Activation-Induced Deaminase in Early Developing B cells and Toll-like Receptor 8 in the Etiopathology of Murine Systemic Lupus Erythematosus

A thesis

submitted by

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In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy
in
Immunology

TUFTS UNIVERSITY
Sackler School of Graduate Biomedical Sciences

Monday, August 11th, 2014

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of pathogenic IgG anti-nuclear antibodies and increased type I interferon (IFN-I) production. Increased granulopoiesis is often observed in human SLE patients; however, the mechanism by which these cells contribute to autoantibody and IFN-I production is unclear. Using 564Igi mice, which model these disease symptoms, our goal was to understand the etiology of SLE.

564Igi mice have rearranged heavy and light chain knock-in genes in the Ig loci encoding anti-RNA autoantibodies on a wild type C57BL/6 background. Similar to human SLE patients, 564Igi mice produce anti-RNA autoantibodies and have expanded neutrophil and monocyte populations. These cells produce IFN-I and have increased expression of activating FcgRIV receptors. Our results indicate that the production of anti-RNA autoantibodies is sufficient to induce an increase in bone marrow, blood and spleen IFN-I-producing neutrophils, thus suggesting a mechanism by which autoantibodies and IFN-I contribute to SLE through the activation of B cells, neutrophils, and monocyte effector cells *in vivo.* (Han et al., 2013)

564Igi mice have high numbers of anergic peripheral B cells and high titers of IgG anti-RNA antibodies, but low titers of IgM anti-RNA in the sera. We found that class-switched pathogenic IgG autoantibodies were produced only when activation-induced deaminase (AID) is expressed in early developing B cells. Furthermore, the absence of AID in early developing B cells results in high titers of IgM anti-RNA antibodies in the sera, indicating that AID contributes to tolerance at least partially through somatic hypermutation (SHM) (Umiker et al., 2014a).

Using 564Igi mice, we also investigated the role for Toll-like receptors (TLRs) in SLE pathogenesis. We observed that TLR signaling through MyD88 is necessary for the SLE-like phenotype of 564Igi mice. IgG anti-RNA autoantibodies were produced in mice with single deletions of *Tlr7*, *Tlr8* or *Tlr9* as well as in mice with combined deletions of *Tlr7* and *Tlr9*. Autoantibodies were not produced in the combined absence of *Tlr7* and *Tlr8*, indicating that TLR8 contributes to the break in B cell tolerance. (Umiker et al. 2014b). We also determined that the dosage of X-linked *Tlr8* plays a major role in the increased incidence of disease in females. Finally, TLR9 suppresses granulopoiesis and IFN-I production by neutrophils. Collectively, we determined that individual TLRs play unique roles in the pathogenesis of SLE. (Umiker et al., 2014b).

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LIST OF ABBREVIATIONS

Aicda^{tg}	AID transgene
AID	Activation Induced Deaminase
AGS	Aicardi-Goutieres syndrome
ANA	Anti-nucelar antibody
APE	Apyrimidinic endonuclease
ASC	Antibody-secreting cell
BAFF	B-cell-activating factor
BAFF-R	B-cell-activating factor receptor
BCR	B-cell receptor
BER	Base excision repair pathway
BIM	BCL-2 interacting mediator of cell death
BM	Bone marrow
BMI	Body mass index
Btk	Bruton tyrosine kinase
C_H	Heavy chain constant region
C_L	Light chain constant region
CLP	Common lymphoid progenitor cell
CR1, 2	Complement receptor 1 and 2
CSR	Class switch recombination
CT	Circle transcripts
DC	Dendritic cell
ds	Double stranded
DSB	Double stranded break
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
FcγR	Fc gamma receptor
FO	Follicular B cell
GC	Germinal center
G-CSF	Granulocyte colony-stimulating factor
GCV	Gene conversion
GWAS	Genome wide association study
H	Heavy chain
HIGM2	Hyper IgM type 2
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IC	Immune complex
Ifit1	Interfereon-induced protein with tetracopeptide repeats
IFNAR	IFN-α/β receptor
IFN-I	Type I interferon
Ig	Immunoglobulin
IRF	Interferon regulatory factor
L	Light chain
L1	Long interspersed nuclear elements
LRR	Leucine-rich repeat

