-Ro and anti-La have been associated with the dermatologic manifestations of cutaneous (Skin) lupus and also with the syndrome of neonatal lupus (Croker, 2005).

SLE development has been shown to be influenced by multiple genetic factors, environmental factors, race and sex hormones. SLE affects predominantly women especially those in their reproductive age. About 90% of lupus patients are females and this therefore suggests that female hormones might play a role in the disease pathogenesis (Croker, 2005). A study measuring anti- nuclear antibodies (ANA) in

both healthy and SLE patients showed a higher levels of ANA in females than in males in all the cases (QZ et al, 2011). African American/Hispanic American women have a 3-4 times higher risk of developing the disease than Caucasians hence implicating race. Again, SLE is reported to be about six to eight times higher in people of West African descent than in Europeans who are all living in the same country(QZ, et al, 2011).

The role of genetics in SLE development has been implicated. A study using of monozygotic twins showed a concordance of 25% in disease development and approximately 2% among dizygotic twins (QZ, et al, 2011). Another study has identified the proteins; complement regulator factor H (CHF) and five CHF- related proteins (CFHR1-CFHR5) encoding for genes located within the chromosome 1q 31-32 loci as being SLE susceptible genes. CHF is a protein which inhibits complement activation by preventing C3 convertase formation and also accelerating its decay. A lupus prone mouse model MRL- lpr has shown that CHF deficiency accelerates lupus nephritis development. This is because an impairment in any of these genes would lead to an impairment in complement and hence susceptibility to various diseases including lupus. Genetic variants in the CFH-CFHRs region are associated with SLE susceptibility (Zhao et al, 2011).

Ten year survival rate for SLE has increased to >90% in the past four decades. This has been possible due to early intervention especially in developed countries. Immunosuppressant's and anti-inflammatory drugs are being used to manage the disease since SLE has no cure. The role of auto-reactive B cell in SLE is crucial, high affinity IgG antibodies have been detected bound to Ro, La and Sm. Several studies have identified anti-DNA and anti-RNA autoantibodies associated with lupus. The

production of autoantibodies reflects loss of B cell tolerance (Croker, 2005). It is imperative therefore that we understand B cell development.

2.2 Viruses and SLE

B lymphocytes are the site for viral persistence (Yu Sun, et al, 2011). Viral infections affect an SLE patients' disease onset, diagnosis, clinical manifestations and prognosis (Hongbo, et al., 2011). The role of environmental factors such as virus in the development of SLE has been studied by scientists for decades. Viral infection locations in SLE mainly occur in gastrointestinal tract, respiratory tract, retina, and skin. There have been reports of SLE patients with intracranial viral infection but this is rare (Hongbo, et al., 2011).

Studies so far indicate a strong association between Epstein –Barr virus (EBV) infection and the development of SLE. It has been shown that EBV can trigger the onset as well as exacerbate SLE (James JA, et al, 1997). Other viruses implicated in either the onset and /or exacerbation of SLE include human herpesvirus 8 (HHV-8), or Kaposi's sarcoma (KS)-associated herpesvirus, human parvovirus B19, human papillomavirus(HPV), cytomegalovirus (CMV), herpes simplex virus and varicella zoster virus (Yu Sun, et al,2011, Nath, et al, 2007 and Sekigawa,et al ,2002).

A study conducted in China on the prevalence of human herpes virus 8 infection in SLE showed an increase in HHV-8 infection in individuals with SLE than the healthy controls. This result implies that there is an association between HHV-8 and the development of SLE(Yu Sun, et al,2011). HHV-8 has been shown to have many similarities with EBV. Some of these include their ability to infect and build up latency in B lymphocytes as well as produce a number of proteins responsible for maintaining viral episomes, promoting B-cell survival and proliferation, inhibiting p53 and Rb, and encoding a large number of cellular homologs (Yu Sun, et al, 2011).

2.3 EBV and SLE

EBV is a human herpes virus that persists in the memory B cells in a latent form in a vast majority of the world's population. A study found out that the first lupus specific autoantibodies arise from particular antibodies directed against Epstein–Barr virus nuclear antigen-1 (EBNA-1) and that infection with EBV is an environmental risk factor for lupus (Harley J. B.et al,2006). Again, it was discovered that reactivated or repeated EBV infection might be linked to SLE development (Parks, et al, 2005). EBV can exist in either the latent or lytic stage and both stages elicit a strong immune response i.e both humoral and cellular immunity. EBV is considered as a cofactor in human autoimmune disease due to its ability to cause infectious mononucleosis. Nearly all adults in the world have been exposed to EBV (Niller, et al., 2011).

During EBV infection, an increase in viral load or a change in both lytic and latent proteins can cause a cross reactivity with cellular antigens which may trigger pathogenic processes. This could result in SLE and other autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (Niller, et al., 2011). Patients with SLE have increased evidence of viral replication and an abnormal immune response to EBV (Kang, 2004).

Studies have shown that SLE patients have higher anti-EBVresponse and EBV DNA burden. There is evidence of homologies between EBV proteins and Sm ribonucleoprotein, which is one of the major targets of humoral immune response in SLE (Bahar, A.E. , et al, 2010). Therefore individuals who are genetically prone to SLE could use mechanisms of molecular mimicry based and epitope spreading on the homologies to induce systemic autoimmunity Molecular mimicry is when autoreactive B and T cells become activated by peptides from virus or any infecting

organism due to similar structure and also similar amino acid sequence as host tissue peptides.

Epitope spreading is also a phenomenon of increasing diversity in the autoantibody repertoire and this usually occurs prior to the development of symptoms (Yao, et al, 1996).

2.4 B cell development

Cells of the immune system as well as all other cellular elements of the blood arise from pluripotent hematopoietic stem cells in the bone marrow. These stem cells give rise to lymphoid progenitor cells and myeloid progenitor cells. The lymphoid cells then produce B cells, T cells and Natural Killer (NK) cells. B and T cells can easily be distinguished from myeloid progenitor cells such as erythrocytes and megakaryocytes due their possession of antigen receptors. They can also be distinguished from each other by their differentiation sites, i.e. B cells in the bone marrow and T cells in the thymus. B and T cells differentiate into antibody-secreting plasma cells and effector T cells respectively (Murphy, *et al*., 2008).

Both B and T cells generate B cell antigen receptor (BCR) and T cell antigen receptor (TCR) by joining Variable (V), diversity (D), and joining (J) segments of the Immunoglobulin loci and TCR loci in a process called V(D)J recombination. D is found only in heavy chain loci. V (D) J is a process initiated by recombination activation gene (RAG) that encodes for RAG1/RAG2 proteins which is expressed only in developing lymphocytes while it assembles their antigen receptors (Boehmer *et al*, 2010). RAG1/RAG2 recognizes recombination signal sequence (RSS). The V, D and J segments are flanked by conserved heptamer and nonamer motifs separated by non -conserved spacers of 12 or 23 base pair which makes up RSS. Recombination

obeys the 12/23 rule because it brings together gene segments flanked by RSSs with spacers of different lengths (Jones, *et al*, 2001). This 12/23 rule is important because it prevents the V genes from rearranging themselves during V (D) J rearrangement.

The process of V (D) J can be divided into the cleavage phase and the joining phase. Cleavage requires RAG 1/RAG 2 proteins while joining needs proteins used in non-homologous end joining (NHEJ) pathway of DNA repair (Jones, et al, 2001). In the cleavage phase, RAG1/RAG 2 recognizes and binds to two RSS and leads to a synapsis between the RSS or signal end and the coding joint, this initiates V (D) J recombination. It introduces small deletions and insertions at the coding joints. During recombination, single-stranded breaks are introduced in the heptamer and coding region. The 3' hydroxyl end of the coding DNA then attacks the phosphodiester bond on the opposite DNA strand in a reaction which results in double-strand DNA break leaving a hairpin sealed coding DNA end and a blunt signal end. The coding ends are then joined quickly after the DNA break. Terminal deoxynucleotidyl transferase (TDT) which is expressed in pro B cells during heavy-chain gene rearrangement adds nontemplated nucleotides (N-nucleotides) at rearranged joint gene segments and this enhances diversity of BCR repertoire (Murphy, et al, 2008).

V (D) J leads to BCR and TCRs with diverse antigen binding specificity to some of whom unfortunately bind self -antigens. This diversity in the repertoires makes the body able to recognize diverse pathogens (Boehmer et al, 2010). V (D) J recombination can also alter antigen receptor specificity and the rearrangement usually occurs in a particular sequence which is regulated by gene accessibility (Nemazee, 2000).

Immunoglobulin loci have three loci capable of undergoing V (D) J recombination. They are Immunoglobulin heavy chain (IgH) for heavy chain, kappa (Igk) and lambda (Igλ) for light chain loci. Igk and Igλ are not expressed in the same B cell, this is called Isotype exclusion. IgH rearrangement occurs before Igk rearrangement (Inlay, 2003). IgH rearrangement occurs by deleting intervening DNA, this is because the orientation of V, D and J gene are the same while Igk and Igλ have a different orientation for Vk or Vλ and their various J elements hence rearrangement occurs usually by inversion (Nemazee, 2000).

IgH variable genes rearrangement happens at the progenitor (pro) B stage. Here, DH rearranges to JH followed by VH to DJH. Here, B cells which have functional μ H-chains are selected for clonal expansion and differentiation while cells lacking functional μ- H chain often attempt rearranging the second allele or are eliminated if non- functional ones are still produced. For a pre B cell whose IgH locus has been rearranged to be selected, it must have a μ-chain which has the ability to associate with surrogate L-chain component λ5 and also mediate trans membrane signals through signal transducers Ig-α/β. This process is followed by cell division which results in cells exiting the cell cycle. L-chain rearrangement then occurs. It is easier for the body to rearrange Igk than Igλ, hence rearranges it first before Igλ. For both Igk and Igλ rearrangement, Vk rearranges to Jk. Feedback regulation mechanism is used by IgH to stop further rearrangement (Inlay, 2003 and Nemazee, 2000).

Both alleles of a B cell has the ability to produce an antibody bearing H and L chain but it uses one allele to produce a single antibody H and L chain at each point in time. The reason for this technique is not known. Allelic exclusion in both heavy and light chain locus during VDJ recombination is one way the body ensures that there is mono specificity. This is important in both self- tolerance and antibody effector function.

Allelic exclusion is the process by which after the body produces a productive VDJ recombination of one allele, the second allele is l

prevented from recombining. Recombination of the second allele occurs if the first V (D) J recombination is unproductive. In Igk locus, demethylation occurs and this process has been shown to promote transcription and it is mono allelic in mature B cells supporting the fact that only one allele can be rearranged at a time (Inlay, 2003). After a productive V (D) J recombination at both heavy and light loci, the BCR is said to be complete and is hence assembled. This signifies the end of proliferation and rearrangement of pre-B cell phase (Neuberger, 1997).

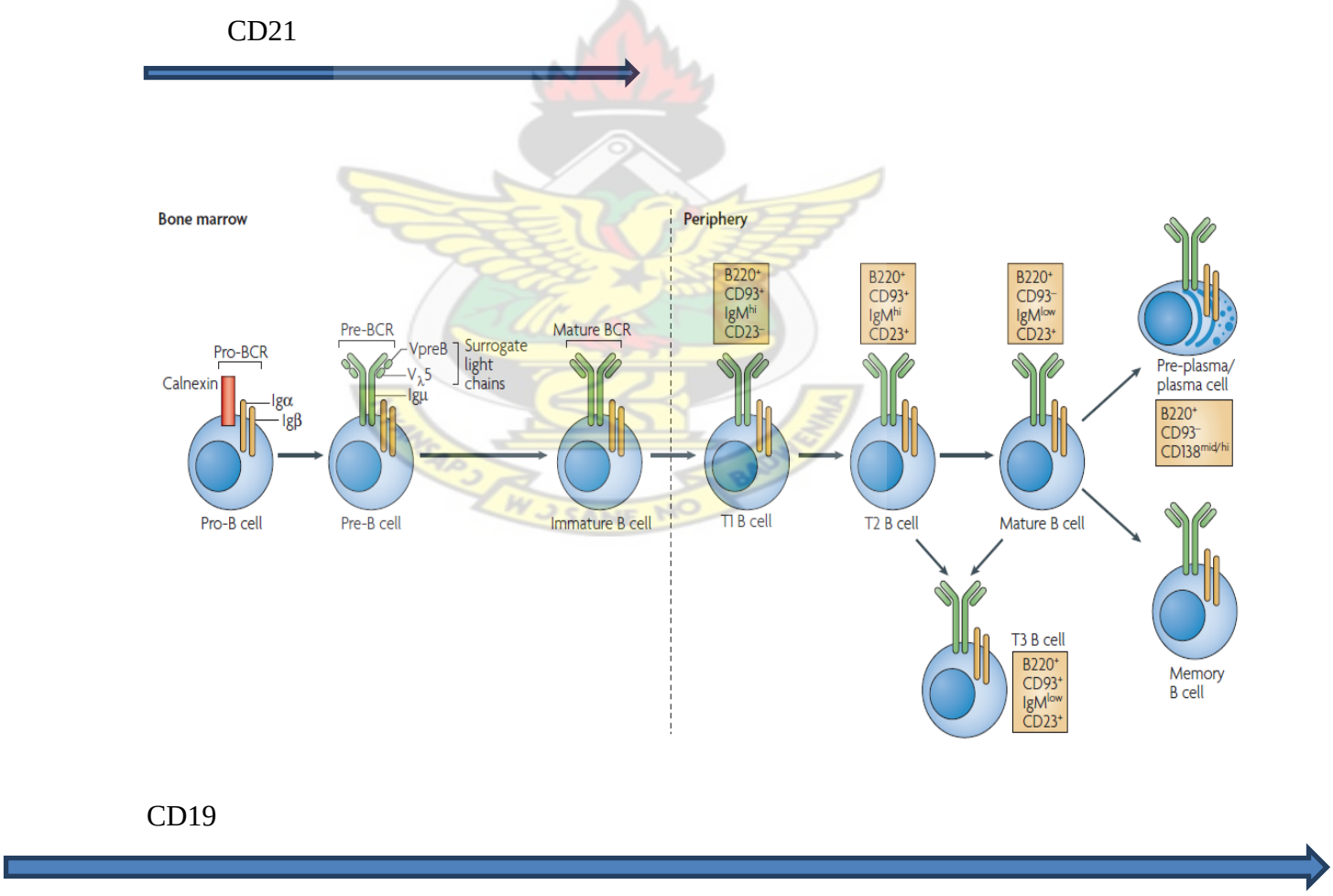


Figure 1: B-cell development stages.

This occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen. Each B cell stage has unique surface marker it express Figure adopted from Cambier, *et al*, 2007.

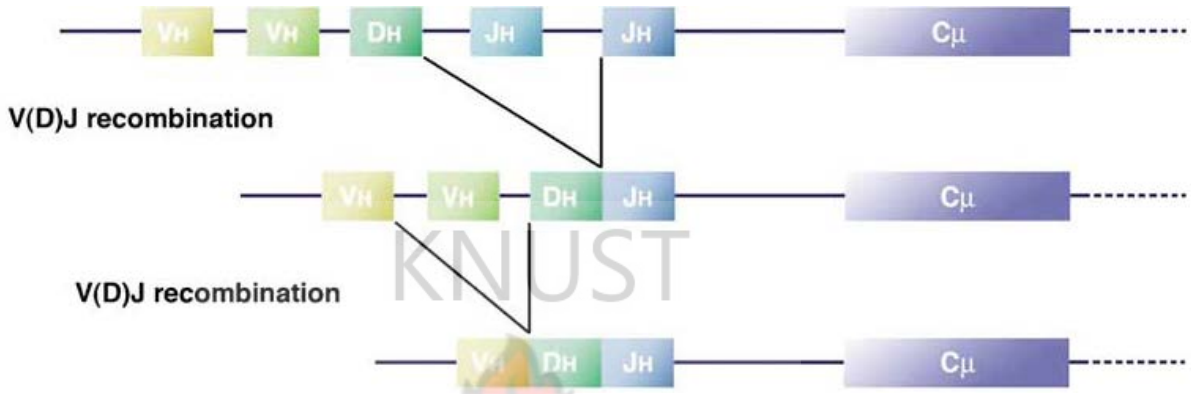


Figure 2: V(D)J recombination in heavy chain loci. Figure adopted from Soulas-Sprauel, P et al, 2007)

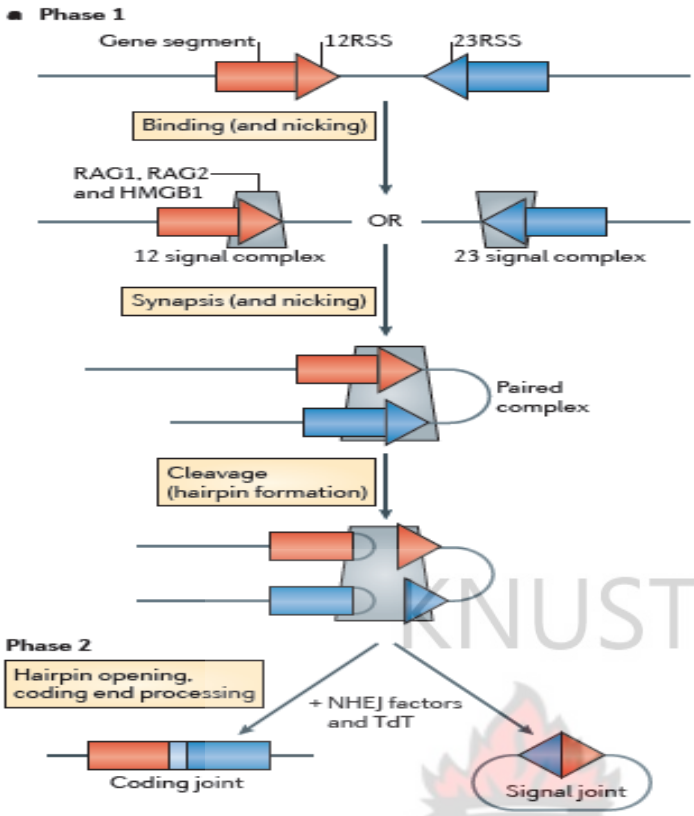


Figure 3: Mechanism of V (D) J recombination. Phase 1 involves the process of Rag1/Rag2 binding the 12 or 23 RSS to form a complex. Synapsis results with introduction of double strand breaks between the gene segments and the RSSs by RAG. Phase two involves re-joining of DNA ends by NHEJ DNA repair factors and also addition of non-template nucleotide (light blue rectangle) by terminal deoxynucleotidyl transferase (TdT). Figure adopted from (Schatz, et al., 2011).

2.5 Cellular Expression of B cells

B cells at various stages in their development express unique surface markers and some intracellular proteins. There have been various studies which have employed these surface markers to better understand both B and T cell development as well as their role in autoimmune diseases. B220 (CD45R) and CD19 are both markers expressed on the surface of all B cells right from the pro-B cell stage to mature B cell i.e. it's expression is throughout B cell development. They are both required for signal transduction in B cells. Some surface markers are unique to certain stages in the development process, this includes AA4.1 which is expressed at Pro-B cell stage to immature B cell stage and also CD21 (CR2) which is expressed on mature B cells (Murphy, 2008).

At the pro B cell stage, surface markers CD45 whose function is unknown, IL-7 receptor, CD43, Kit (CD117) and CD34 are expressed. Other receptors CD24 and IL2 receptor CD25 are expressed at the late Pro B stage and CD10 is expressed at both early and late pro-B stage. Kit, IL-7 receptor and CD24 are growth factor receptors (Murphy, 2008 and Nature reviews Immunology 7, 2007).

The Pre B-cell stage lacks the expression of Kit and IL-7 receptor but it expresses the enzyme BP-1 which is an amino peptidase. CD38, a B cell activation marker is expressed at the late pro-B and pre-B stage. CD20 and CD40 are both expressed at the late pro-B stage to when the B cells are mature. The expression of these specific surface markers at different stages of B cell differentiation makes it possible to study B cells employing the surface markers (Murphy, 2008 and Nature reviews Immunology 7, 2007).

2.6 Antibody Production

B cell activation is induced when a BCR recognizes epitopes of chemical structures such as viral particles, bacterial cells, polysaccharides, glycoproteins and proteins and binds to it. It then signals its interior organs and delivers the bound antigen to intracellular sites where degradation to form peptides occur. This is followed by a cascade of events. B cell is an efficient antigen presenting cell (APC) and its activation has two main pathways, T-dependent and T- independent.