MHC	Major histocompatibility complex
MLV	Murine leukemia virus
MMR	Mismatch repair
Mx1	Mixovirus resistance 1
MZ	Marginal zone B cell
NK	Natural killer cell
NZB	New Zealand Black
Oas1a	2'-5' oligioadenylate synthetase 1A
PAMPs	Pathogen-associated molecular patterns
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
Pol β	DNA polymerase β
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
S	Switch region
S1P1r	Sphigosine-1-phosphate-1 receptor
Sca1	Stem cell antigen-1
SCF	Stem cell factor
SHM	Somatic hypermutation
SHP1	SH2-somain-containing protein tyrosine phosphatase
SLE	Systemic Lupus Erythematosus
Sm	Smith antigen
Sp	Spleen
ss	Single stranded
SSB	Single stranded break
SWR	Swiss Webster
PTPN22	Protein tyrosine phosphatase non-receptor 22
T1, T2, T3	Transitional B cells 1,2,and 3
TIR	Toll-IL-I receptor domain
TLR	Toll-like receptor
Tlr7^{tg}	Multiple copy of Tlr7 transgene
UNG	Uracil DNA glycosylase
Yaa	Y-chromosome linked autoimmune-accelerator

INTRODUCTION

I.1. Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder that affects multiple organs and tissues. In SLE the immune system responds to self-antigen, leading to inflammation and tissue damage. Approximately 90% of SLE patients are female (Rahman and Isenberg, 2008). The diagnosis of SLE tripled in the latter half of the 20th century (Abu-Shakra et al., 1995).

The symptoms of SLE are multi-faceted and unpredictable. Patients often have periods of remission followed by flares. Dermatological, musculoskeletal, hematological, cardiac, pulmonary, renal, and neurological manifestations commonly occur in patients (Rahman et al., 2008). For individual patients, symptoms can vary and change over time. Symptoms can include severe fatigue, swollen joints, hair loss, a typical “butterfly” rash on the cheeks and nose, as well as a tingling and discoloration in the fingertips called Raynaud’s syndrome (Rahman et al., 2008).

A functioning immune system maintains a highly orchestrated balance. While it must be finely tuned to respond to a diverse set of pathogens. It must avoid targeting host tissues. SLE is an autoimmune disorder in which the immune system has become dysregulated and produces antibodies that are specific for self-antigen. In SLE, autoantibodies primarily recognize nucleic acids or molecules associated with nucleic acids (Rahman et al., 2008).

SLE is characterized by immune system abnormalities that involve both innate and adaptive immunity. Autoimmune responses in SLE result in B-cell activation with an increase in antibody producing plasma cells, hypergammaglobulinaemia, and immune complex (IC) formation (Choi et al., 2012). This leads to aberrant levels of inflammation, organ damage, and pathology. The kidney is often targeted in SLE leading to glomerulonephritis. Renal biopsies display mesangial cell proliferation, inflammation, basement membrane pathology, and IC deposition (Choi et al., 2012).

A hallmark of SLE is the production of autoantibodies. Secreted autoantibodies aggregate to form ICs with nucleic acid-associated antigen. ICs have long been thought to be the main cause of glomerulonephritis as they are deposited in the kidneys of SLE patients. ICs bind to the glomerular membrane, and cause tissue damage upon the clogging of blood flow, complement activation, and cytokine or chemokine production (Berden et al., 1999). These mechanisms initiate the infiltration of other immune cells including macrophages and neutrophils. The infiltrating cells promote inflammation and tissue damage (Bergtold et al., 2006; Choi et al., 2012).

I.2 B-cell Development

B cells are responsible for all antibody production in the immune system. The following sections describe the developmental processes that lead to functional antibody producing B cells, including important steps in B-cell developmental stages and molecular markers for each stage. In Figure I.1, the

major stages of B-cell development are presented with indicated cell-surface markers.

After birth, all B-cell development begins in the BM. Hematopoietic stem cells (HSCs) can differentiate the three main lineages that differentiate into immune cells: lymphoid, myeloid, and erythroid. Common lymphoid progenitor cells (CLPs) differentiate into all lymphocytes and natural killer (NK) cells. but not into myeloid or erythroid cells (Nagasawa, 2006).