In T-dependent (TD) activation, B cell activation requires T-cell help. B cells display antigens on their surface recognizable by T-cells. Another APC, dendritic cells can also activate B cells by moving to the site of infection. When a B cell binds to an antigen and it becomes internalized, it is returned to the cell surface as peptides bound to major histocompatibility complex II (MHC II) molecule which attracts antigen specific helper T cell (CD4) and sends signals to B cells which leads to its activation. It then proliferates and differentiates into antibody producing plasma cells. The antibodies secreted have the same specificity as the BCR which triggered its secretion. Dendritic cells also present antigen to naïve T cell via MHC II (Murphy, 2008 and Engel et al, 2011)

T-independent (TI) activation does not require T cell help. Certain common components of TI antigens which include polysaccharides, glycolipids, and nuclear acids which are self tolerant are recognized directly by the B cell and leads to its activation. Again when a B cell binds repeating epitopes of e.g. a bacterial cell, there is a formation of crosslinking of BCRs which can lead to its activation. The role of TLRs in this type of B cell activation has been identified, its activation gives an important signal that leads to the secretion of antibodies. The TI B cell activation can be divided into two types; Type 1 and Type 2. Type 1 leads to activation and

proliferation by stimuli such as lipopolysaccharide (LPS) or CpG that elicit polyclonal B-cell activation via Toll-like receptors and Type 2 is important against capsulated and cell-wall polysaccharides expressed by some bacterial pathogens. This requires massive crosslinking of BCRs which interacts with Brutons tyrosine kinase (BTK) and leads to prolonged calcium flux which results in B cell activation and proliferation. This induces antigen specific B cell response. Both TD and TI B cells are all capable of responding to (MZ) marginal zone B cells (Vos et al. 2000 and Murphy, et al., 2008). In lupus-like mouse model, 564Igi, autoantibody production has been shown to be T-independent (Berland, *et al*, 2006).

2.7 Tolerance and Break in Tolerance

Tolerance in both B and T cell development is crucial. It is the body's ability to recognize self from non-self antigens and checkpoints starts early during B cell development. There are several checkpoints during B cell development to ensure that BCRs produced are self-tolerant. Tolerance is described as being central tolerance when it occurs at the central lymphoid organs. Here, B and T cells that recognise self-antigens is either deleted by apoptosis or undergoes receptor editing. It is known that almost 50-70% of early immature B cells are self-reactive and the body there utilizes tolerance mechanism to correct this (Cambier et al, 2007). Some of these early self - reactive B cells not only bind to self but also exhibit polyreactivity i.e. they have the ability to bind several antigens. Some of the heavy chain V domain of the auto reactive antibodies has aromatic amino acid residues which can either be positively charged or not (Engel, et al 2011).

When a BCR is noticed to be self- reactive, i.e. if the intracellular signaling and receptor crosslinking exceeds a certain threshold, the body puts in measures to

recover from the self- reactivity. This include preventing CD62 ligand which is needed for B cells to enter the lymph nodes from being expressed, B-cell-activating factor (BAFF) which is a circulating cytokine required to sustain peripheral B-cell survival are poorly induced and the continual expression of RAG1/RAG2 proteins (Goodnow, *et al*, 2005).

Receptor editing which involves secondary rearrangement of mostly light chain loci provides a new BCR which is usually non-self- reactive. Igk is frequently rearranged because rearranged VkJk gene segments are usually flanked by Vk and Jk segments which have not been rearranged (Radic, et al, 1996 and Halverson, *et al*, 2004). Igλ rearrangement occurs if Igk recombination is non- productive or is productive but still auto-reactive. Rearrangement also occurs at a much lower frequency in the IgH locus (Halverson, *et al*, 2004). If this approach fails to correct self-reactivity, the cell undergoes deletion by apoptosis. This clonal deletion may result partly because of growth factor withdrawal due to the low expression of BAFF and also due to increasing levels of pro –apoptotic factor BIM(BCL-2-interacting mediator of cell death) which inhibits B cell survival proteins from BCL-2 family. A study has shown that deficiency in BIM leads to an increase in production of anti-DNA autoantibodies (Goodnow, et. al, 2005). BAFF triggers an increase in NFkB2 which maintains peripheral B-cell survival through BCL-2 expression and it also induces PIM2 a serine-threonine kinase which has potent pro-survival effects by phosphorylating and inhibiting the activity of BAD, a pro-apoptotic protein. B cells expressing a functional BCR which is not self-reactive enters the periphery as transitional B cells (Cambier et al, 2007).

Despite receptor editing and deletion, some self-reactive B cells escape and enter the periphery. Here, another tolerance mechanism known as peripheral tolerance is available. At the periphery, anergy occurs. Anergy is a condition in which the self – reactive B cell is unresponsive to antigens though they survive. Anergic B cells are lethargic; they cannot sustain activation after mounting an initial response to antigen because they do to receive secondary signals. Anergic B cells can be differentiated from naïve B cells by their life span and by their anatomical location (Cambier et al, 2007). Anergic B cells are excluded from B cell follicles and marginal zone (MZ) in the spleen. Transgenic mice carrying the autoimmune immunoglobulin 546 has been shown to undergo normal B cell tolerance mechanism of receptor editing, deletion and anergy just like non autoimmune C57BL/6 mice, but auto-reactive B cells are still present (Berland, et al, 2006). This implies that some developing B cells though auto-reactive escape tolerance and proceeds to become mature B cells. They are able to produce autoantibodies characteristic of SLE and other autoimmune diseases. The mechanism used by B cells to escape tolerance is unknown.

2.8 Class Switch Recombination

Class switch recombination (CSR) requires Activation-induced cytidine deaminase (AID) in order to happen. AID is an enzyme which has similar sequence as RNA editing enzyme APO-BEC-1 but they differ in function since AID works directly on single stranded DNA. The expression of AID induces CSR which is an isotype switching process where the constant region of the immunoglobulin changes from IgM antigen receptors to IgG, IgA, IgE (Chaudhuri et al, 2003). This isotype switching results in a change in effector function since the constant region of the heavy chain determines the effector function of Ig. The specificity of the variable

region however is maintained. CSR is guided by stretches of repetitive DNA called switch(S) regions. S regions consist of repeats of short G-rich sequences which are 20–80 bp which are located upstream of each CH gene with the exception of Cδ region (Stavnezer, 2011).

CSR has been shown to occur in both developing and mature B cells. Generally, CSR is known to occur in mature B cells, a study recently has shown that not only is AID expressed in developing B cell but CSR and somatic hypermutation (SHM) occurs as well. The expression of AID was T cell independent. The study showed the presence of circle transcript (CT) and postswitch transcripts (PST) in developing B cells. CT is AID dependent and its presence indicates ongoing CSR while the presence of PST which occurs after the association of the germline μ promoter with a particular CH of the switched isotype were both seen in developing B cell (Hans, et, al., 2007).

Studies in both human and mice have shown that, a deficiency in AID results in hyper-IgM phenotypes (Stavnezer, 2011).AID is also required for somatic hypermutaion (SHM) which is the introduction of point mutations at high frequency into IgH and IgL of the immunoglobulin variable gene region. This allows for selection of B cells that secrete higher-affinity immunoglobulin. Both CSR and SHM involves the process of chromatin opening of target sites i.e. Switch and Variable regions respectively which is transcription associated, DNA cleavage, repair and ligation.

The mode of action of AID is to introduce DNA double-strand breaks and repair by means of end joining. AID acts by removing the amino group in cytidine (C) base and replaces it with uridine(U). This mismatch would be corrected by either a base - excision repair (BER) which is a ubiquitous repair pathway that repairs damaged

DNA bases. This is done by uracil-DNA glycosylase (UDG) which removes the uracil. The removal of U creates sites which can undergo replication using error-prone trans-lesion polymerases. The mismatch can also be used as a template for DNA synthesis which can cause a C:G transition mutation to T:A base pair since the U is analogous to Thymidine (T) in DNA and T pairs with Adenosine(A).The process of CSR involves BER introducing single stranded breaks (SSB) into the switch region (S), this region contains a lot of AID hotspots target. If the S region where the break occurred is on opposite sides and it is close to each other, double stranded breaks (DSB) occur. However if the SSB occurs at regions far apart mismatch repair (MMR) another DNA repair pathway is required (Stavnezer, 2011). The repair mechanisms employed by the cells leads to the generation of DNA breaks needed for the recombination events involved in CSR and SHM (Imai et al., 2003).

There are five main classes of immunoglobulins which are distinguished by the structure of their heavy chain constant (CH) region. Each CH region has a different affinity for the proteins it binds. Such proteins include complement and Fc receptors. It also affects the stability of the antibody. IgM is the first Ig to be produced during B cell development, It has the ability to form pentamers(macroglobulin) and has a short life span of 10days in the serum. It activates compliment and is not transferred across the placenta. When B cell is activated by an antigen, IgM is the first produced and through CSR, antibodies of the various isotypes such as IgG, IgA and IgE are produced having the same specificity as the BCR. All the various classes of Ig can occur as transmembrane antigen receptor or secreted antibody. IgG has various subclasses which are IgG1 to IgG4 in humans and IgG1, IgG3, IgG2a, IgG2b in mouse. It has a much higher half-life in serum than IgM. It is transferred through the placenta hence providing immunity to the fetus and activates the classical pathway of

complement. IgA, IgE and IgG have shorter life span than IgM. They do not activate complement with the exception of IgA1 which activates complement through the alternative pathway (Murphy et al, 2008). Class switched IgG is most important in SLE due to its role in the disease. They have also been implicated in tissue damage. In murine SLE, IgG2a and IgG2b (IgG1 and IgG3 in humans) are the most abundant autoantibody subclass detected in the sera.