CLPs

CLPs have a limited lineage potential compared to multipotent stem cells. CLPs express stem cell factors that are used as cell markers in their identification. For instance, CLPs express c-Kit, also known as CD117. c-Kit recognizes steel factor - also known as stem cell factor (SCF) - providing the CLP key cell survival proliferation and differentiation signals (Ronnstrand, 2004). Stem cell antigen-1 (Sca1) is also a marker of CLPs. Sca1 is a GPI-anchored cell surface protein that associates with the lipid domain of developing lymphocytes (Ronnstrand, 2004). CLPs are often defined as $Sca1^{+}KIT^{+}FLT3^{+}$. Microenviornmental niches in BM stromal cells influence the differentiation of CLPs into pre-pro-B cells (Nagasawa, 2006).

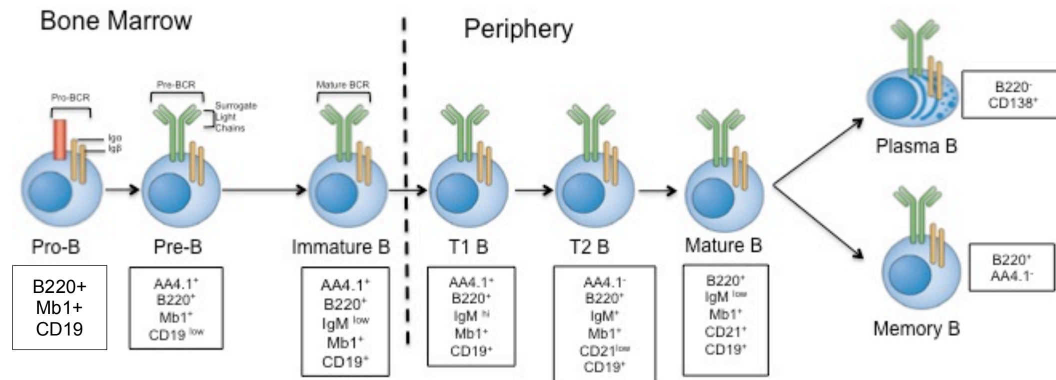


Figure I.1. **B-cell Development**. BM and peripheral B cell development is displayed starting with pro B cells. Important cell surface markers are in boxes below cells. The pro BCR is made up of calnexin and the Ig alpha/beta heterodimer. Pre-BCR has a rearranged H chain and a surrogate light chain. Immature B cells express a fully functional IgM BCR. Further development occurs in the periphery leading to plasma cell and memory B cell differentiation. B220 and mb1 are pan B cell markers. CD19 expression occurs only at low levels in pre-B cells, but it is expressed highly at later cell stages. AA4.1 is not expressed on mature B cells, but CD21 is expressed only in peripheral B-cell development. Figure is adapted from (Cambier et al., 2007).

Pre-pro B cells

Pre-pro B cells are B220^{+} and no longer express c-Kit or Sca1. Pre-pro B cells can give rise to many cell types including T cells, NK cells, and dendritic cells (DCs) (Hardy et al., 2007). The potential to differentiate into non-B cells is completely extinguished in later stages, when CD19 is expressed (Welner et al., 2008).

There are many factors that are critical for the development of B cells. CXCL12 is a chemokine that interacts with the chemokine receptor CXCR4 (Egawa et al., 2001). In mice deficient for CXCL12 there are very low numbers of pre- and pro-B-cells in the BM (Egawa et al., 2001). CXCL12 directs the

chemotaxis of B-cell precursors to the appropriate microenvironment in the BM to induce B-cell differentiation (Egawa et al., 2001).

Pro-B cells

Pro-B cells, expressing the pro-BCR, represent the next stage in B-cell development. The pro-BCR consists of calnexin and a heterodimer of Igα (or mb1) and Igβ. The Igα/β heterodimer contains an extracellular domain and a cytoplasmic tail (Wang and Clark, 2003). The Igα/β heterodimer is important in the signal transduction of a fully functional BCR in later B cell stages. However, it is important to note that the Igα/β heterodimer is also present very early in B-cell development (Wang and Clark, 2003). Calnexin is believed to allow for tonic signaling, which keeps early developing B cells alive as they wait for other signals for further B cell differentiation (Wang and Clark, 2003).