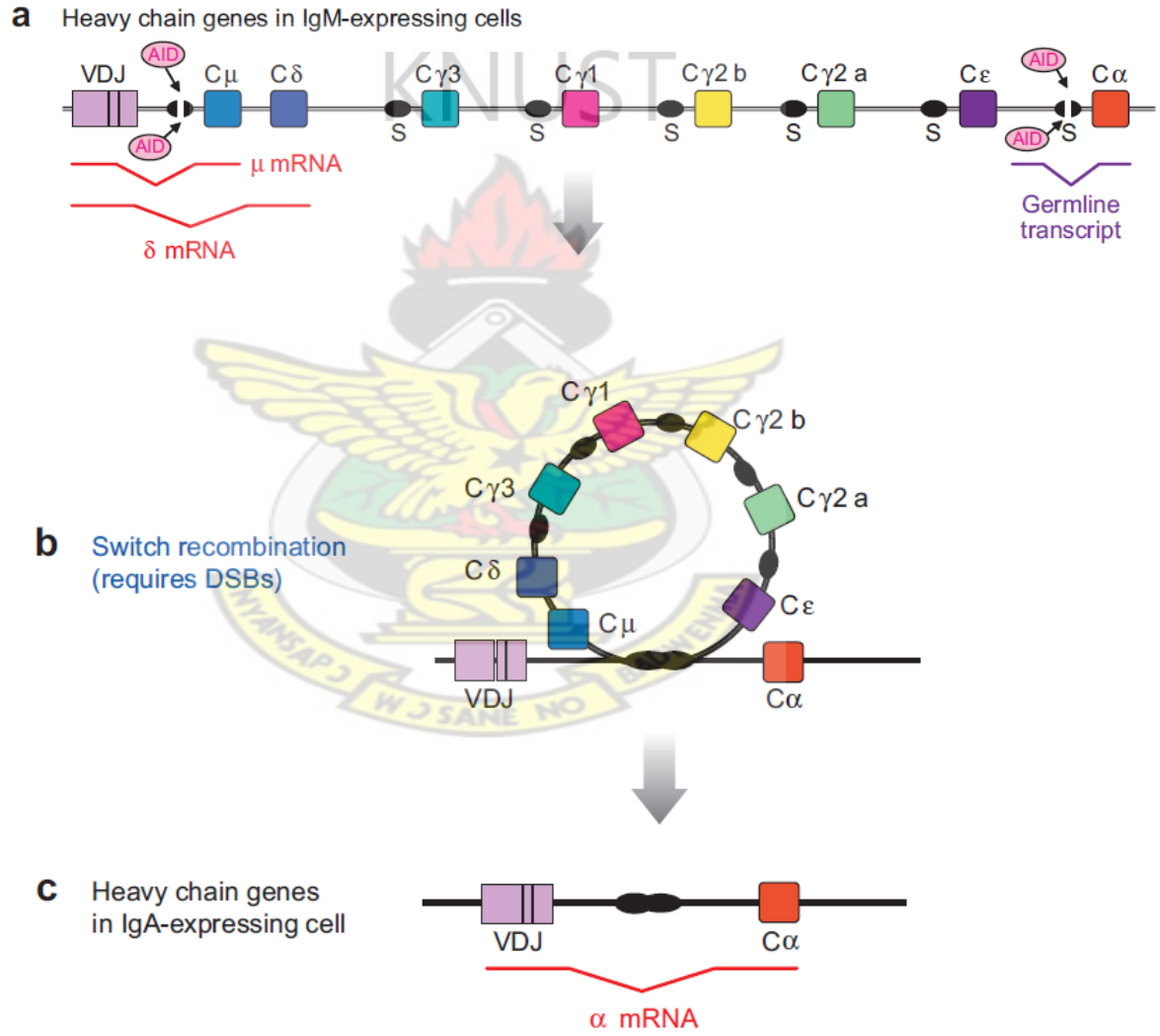


Figure 4: Diagram of Ig class switch recombination (CSR) to IgA. (a) The mouse IgH locus in B cells expressing IgM and IgD (by alternative RNA transcription/processing). (b) During CSR, activation-induced cytidine deaminase

(AID) deaminates dC residues in strands of transcriptionally active S regions initiating a process that results in double-strand DNA breaks (DSBs) in S regions.

(c) The IgH locus after CSR to IgA. Splicing diagrams of the μ, δ mRNAs and the germline α transcript. Similar germline transcripts are induced from unrearranged Cγ, Cε, and Cα genes, depending on the cytokine stimulation received by the B cell (Figure adopted from Stavnezer, et al, 2008).

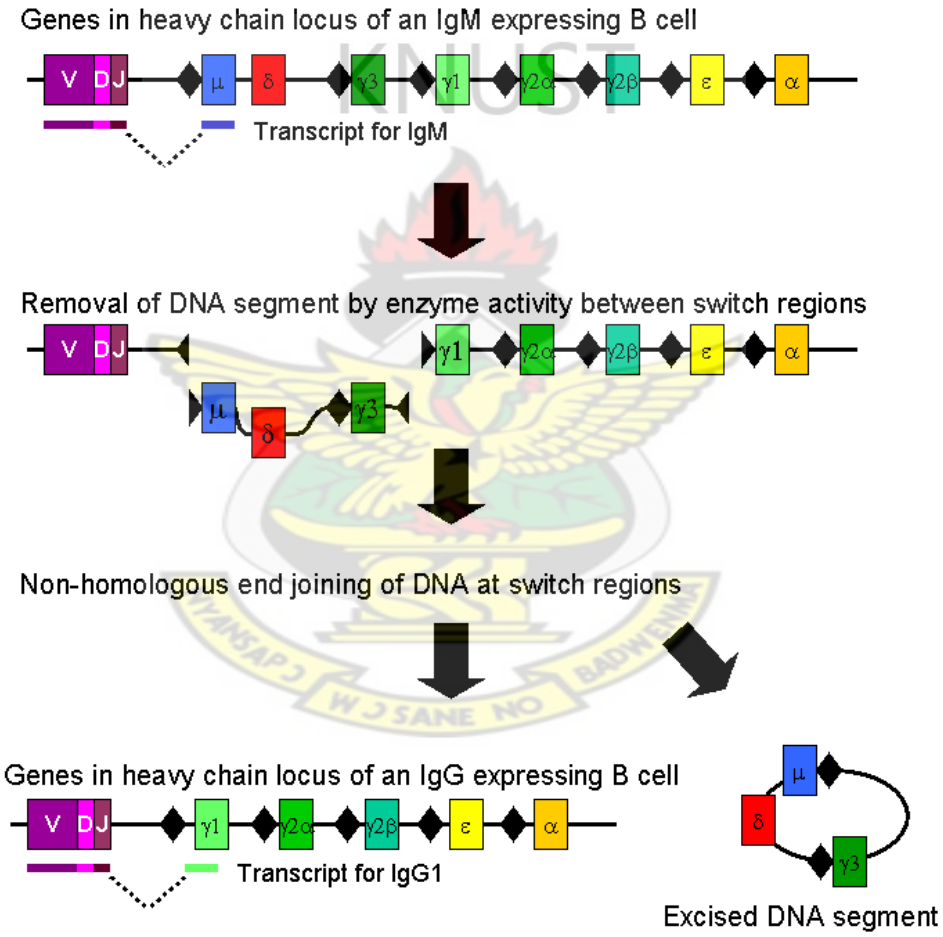


Figure 5: Diagram of IgM class switch recombination (CSR) to IgG1 Figure adopted from Thesis of unpublished work.

2.9 Mice

564Igi mice was created using 564Ab from a hybridoma derived from SNF -1 mice which is an F1 progeny derived by crossing NZB an autoimmune mouse and SWR which is a normal mouse. SNF-1mouse produces high levels of IgG autoantibodies and develops glomerulonephritis as observed in SLE patients. The heavy and light chain genes of 564Ab were targeted to that of C57BL/6 a non- autoimmune mouse by back crossing mice having either heavy or light gene to C57BL/6 for about 10 generations and then intercrossing to generate mice having inserted 564 heavy and Light chain genes. This mouse generated produces antibody against RNA, single stranded DNA and nucleosomes. 564Igi mice also develop glomerulonephritis observed in SLE patients and tolerance has been shown to occur normally in this mouse. 564Igi Rag-/- mice are unable to undergo V(D)J recombination and hence express only pre rearranged 564 transgene while in the presence of rag, normal receptor editing occurs producing some Id negative antibodies in 564Igi mice (Berland, et al, 2006).

Conditional gene targeting allows a particular gene of interest to be studied and a common enzyme used for such a technique is cre recombinase. This enzyme is derived from a bacteriophage and it recognizes a 34 base pair motif called lox P sites. Flanking a target gene segment with sequences recognized by recombinases allows for conditional gene targeting (Rajewsky,et al, 1996).

This cre recombinase model system was employed to create two kinds of mice to regulate the expression of AID in B cell at specific stage in its development. To ensure the conditional expression of AID, a conditional transgenic mouse whose AID expression is blocked by GFP flanked by two loxP sites. The expression of AID is

only possible by the expression of cre recombinase which recognizes the loxP sites and hence deletes the GFP cDNA from the transgene. To create this mouse, a GFP-AIDtg mouse is crossed with a mouse which has the cre gene knocked into the downstream of either a CD19 or CD21 promoter (Muto, et al, 2006, Kraus, et al, 2004). A 564Igi mouse on an AID deficient background, having AIDtg which is flanked by GFP with two loxP sites and either CD21 or CD19 promoter is obtained from a cross between 564Igi.AIDtg mouse and CD19cre or CD21cre mouse.

This mouse model allows conditional expression of AID and hence its activity at the various stages can be studied. AID expression in CD19cre mice starts from the pre B stage to the mature B stage and CD21cre mice AID expression is restricted to mature B cell stage because the expression CD21 proteins starts when immature, transitional B cells differentiate into mature B cells(Muto, et al, 2006, Kraus, et al,2004).

A.

B

Cre Expression

developmentally controlled by the genomic CD21 locus

Figure 6: The conditional AID transgene construct that expresses AID after Cre-dependent recombination. The floxed GFP located upstream of AID cDNA is regulated under the transcriptional control of the constitutive and ubiquitous promoter pCAG. Figure is adopted from muto et al, 2006. (B) Cre transgene mediating Cre expression in a stage specific manner. (CD21-cre) Figure adopted from Kraus, et al, 2004.

CHAPTER THREE

3.0 MATERIALS AND METHODS

The various experiments with the mice were done with proper clearance from Tufts/ NEMC IACUC. They were performed at Tufts University, Immunology Program, Boston, MA, USA.

3.1 Genotyping

This was done using DNA from 564Igi mice. To obtain the DNA, 2-3cm of tail from mice about 3weeks old is cut and incubated overnight in 180µl of Viagen Direct PCR (Tail) lysis buffer and 20 µl of Qiagen Proteinase K shaking at 55 degree Celsius (^{o}C) . The crude lysates was heat inactivated at 85 ^{o}C for 45 mins after overnight incubation. The lysates was then centrifuged for 30secs at 3000rpm and then used in Polymerase chain reaction (PCR). The reagents used for the PCR is from Invitrogen i.e Tag polymerase(5U/µl), 50mM $MgCl_2$, 10X PCR buffer , 10mM dNTP . To determine which mice where 564Igi mice the following primers were used to determine which had 564Igi heavy chain and light chain. Heavy chain forward primer, 564VH-F has the sequence 5'TGG AGC TAT ATC ATC CTC TTT 3' and reverse primer, JH4Eµ-R has the sequence 5'CAC AGA TTC TTA GTT TTT CAA 3'. For Light Chain 564Vκ-F, forward primer sequence is 5'CAA GTG CAG ATT TTC AGC TTC 3' and reverse primer Jκ5-R is 5'CAG CTT GGT CCC AGC ACC GAA 3'. The expected size for both heavy and light chain is 700bp and 550bp respectively.

To determine endogenous heavy and light chain, reverse primers of both heavy and light is the same as that of 564Igi but the forward primers are JH2 5' TCA GGT CAT GAA GGA CTA GGG ACA C3'and Jk23F 5' AGT AAC TAA ACT AGG GGA

AGA GGG 3' for heavy and light chain respectively. The expected product size for heavy endogenous is 1100bp and light endogenous is 800bp.