IL-7 is a cytokine that is critical for the development of lymphocytes and can induce differentiation (Peschon et al., 1994). IL7R-α, the receptor for IL-7, is expressed on CLPs and in early B-cell developmental stages. In both IL7 and IL7R-α deficient mice, there is a deficiency in pro-B cells, but not pre-pro-B cells. Therefore, IL-7 is critical for the differentiation of pre-pro-B cells into pro-B cells (Peschon et al., 1994; von Freeden-Jeffry et al., 1995).

The first wave of expression for the recombination-activating genes 1 and 2 (*Rag1* and *Rag2*) occurs in the pro-B cell stage. During this stage, the heavy chain locus of the antibody gene is rearranged. The antibody chain product of the rearranged H chain gene is then expressed in pre-B cells (Schatz et al., 2011). A

more detailed description of RAG mediated antibody gene rearrangement will be presented in a later section of the introduction.

Pre-B cells

Upon IL-7R signaling in a pro-B cell, proliferative expansion of these cells occurs. This leads to the differentiation to pre-B cells, marked by the expression of a pre-BCR (Goetz et al., 2004).

The pre-BCR is comprised of a heavy chain and a “surrogate” light chain. The surrogate light chain contains two separate chains: λ5 and VpreB (Karasuyama et al., 1990). Though the pre-BCR is not considered a fully functional BCR, it does have the ability to signal when activated. Signaling through the pre-BCR is critical for further B-cell differentiation (Gong and Nussenzweig, 1996). Pre-BCR signaling is important as it helps to expand the population of B cell precursors. The remaining stages in the differentiation of mature B cells do not involve significant cell division (Rickert, 2013).

A deficiency of λ5 in mice blocks B-cell development at the pre-B cell stage (Kitamura et al., 1992). This implies that the pre-BCR has an important function in B-cell development. The pre-BCR can signal in a cell-autonomous manner. A domain in the λ5 portion of the surrogate light chain can provide auto-crosslinking of the pre-BCR (Ohnishi and Melchers, 2003). Once crosslinking has occurred, SRC kinases phosphorylate the Igα/β heterodimer. Leading to phosphorylation of ZAP70 and SYK. This leads to the activation of the protein BLNK, which recruits both PLCγ2 and BTK. These proteins induce calcium influx

and activate the RAS/RAF/MEK/ERK pathway. Such signaling events lead to the proliferation of pre-B cells (Ohnishi and Melchers, 2003).

During the pre-B cell stage, a second wave of Rag mediated rearrangement can occur in the light chain locus (Grawunder et al., 1995). At this point, the pre-B cell can differentiate into an immature B cell. In immature B cells, a mature BCR comprised of rearranged heavy and light chains, are expressed on the membrane.

Immature B cells

Fully competent IgM BCRs are first expressed on the surface of immature B cells. Immature B cells are often identified by a monoclonal antibody to CD93, called AA4.1. CD93 is down-regulated upon B-cell maturation in the spleen, but the function of CD93 is unclear. Some have suggested that CD93 is a receptor for the complement component, C1q, while others presented evidence that CD93 is involved in cell-cell adhesion (Chevrier et al., 2009). CD93 is reintroduced later in B-cell development on the surface of antibody producing plasma cells. CD93 deficiency leads to a loss of IgG production as well as a loss of $CD138^+$ plasma cells in the BM (Chevrier et al., 2009). Immature B cells leave the BM and enter the blood where they differentiate into transitional B cells (Osmond and Batten, 1984). (Pereira et al., 2009)

Transitional B cells and mature B cells

BM immature B cells further differentiate into mature B cells through three additional transitional steps. All three stages of transitional B cells (T1, T2, and T3) express CD93. T1 and T2 cells are found circulating in the blood and express CD24 (heat stable antigen) and CD38 (cyclic ADP ribose hydrolase). They are also found in small numbers in the BM, implying that the transitional stages can occur before migration out of the BM (Agrawal et al., 2013). The main difference in surface expression between the three transitional B cell stages is in the expression of surface IgM and IgD. T1 B cells are IgM^{+} IgD^{low}, but T2 B cells are IgM^{+} IgD^{high} (Agrawal et al., 2013).

CD21 (complement receptor 2 [CR2]) and CD35 (CR1) are products of one gene that undergoes alternative splicing to encode the two different receptors (Kurtz et al., 1990). CD21 is expressed at low levels in the transitional B cell stages. As B cells enter the mature B cell stage they express high levels of CD21 (Kurtz et al., 1990).

In the spleen, fully developed mature B cells are separated into two types: follicular (FO) and marginal zone (MZ) B cells. FO and MZ B cells have distinct localization, cell-marker surface expression, and function (Lopes-Carvalho et al., 2005).

MZ B cells are IgD^{-} and IgM^{+}. As their name implies, MZ B cells are located within the marginal zone of spleens. Within the marginal zone are many immune cell types including macrophages and DCs. MZ B cells are thought induce the primary antibody response to pathogens as they enter the spleen through the circulatory system (Lopes-Carvalho et al., 2005). The MZ is located

in a region of the spleen with a low rate of blood flow. This low blood flow rate allows more time to detect blood-born pathogens (Lopes-Carvalho et al., 2005). In response to blood-born pathogens, a specific CD11c^{lo}Mac-1^{hi} DC subset contributes to a T cell-independent process of plasma cell differentiation by MZ B cells (Balazs et al., 2002). MZ B cells can also participate in germinal center (GC) reactions. These are T cell-dependent reactions that occur at the T-B cell border of the spleen. (Song and Cerny, 2003)

Follicular (FO) B cells are the major contributors to GC-dependent class switched antibody responses to pathogens. The primary function of FO B cells is to differentiate into either antibody producing plasma cells or memory B cells. FO B cells undergo GC reactions in both the spleen and the lymph node. Unlike MZ B cells, which stay in the spleen, FO B cells recirculate into the blood stream and into the lymph node (Gray et al., 1982). FO B cells continuously recirculate into the secondary lymphoid organs and respond to antigen challenge. Upon recognition of antigen, B cells encounter T cells at the B-T cell border. The chemokine receptor CCR7 attracts B cells to the B-T cell border and CD40/CD40L interactions between B and T cells are critical for B-cell activation. This initial B-cell activation leads to affinity maturation and B-cell differentiation into plasma cells and memory B cells (Gatto and Brink, 2010).

Another category of mature B cells is B-1 cells. B-1 cells are found in low numbers in the spleen and lymph node, but populate the lamina propria and peritoneal cavity. In general, the specificity of B-1 cells is for common foreign antigens or self-antigens. B-1 cells can be derived from fetal B cells (Berland and

Wortis, 2002; Hardy and Hayakawa, 1994). Additionally, self-renewal of B-1 B cells allows them to remain in an animal for its entire life. Alternatively, B-1 cells can also be derived from conventional B cells (Berland and Wortis, 2002; Cong et al., 1991).

B-1 B cells are the primary source of natural antibodies. These antibodies recognize antigens from common pathogens, are often poly-reactive, and weakly self-reactive. Models have demonstrated that B-1 cells can play a role in the development of autoimmunity including Sjorgens syndrome, SLE and rheumatoid arthritis (RA). Elevated numbers of B-1 cells have been associated with SLE-like symptoms in (NZBxNZW) F_1 mice (Berland and Wortis, 2002; Hayakawa et al., 1983).

I.3 Mechanisms of B-cell Tolerance

The immune system has evolved to create a diverse set of receptors that sense almost any chemical entity. This requires a large amount of receptor diversity. One means of acquiring receptor diversity is RAG-mediated V(D)J recombination. V(D)J recombination assembles antibody sequences from a set of variable (V), diversity (D), and joining (J) gene segments. Various combinations of V, D, and J segments in H chain genes, and V and J segments in light chain genes, lead to a diverse set of antibody gene sequences.

RAG-mediated V(D)J recombination occurs in both B and T cell development (Chen et al., 1997; Oettinger et al., 1990; Schatz et al., 1989). After the initial V(D)J recombination 20-50% of the Ig receptors recognize self-antigens

(Laufer et al., 1996; Wardemann et al., 2003). The immune system has developed mechanisms of self-tolerance to prevent these autoreactive receptors from causing autoimmunity. This section will focus on how B cells become tolerance to self-reactive BCRs, thus preventing autoantibody production.

Central B-cell Tolerance

Central B-cell tolerance occurs while developing B cells progress through B-cell stages in the BM. Central B-cell tolerance occurs using three strategies: receptor editing, cell deletion, and induction of B-cell anergy (Goodnow et al., 2005).