The PCR master mix for 564Igi heavy and endogenous heavy chain was prepared using 14.8µl dH_2O, 2µl 10X buffer, 0.4µl 10mM dNTP, 0.8µl 50mM MgCl2, 0.4µl each of 10µM 564VH-F and 10µM JH4Eµ-R (10µM JH2-F for endogenous heavy chain forward primer), 0.2µl Taq polymerase (5U/µl) and 1µl genomic DNA from crude lysates. 564Igi light and endogenous light chain was prepared using 15.3µl dH_2O, 2µl 10X buffer, 0.4µl 10mM dNTP, 0.5µl 50mM MgCl2, 0.4µl each of 10µM 564Vk-F and 10µM Jk5-R (10µM Jk23-F for endogenous light chain forward primer), 0.1µl Taq polymerase (5U/µl) and 1µl genomic DNA from crude lysates. All the master mixes are put into PCR tubes and run using a thermal cycler with PCR cycle of 94 ^oC for 3 min, 40 cycles of 94 ^oC for 30 sec, 55 ^oC for 30 sec, 72^oC for 2 min and 72 ^oC for 15min holding at 4^oC. 564Igi homozygote and C57BL/6 are used as controls.

Genotyping of CD21 and CD19 mice was done by Tag center transnetyx automated genotyping using the primers Cre-R1 TTC CAT GAG TGA ACG AAC CTG G and Cre-R2 TTA CCG GTC GAT GCA ACG AGT for cre recombinase genotyping and AID-S1: CGG CAT GAG ACC TAC CTC TGC TAC, AID-R1: CCA GGC TTT GAA AGT TCT TTC ACG, IMR0042: CTA GGC CAC AGA ATT GAA AGA TCT and IMR0043: GTA GGT GGA AAT TCT AGC ATC ATC C for AIDtg gene genotyping.

3.2 Enzyme linked Immunosorbent Assay (ELISA)

In this assay, Immulon 1 B, 96 well plate was coated with 50ul per well 1ug/ml anti-idiotype in 1X borate buffer and incubated at 4^oC overnight. After overnight

incubation, the liquid is flipped out and the wells are blocked with 1% BSA/0.1% NaN3/Borate buffer and incubated for 30mins at room temperature. It was then washed three times using 0.1% Tween20/Borate buffer and tapped dry. 50ul per well sera from CD21, CD19 and control mice was added and incubated at 4oC overnight. Sera were serially diluted from 1:30 to 1: 30000 in 1% BSA/0.1% NaN3/Borate buffer. Plates were washed five times with 0.1% Tween20/Borate buffer. Alkaline phosphatase (AP) conjugated secondary antibody (IgM-AP, IgG2a-AP, IgG2b-AP) was diluted at 1: 1000 and added at 50ul per well. It was then incubated at 37^{o}C for 1hr followed by washing and addition of 50ul per well of substrate (4-nitrophenyl phosphate disodium salt hexahydrate) which was prepared by using 1mg/ml substrate tablet in ELISA buffer. It was kept at room temperature and data was acquired in a Spectra Max 340 ELISA plate reader (Molecular Devices) at the optical density of 405 nm (OD_{405}) after 1hr, 3hrs, 5hrs and 12hrs.

Total IgG1 antibodies was also determined using the same procedure described above but in this instance the plates were coated with IgG1 goat anti mouse antibody at 1:1000 dilution.

3.3 RNA ELISA

Nunc maxisorp flat bottom 96 well plate was coated with 100ul Poly-L-Lysine, placed in a humidified chamber under foil and incubated at room temperature for 4hrs. The plates were emptied and tapped dry followed by coating with 10µg/ml RNA diluted in 1X borate buffer. Each well was coated with 50ul and incubation was done at 4^{o}C overnight. The procedure after this is the same as the ELISA described above, the only exception is the use of goat blocking buffer instead of BSA to dilute sera and secondary antibodies.

3.4 HEp-2 Staining

HEp-2 staining employs the use of premade slides fixed with antinuclear antibody test antigen substrate (ANA substrate slides). CD19, CD21, C57BL/6 and 564Igi Sera were diluted in 1X PBS. 1:50 and 1:100 dilutions were used. 20µl of diluted samples were loaded to designated wells on slides and incubated for 30-45mins at room temperature in 100% humidified chamber. Slides where washed twice with 1X PBS and submerged in 1X PBS for 10mins. Slides were rinsed with dH2O and tapped dry. 20ul of Secondary antibody (IgG A488) which was prepared in 1X PBS using 1: 2000 dilution was added to each well on the slide and incubated for 30mins at room temperature. The slides were then twice washed with 1X PBS and submerged in the same buffer for 10mins. Anti-fading reagent was put in each well and slide was covered with cover slides. Manicure was applied to each corner of cover slides to fix it on slide. The slide was then allowed to dry and viewed using a fluorescent microscope at a magnification of 100X.

3.5 Statistical Analysis

Student t test was used to determine significance of the various groups in the results.

CHAPTER FOUR

4.0 Results

4.1 Total IgG1 ELISA

To verify that class switch recommendation (CSR) was not compromised in the transgenic mice, total IgG1 ELISA was performed using sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21 (CD21) and 564.AIDtg.CD19 (CD19). The Results as shown in Figure 7 shows controls C57BL/6, 564Igi WT as well as CD21 and CD19 mice have normal class switching. While AID-/- and 564Igi AID-/- have no class switching. N is the total number of mice used in the experiments. Error bars represent Standard error of the mean (SEM). There is no significant difference between B6, 564Igi WT, CD21, and CD19. P> 0.05

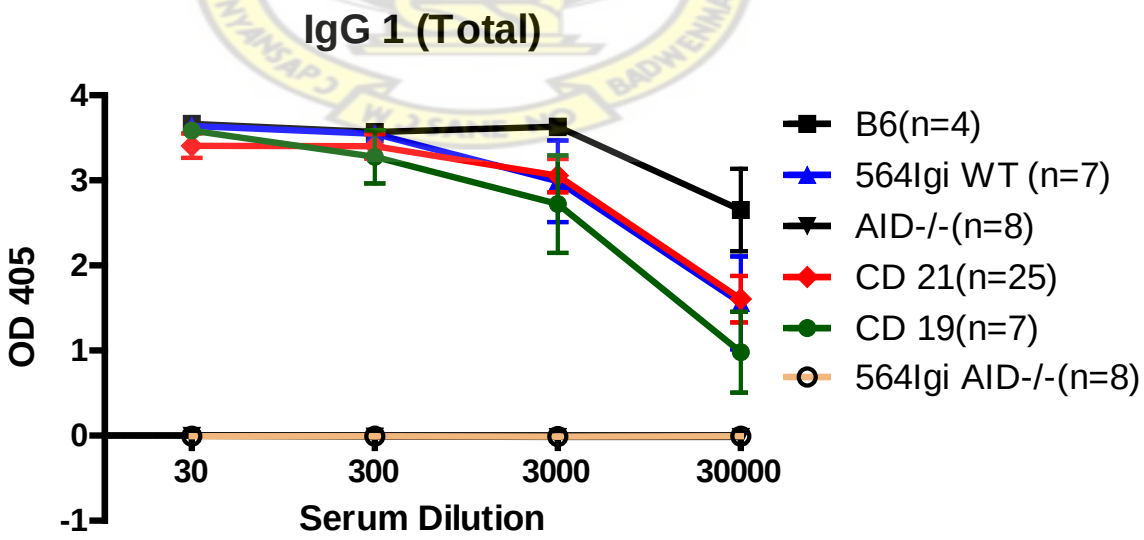


Figure 7. Class Switch Recombination is not affected in CD21 and CD19 mice

4.2 Idiotype and RNA ELISA

These two main types of ELISA was done to determine the group of mice that were Idiotype positive as well as those that had Anti-RNA antibodies of the various antibody classes used. Most 564Igi CD21cre do not have RNA binding auto-antibodies.

A. Figure 8A shows Idiotype ELISA which was done to determine the mice that were IgM Idiotype positive. Sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21 (CD21) and 564.AIDtg.CD19 (CD19) was used. Id+ IgM antibodies were measured. N is the total number of mice used. Error bar represent SEM. The result shows B6 does not have Id+ IgM antibodies, all others have varying degree of Id+ IgM antibodies.

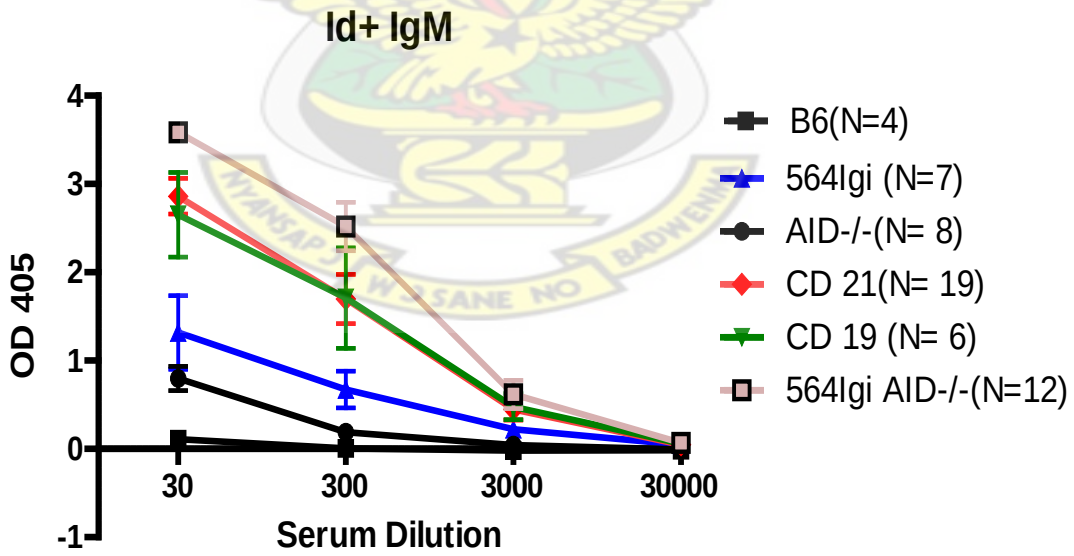


Figure 8A. Idiotype Positive IgM

B. Figure 8B shows RNA binding ELISA which was performed using sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21 (CD21) and 564.AIDtg.CD19 (CD19). Anti- RNA IgM antibodies were measured. N is the total number of mice used. Error bar represent SEM. The various types of mice have varying degree of anti-RNA IgM antibodies.