If a receptor on a B cell is self-reactive, one strategy employed to prevent autoimmunity is receptor editing. On an immature B cell, if a BCR exceeds a threshold for binding strength to antigen, the development of the B cell is halted and the BCR is internalized (Goodnow et al., 2005; Hartley et al., 1993). At this point, the homing receptor, CD62 ligand, is down-regulated. Without CD62 ligand the B cell will not enter the lymph node (Goodnow et al., 2005; Hartley et al., 1993). Lastly, recombination-activating genes RAG1 and RAG2 continue to be expressed in autoreactive B cells. The expression of RAG leads to a secondary rearrangement of the BCR. Secondary rearrangements occur in light chain genes (Chen et al., 1997; Hikida and Ohmori, 1998) and heavy chain genes (Chen et al., 1995a; Kobrin et al., 2001). Once editing occurs the receptor may have lost self-reactivity.

An autoreactive B cell may be targeted for deletion if receptor editing fails to result in a loss of self-reactivity. B cells expressing anti-DNA receptors are deleted in the BM during the pre-B to the immature stages, and in the spleen during transitional stages (Chen et al., 1995a). There are many mechanisms that contribute to the programmed cell death of self-reactive B cells.

B-cell-activating factor (BAFF), a pro B-cell survival cytokine, signals through the BAFF receptor (BAFF-R). Immature B cells that are self reactive down regulate BAFF-R (Mackay et al., 2003). In theory, this could decrease the survival of immature B cells that express autoreactive BCRs. Thus, a decrease in BAFF-R is an example of intrinsic B-cell tolerance. Interestingly, it has been shown that an increase in BAFF can overcome B-cell tolerance checkpoints, leading to autoantibody production (Lesley et al., 2004; Thien et al., 2004).

BCR-mediated apoptosis also occurs during the process of autoreactive B-cell deletion. An internalized BCR can signal to increase the levels of the pro-apoptotic protein BCL-2 interacting mediator of cell death (BIM). BIM inhibits the BCL-2 family of pro-survival factors thereby promoting apoptosis (O'Connor et al., 1998).

Several changes in autoreactive B cells may occur leading to a state of anergy. For example, BCR transport out of the endoplasmic reticulum (ER) is blocked in autoreactive B cells leading to a decrease in BCR surface expression (Bell and Goodnow, 1994). The BCR that remains on the surface of autoreactive B cells does indeed induce NF-kB at the same rate as non-autoreactive BCRs

(Lesley et al., 2004); yet, autoreactive B cells also signal to induce BIM production as discussed above.

The threshold for BCR activation is finely tuned, meaning a certain level of antigen binding is required for the activation of a B cell through the BCR. The SH2-domain-containing protein tyrosine phosphatase (SHP1) increases the threshold for B-cell activation. SHP1 is always expressed in B cells; however, in autoreactive B cells SHP1 is actively recruited to the BCR. SHP1 increases the threshold for BCR activation and thereby contributes to the anergic state of the autoreactive B cell (Goodnow et al., 2005; Ravetch and Lanier, 2000).

Peripheral B-cell tolerance

Self-reactive B cells that enter the peripheral tissues will in most cases be rendered unresponsive through anergy. B-cell anergy is the state for which a B cell is unable to respond to its specific target antigen. Anergic B cells do not proliferate, express activation markers, or secrete Ig after stimulation with antigen (Goodnow et al., 1988; Nossal and Pike, 1980).

Some autoreactive B cells do escape central tolerance. Various self-antigens are not present in the BM or do not bind BCRs at high affinity. BCRs that bind such self-antigens can escape central tolerance. To prevent autoimmune responses, a second defense against autoreactive antibody production exists known as peripheral B-cell tolerance. Peripheral B-cell tolerance occurs when autoreactive B cells enter the lymphoid organs (Russell et al., 1991).

At present, mechanisms of peripheral tolerance are not entirely clear. Both positive and negative selection processes have been proposed as mechanisms. Self-antigen recognition has been shown to induce cell death in the periphery (Russell et al., 1991). However, recognition of antigen is known to be important for splenic B-cell development (Gu et al., 1991). Thus, it is possible that autoantigen is important for the development of mature B cells, especially in germ-free mice (Loder et al., 1999).