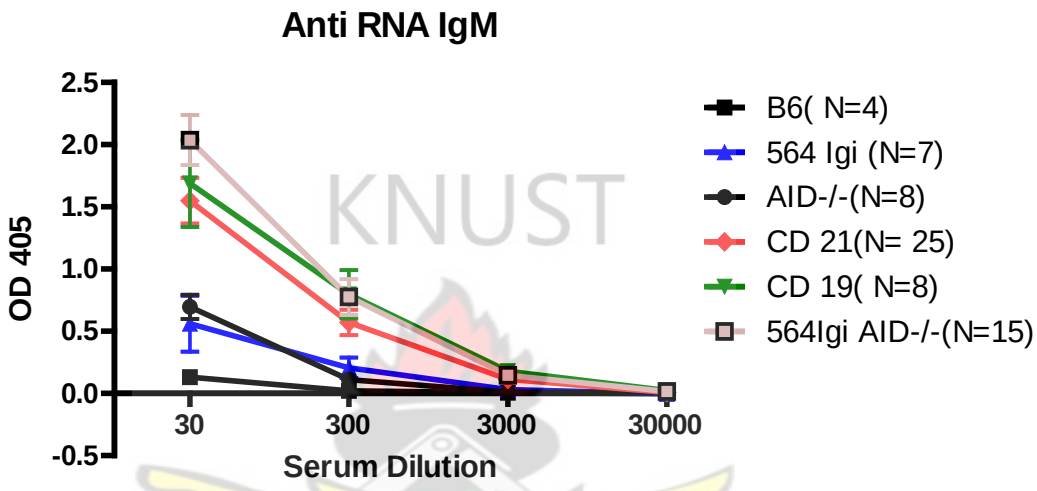


Figure 8B. Anti RNA IgM

C. 564 Idiotype ELISA was performed using sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21 (CD21) and 564.AIDtg.CD19 (CD19). Id+ IgG2a antibodies were measured. N is the total number of mice used. Error bar represent SEM. The results as shown in Figure 8C shows no Id+ IgG2a antibodies in B6, AID-/- and 564Igi AID-/-, and varying degree of antibodies in CD21 and CD19 mice.

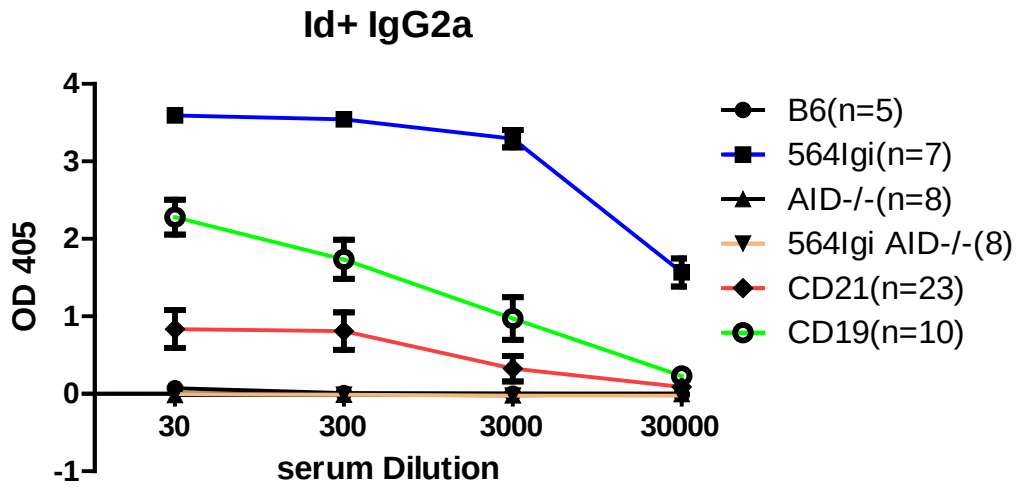


Figure 8C. Idiotype positive IgG2a

D. Figure 8D shows RNA binding ELISA which was performed using sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21 (CD21) and 564.AIDtg.CD19 (CD19). Anti- RNA IgG2a antibodies were measured. N is the total number of mice used. Error bar represent SEM.

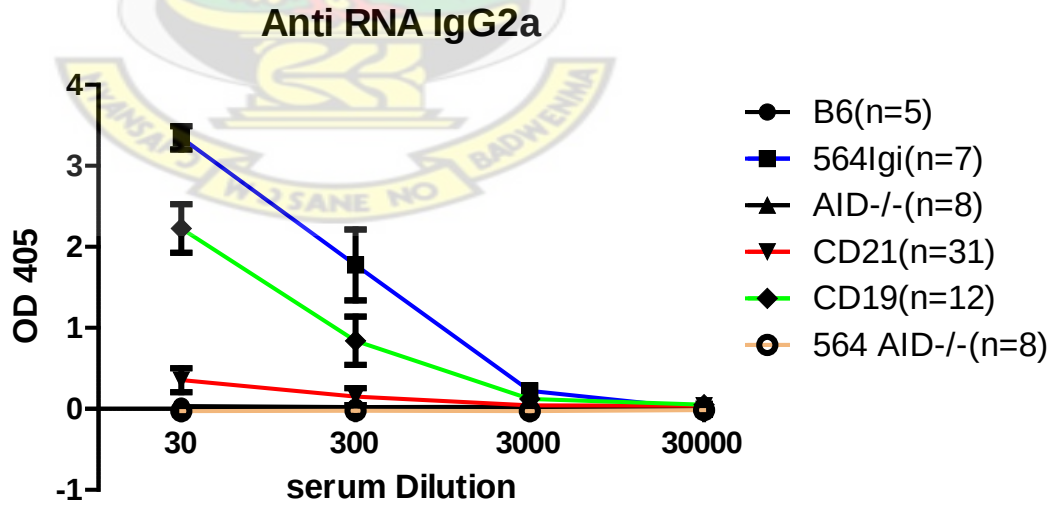


Figure 8D. Anti RNA IgG2a

E. Figure 8E shows 564 Idiotype ELISA which was performed using sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21

(CD21) and 564.AIDtg.CD19 (CD19). Id+ IgG2b antibodies were measured. N is the total number of mice used. Error bar represent SEM.

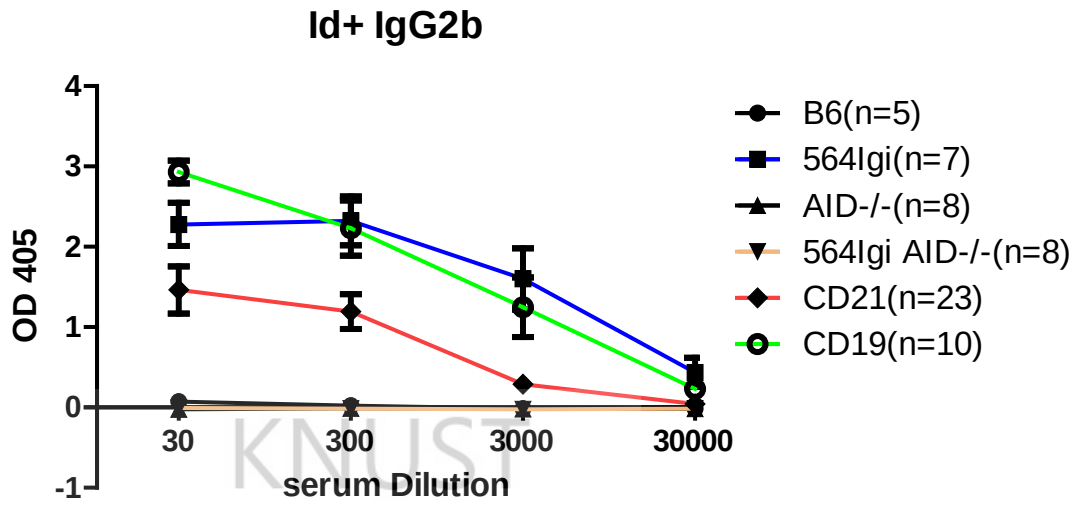


Figure 8E. Idiotype Positive IgG2b

F. Figure 8F shows RNA binding ELISA which was performed using sera from C57BL/6, 564IgiWT, AID-/-, 564Igi AID-/-, 564.AIDtg.CD21 (CD21) and 564.AIDtg.CD19 (CD19). Anti- RNA IgG2b antibodies were measured. N is the total number of mice used. Error bar represent SEM

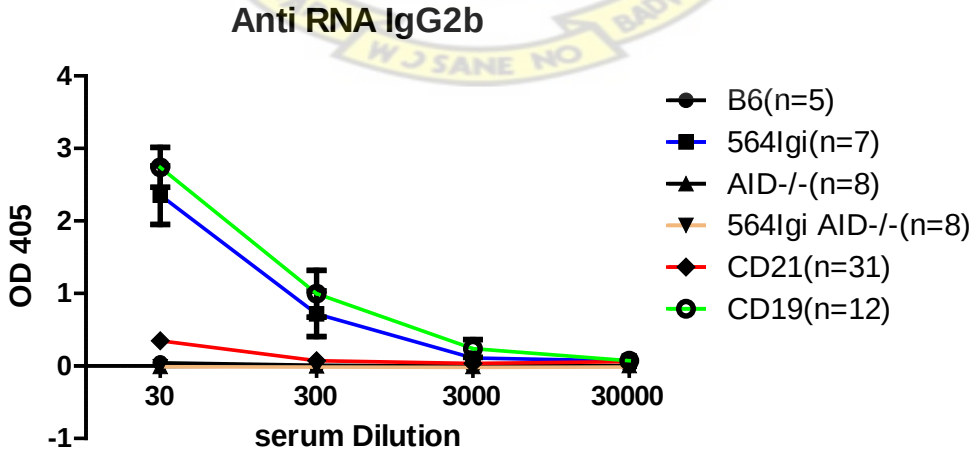


Figure 8F. Anti RNA IgG2b

4.3 Hep 2 staining

HEp 2 staining was done using sera from 564Igi WT, C57BL/6, 564Igi AID-/-, 564.AIDtg.CD21cre (CD21) and 564.AIDtg.CD19cre (CD19) to determine IgG auto-reactive antibodies. Three independent experiments were conducted. A total of 3 mice per strain were used in each of the experiments as shown in Figure 9A to E.

A.

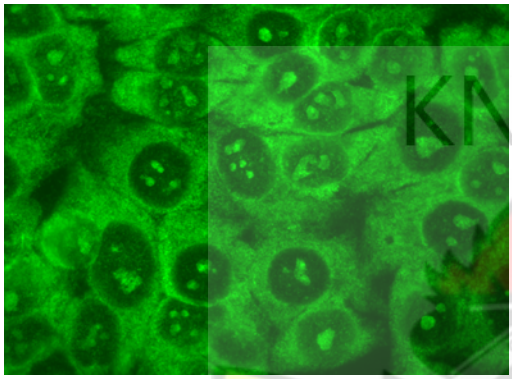


Figure 9A. 564Igi WT

B.

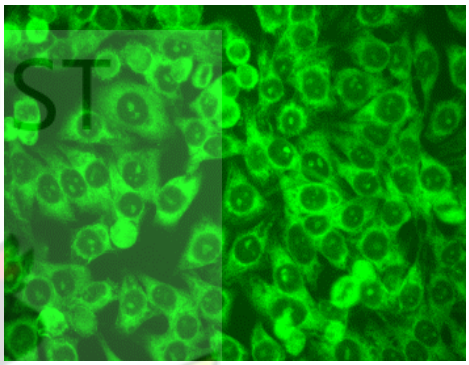


Figure 9B. CD19cre

C.

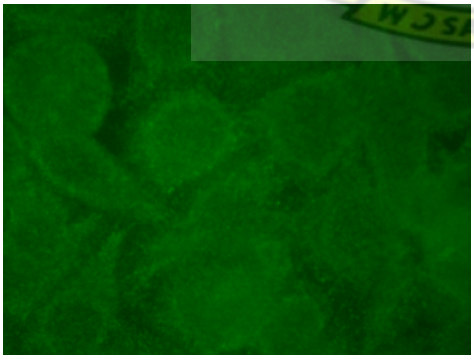
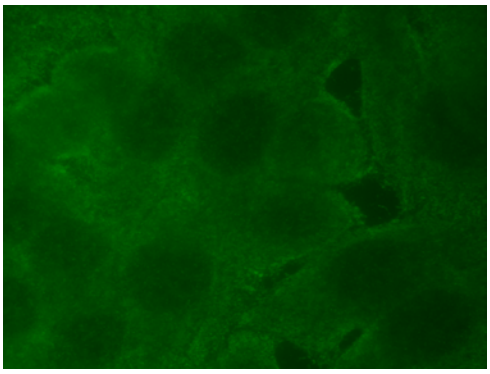


Figure 9C. C57BL/6

D. E.



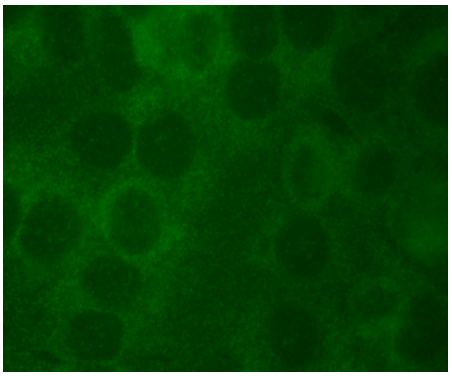


Figure 9D. 564Igi AID-/- Figure 9E. CD21cre

Figure 9. Most 564Igi CD21cre mice do not have nucleoli binding autoantibodies

4.3 GENOTYPING

This was done to determine the genotypes of the mice used in the experiments and to ensure that the right group of mice was used for the experiment.

A. Heavy Chain endogenous gene and 564Igi heavy chain gene respectively as shown in I and II. A mouse is positive for 564Igi heavy chain gene if it has no endogenous band but has 564Igi band. It is negative if it has endogenous band but no 564Igi band. It is heterozygous if it has both endogenous and 564Igi band. As shown in I and II below.

I.

Figure 10AI. Heavy Chain endogenous gene

II.

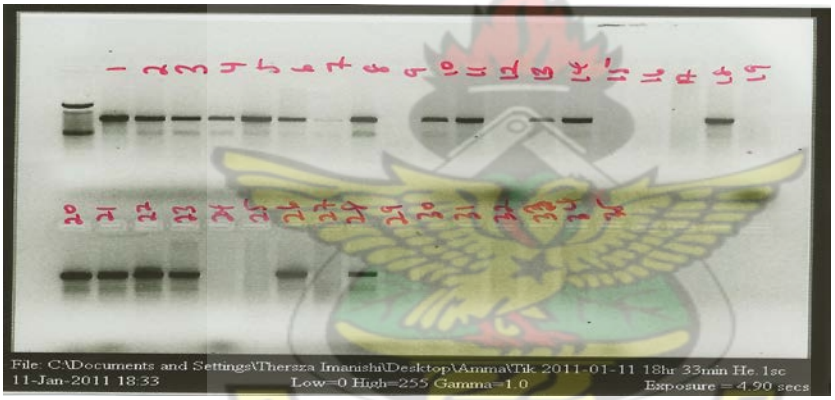


Figure10AII. 564Igi heavy chain gene

B. Light Chain endogenous and 564Igi light chain gene respectively as shown in I and II. A mouse is positive for 564Igi light chain gene if it has no endogenous band but has 564Igi band. It is negative if it has endogenous band but no 564Igi band. It is heterozygous if it has both endogenous and 564Igi band.

I.

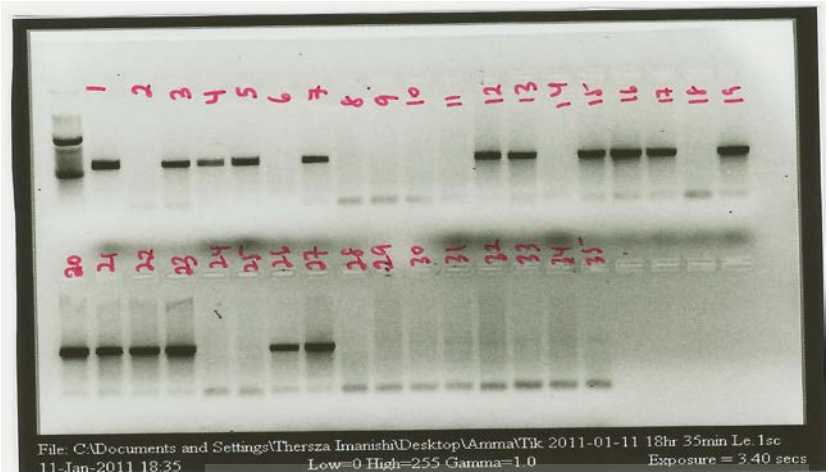


Figure10BI. Light Chain endogenous

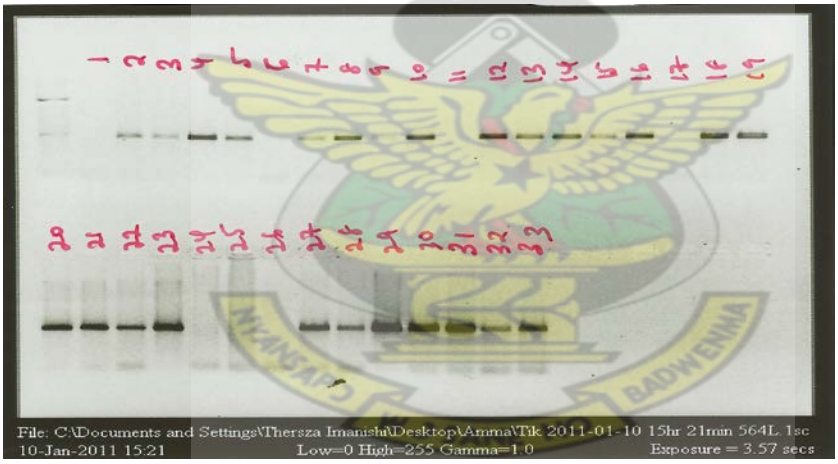


Figure10BII. 564Igi light chain gene

Figure 10: 564Igi Heavy and Light Chain products from PCR run.

CHAPTER FIVE

5.0 Discussion

CSR has been mostly shown to occur in mature B cells when they are responding to an antigen. This is mediated by AID which is generally found to be highly expressed in B cells responding to antigen stimulation. It has recently been shown that, developing B cells can also undergo CSR and SHM. The study observed AID expression in pre B and immature B cells indicating that AID mediated processes of CSR and SHM would occur. The presence of circle transcript (CT) in immature B cells which also disappear rapidly was observed in the developing B cells. The production of CT is dependent on AID and their presence suggests that class switching is ongoing. The presence of postswitch transcripts (PST) was also observed in cDNA of pre B and immature B cells indicating that CSR has occurred. PST is the association of the germline μ promoter with a particular CH of the switched isotype. The study showed that the expression of AID in developing B cell was T cell independent (Hans, et al, 2007). AID expression as well as active and functional CSR and SHM in developing B cells led to the question of finding out which B cells were actually making class switched idiotype positive and anti-RNA autoantibodies.

Conditional gene targeting is an important method to studying a particular gene function and specific type of cell. To determine whether the cre recombinase model system that was used in our study is effective and that conditional expression of AID in CD19cre and CD21cre mice was occurring as expected, total IgG1 antibodies was tested using ELISA to determine if the cells were undergoing normal class switching. As negative controls C57BL/6 on an AID deficient background (AID-/-), 564Igi on AID deficient background which is either AID transgene (AIDtg) positive or

CD19/CD21cre positive but not both (564Igi .AID-/-) was used. Figure 7 shows class switched antibodies in CD21cre mice, CD19cre mice as well as 564Igi and C57BL/6 mice. One way ANOVA shows no significant difference between the antibodies produced by these mice ($P< 0.05$). AID-/- mice and 564Igi AID-/- show no class switching displayed by lack of IgG1 antibodies. Figure 7 shows that the expression of cre induces AID expression since 564Igi AID-/- mice could not produce IgG1 antibodies because they lacked either the AIDtg or cre. This was not the case of CD19cre and CD21cre mice which had both the AID transgene and cre. The result supports literature concerning the conditional expression of AID (Muto, et al, 2006) and lack of class switched IgG1 antibodies in AID-/- mice confirms that AID is indeed needed for class switching to occur. A study using cre mice and another using 564Igi mouse showed normal B cell development is not disrupted in these transgenic mice both in the bone marrow and secondary lymphoid organs. The B cells also displayed normal weight, size and percentage indicating that conditional expression of AID does not affect B cell development (Muto, et al, 2006 and Kraus, et al, 2004).

564Igi mice undergo normal receptor editing and anergy. A study of the 564Igi mice showed evidence of receptor editing and anergy. A number of B cells from these mice expressed endogenous b allotype indicating that they had undergone receptor editing changing the specificity of the light chain. Again, the B cells that failed to undergo receptor editing became anergic showing evidence of an increase in the expression of CD5 and also exclusion from follicular and MZ B cells in the spleen (Berland, et al, 2006). Using ELISA, anti-idiotype antibody was used to determine B cells which had targeted insertions of both heavy and light chain genes of the 564 antibody. These antibodies are called idiotype positive (Id+) and the isotypes measured gives an indication of the type of antibody.

In CD19cre mice AID is expressed right from the pre B stage through to the mature B cell stage because the cre prompt0r of the mice is CD19. The early expression of CD19 in B cells allows cre recombinase to be expressed early which turns on the expression of AID. In CD21cre mice AID expression is restricted to mainly mature B cells hence these mice cannot undergo any CSR from the pre-B but only when B cells are matured. The expression of CD21 actually begins at the late immature stage and persists to mature B cell stage.

Figure 8 c and e, showed that all the CD19cre mice produced idiotype positive (Id+) IgG2a and IgG2b antibodies. The CD21cre mice however, showed that two-thirds were not producing Id+ IgG2a and IgG2b antibodies but one-third were producing. IgG1 total antibodies measured indicated that normal CSR was occurring, this implies that though CD21cre mice were also undergoing normal CSR, two-thirds was not making Id+ class switched antibodies i.e. IgG2a and IgG2b. This indicates that the production of class switched Id+ antibodies was occurring in immature B cells due to the fact that CD19 is expressed at various B cell stages i.e. both immature and mature B cells produced Id+ IgG2a and IgG2b antibodies and CD21 which is expressed mainly in mature B cells had two-thirds having Idiotype negative (Id-) IgG2a and IgG2b antibodies. The one-third CD21cre mice which were Id+ might be those B cells which were still in that late immature B cell stages which had not yet differentiated into mature B cells. Though normal receptor editing and anergy is known to occur in 564Igi mice, both 564Igi CD19cre and 564Igi CD21cre mice still had Id+ IgM antibodies.

Lupus is characterized by production of anti- nuclear autoantibodies and this makes the 564Igi a good model for studying the disease. 564Igi mice produces antibody which recognizes single-stranded DNA, single stranded RNA and nucleosomes. The

production of these autoantibodies can lead to kidney damage as observed in some SLE patients (Berland, et al, 2006). RNA binding ELISA results indicated that 84% of 564Igi CD21cre mice did not produce IgG2a RNA binding antibodies (RNA-) and 58% did not make IgG2b RNA binding antibodies.564Igi CD19cre mice however, made RNA binding IgG2a and IgG2b antibodies. Figure 8d and f therefore implies that the mature B cells were not producing the anti-RNA IgG2a and IgG2b antibodies since most of them did not have the RNA binding antibodies. This is because if all B cells express CD19 surface markers i.e. both immature and mature B cells and 564Igi CD19cre mice which starts expressing AID very early during B cell development make anti-RNA switched antibodies and CD21 expressed on mature B cells had most of the 564Igi CD21cre mice mostly not making IgG2a and IgG2b anti-RNA antibodies, this gives an indication that it is the immature B cells which are making these anti-RNA switched antibodies. The 564Igi CD21cre mice which did make IgG2a and IgG2b anti RNA antibodies might be those which were in the late immature B cell stage where the expression of CD21 surface marker actually begins. Two way ANOVA analysis of 564Igi CD21 and CD19 cre mice showed a significant difference ($P<0.05$) in the production of IgG2a and IgG2b anti- RNA antibodies of the two different mice group.

Some of the 564Igi CD21cre mice which were Id+ did not make IgG2a and b RNA binding antibodies (Id+ RNA-), though these mice have 564 heavy and light chain gene, an anti-RNA binding antibody. Their inability to make these switched anti-RNA antibodies might be due to point mutations at the RNA binding sites. The use of hybridomas and sequencing of clones obtained from these hybridomas can be used to determine if there have been point mutations (Petterson, et al, 1975).

Both Id+ and anti-RNA IgM of 564Igi AID-/- was much higher than AID-/- due to their possession of 564 heavy and light chain gene which has a higher specificity for auto-antigens. 564Igi WT produced a much lower IgM antibodies, this is synonymous to literature (Hans et al, 2007). Studies have shown that 564Igi WT mice usually produce IgM antibodies which are mostly anergic (Berland, et al, 2006).

Antinuclear antibodies (ANA) are found in greater than 98% of lupus patient. Immuno fluorescent staining using ANA test kit with human HEp-2 stabilized substrate which gives excellent sensitivity and very low background can be used to detect the presence of ANA in the sera of mice. The nuclear antigen is provided by human HEp-2 cells which are grown directly on microscope slides. IgG A488 is the fluorescent antibody used in this experiment. Figure 9 indicated that 564Igi CD21cre mice had no nucleoli or cytoplasmic staining hence no anti- nuclear IgG antibodies. 564Igi CD19cre mice however showed staining at both the nucleoli and the cytoplasm therefore indicating the presence of anti- nuclear IgG antibodies. 564Igi WT showed nucleoli and cytoplasmic staining. C57BL/6 which is the negative control had neither nucleoli nor cytoplasmic staining. 564Igi AID-/- mice also showed the absence of ANA. This gives evidence that the cre recombinase system is indeed efficient and that in order to restore AID expression in the transgenic mice it required both the cre and the AID transgene. This results provides the evidence that class switched antibodies are absent in the mature B cell indicating that it is the immature B cells which are providing these class switched antibodies. Figure 10 showed the various mice used for the work were either homogenous for 564Igi gene or were heterogeneous for 564Igi i.e. having both endogenous heavy or light chain and 564 knock - in heavy or light chain gene. Having either genotype did not affect its production of auto antibodies. What mattered in the CD19 or CD21 mice was not

been homogenous or heterogeneous but rather being positive for cre gene as well as having the AID transgene and CD19 or CD21 promoter.

CHAPTER SIX

6.0 Conclusion and Recommendation

Production of IgG autoantibodies is a characteristic of SLE, understanding CSR and AID expression which has been shown to occur in both mature and immature B cell is important in understanding these pathogenic IgG autoantibodies. The mouse model 564Igi.AID-/-.AIDtg CD21/CD19 cre has permitted the conditional expression of AID in order to better understand the stage where class switched autoantibodies is produced.

From the results, it can be concluded that idiotype positive and anti-RNA class switched autoantibodies are made at the immature stage of B cell development. This is because most of the 564Igi.CD21cre mice unlike 564Igi.CD19cre mice do not have RNA binding antibodies. This implies that before self- reactive B cells reach the mature B cell stage, they would already have undergone CSR thereby escaping tolerance mechanism. They can therefore bind self antigens and these then results in ICs which could be deposited in vital organs if it continues to persist.

I would recommend for future direction, further work with the 564Igi CD19/21 cre mice in order to increase data collected and get further insights into SLE pathogenesis. Hybridomas can be made and cloned to further understand the few exceptional CD21cre mice that were Id+ and had RNA binding antibodies. Sequencing of these clones would give a definite answer of if there have been any point mutations in the gene. Another mouse type 564Igi Mb1 cre has been shown to have a much higher efficiency than 564Igi CD19cre. This could also be used to study this conditional expression of AID to compare the results with 564Igi CD19cre mice.

I would also recommend that more studies be done into SLE in Ghana and also on other autoimmune diseases in order to provide improved treatment for those that need it to reduce mortality.

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APPENDICES

Genotype of Mice used in Experiment

Mouse Tag	H	L	CD19 KO	CD19 WT	CRE	eGFP	Mouse Type
G55	+	+	-	+	+	+	CD21 cre
G95	+	+	-	+	+	+	CD21 cre
G96	+	+	-	+	+	+	CD21 cre
G217	+	+	-	+	+	+	CD21 cre
G218	+	+	-	+	+	+	CD21 cre
G219	+	+	-	+	+	+	CD21 cre
G234	+	+	-	+	+	+	CD21 cre
G236	+	+	-	+	+	+	CD21 cre
G237	+	+	-	+	+	+	CD21 cre
G275	+	+	-	+	+	+	CD21 cre
G278	+	+	-	+	+	+	CD21 cre
G321	+	+	-	+	+	+	CD21 cre
G324	+	+	-	+	+	+	CD21 cre
G329	+	+	-	+	+	+	CD21 cre
G332	+	+	-	+	+	+	CD21 cre
G426	+	+	-	+	+	+	CD21 cre
G428	+	+	-	+	+	+	CD21 cre
G450	+	+	-	+	+	+	CD21 cre
G557	+	+	-	+	+	+	CD21 cre
G558	+	+	-	+	+	+	CD21 cre
F959	+	+	-	+	+	+	CD21 cre
F963	+	+	-	+	+	+	CD21 cre
F964	+	+	-	+	+	+	CD21 cre
F968	+	+	-	+	+	+	CD21 cre
F969	+	+	-	+	+	+	CD21 cre
F970	+	+	-	+	+	+	CD21 cre
F973	+	+	-	+	+	+	CD21 cre
F974	+	+	-	+	+	+	CD21 cre
F975	+	+	-	+	+	+	CD21 cre
G90	+	+	+	+	+	+	CD19 cre
G92	+	+	+	+	+	+	CD19 cre
G94	+	+	+	+	+	+	CD19 cre
G248	+	+	+	+	+	+	CD19 cre
G276	+	+	+	+	+	+	CD19 cre
G412	+	+	+	+	+	+	CD19 cre
G521	+	+	+	+	+	+	CD19 cre
G525	+	+	+	+	+	+	CD19 cre
G526	+	+	+	+	+	+	CD19 cre
G528	+	+	+	+	+	+	CD19 cre
F943	+	+	+	+	+	+	CD19 cre
Mouse Tag	H	L	CD19 KO	CD19 WT	CRE	eGFP	Mouse Type
F944	+	+	+	+	+	+	CD19 cre

G232	+	+	-	+	-	+	564Igi AID-/-
G235	+	+	-	+	+	-	564Igi AID-/-
G249	+	+	-	+	-	+	564Igi AID-/-
G266	+	+	-	+	+	-	564Igi AID-/-
G267	+	+	-	+	+	-	564Igi AID-/-
G269	+	+	-	+	+	-	564Igi AID-/-
G274	+	+	-	+	+	-	564Igi AID-/-
G322	+	+	-	+	-	+	564Igi AID-/-
G326	+	+	-	+	-	+	564Igi AID-/-
G378	+	+	-	+	-	+	564Igi AID-/-
G386	+	+	-	+	-	-	564Igi AID-/-
F329	+	+	-	+	-	+	564Igi AID-/-
F865	+	+	-	+	-	+	564Igi AID-/-
F945	+	+	-	+	-	+	564Igi AID-/-
F961	+	+	-	+	+	-	564Igi AID-/-

One-way ANOVA Analysis using GRAPHPAD PRISM

IgG1 Total

P value	0.7094
P value summary	ns
Are means signif. different? (P < 0.05)	No

Two-way ANOVA Using GRAPHPAD PRISM

Anti- RNA IgG2a for CD19cre and CD21cre mice

Source of Variation	P value
Interaction	< 0.0001
Column Factor	< 0.0001
serum Dilution	< 0.0001

Source of Variation	Significant?
Interaction	Yes
Column Factor	Yes
serum Dilution	Yes

Two-way ANOVA USING GRAPHPAD PRISM

Anti-RNA IgG2b for CD19cre and CD21cre mice

Source of Variation	P value
Interaction	< 0.0001
Column Factor	< 0.0001
serum Dilution	< 0.0001

Source of Variation	Significant?
Interaction	Yes
Column Factor	Yes
serum Dilution	Yes