Finally, peripheral B-cell tolerance is complicated by the fact that mechanisms of tolerance are distinct between B cell subsets. For example, B-1 B cells are less susceptible to peripheral tolerance driven by apoptosis compared with other mature splenic B cells (Ait-Azzouzene et al., 2006). In contrast, transitional B cells are often targeted for deletion when self-reactive (Carsetti et al., 1995).

In SLE, the mechanisms of both central and peripheral tolerance are compromised (Yurasov et al., 2005a; Yurasov et al., 2005b). The next section of this introduction discusses genetic elements that contribute to the development of SLE.

I.4. Genetic Factors in SLE

Genetic predisposition for SLE in humans

Epidemiological studies suggest a contribution of genetic factors in the development of SLE. Many families have multiple relatives diagnosed with SLE. As such, 10-12% of SLE patients have a first or second-degree relative that is also diagnosed with SLE (Ramos et al., 2010). Monozygotic twins with at least one SLE diagnosis have a SLE concordance rate of 24%, while SLE diagnosed in a dizygotic twin has a concordance rate of 2% (Deapen et al., 1992). Genetic predispositions to SLE have been established; however, there is no single casual gene for SLE. In addition, there are important non-genetic factors contributing to the development of this disease.

Previously, genes associated with SLE were discovered by linkage analysis and family studies. More recently, genome wide association studies (GWAS) have increased the understanding of the complex genetic basis for SLE. Many GWAS studies have been performed in various ethnic groups. Using this technique, over 40 genetic loci have been identified that are associated with susceptibility (Cui et al., 2013). The following section discusses selected categories of SLE susceptibility genes that are most relevant to the present study including: Human leukocyte antigen, Fc gamma receptors, genes involved in IC and self-antigen clearance, the IFN-I pathway, Toll-like receptor signaling, and B-cell signaling.

Human leukocyte antigen (HLA) regulation

The major histocompatibility complex (MHC) was the first genetic region associated with SLE. Within the MHC are the classical human leukocyte antigen (HLA) regions. Specific alleles of HLA regions have been associated with SLE. (Ramos et al., 2010). The HLA is the densest portion of the genome, with many genes distributed over the region. A high percentage of these genes have diverse immune functions, especially in the class III HLA region. In many GWAS, variations in the HLA regions have been identified as the strongest genetic factor for susceptibility in SLE (Cui et al., 2013) (International Consortium for Systemic Lupus Erythematosus et al., 2008) (Kozyrev et al., 2008a) (Han et al., 2009) (Graham et al., 2008). Nonetheless, dissecting specific genetic elements within the MHC that associate with SLE is still an area of interest.

One possibility is that specific MHC complexes expressed on antigen presenting cells promote autoimmunity. Certain self-peptides could preferentially bind to specific MHC complexes. If the pMHC complex is recognized by a self-reactive TCR, this could promote autoimmunity.

Fc gamma receptors

Human Fc gamma receptors (FcγR) can recognize the Fc portion of IgG antibody. In both mice and humans there are two distinct functional classes of FcγRs: activating and inhibitory. FcγR2B is the only inhibitory IgG receptor in mice and is expressed on many immune cells including B cells and neutrophils. There are several activating FcγRs found in mice; however none on murine B cells (Nimmerjahn and Ravetch, 2006). In humans there are five activating Fcγ

receptors: FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB. There is one inhibitory Fcγ receptor, FcγRIIB (Nimmerjahn and Ravetch, 2006).

Many genes in the FcγR family have been associated with SLE. Polymorphisms in *FcγI* (CD64), *FcγII* (CD32)*, and FcγIII* (CD16) have strong associations with SLE (Brown et al., 2007). FcγRs have been implicated in the clearance of ICs. Alleles of FcγRs with low affinity binding to IgG reduce the ability of the immune system to phagocytose and thereby clear ICs (Wu et al., 1997) (Salmon et al., 1996) Alternatively, high affinity alleles of FcγRs have also been shown to be associated with SLE (Kyogoku et al., 2002). In this case, FcγR signaling can increase clinical manifestations by driving inflammation that leads to tissue damage. This finding demonstrates the fine balance of the immune system. In another example, the expression of two alleles of the same gene CD16 - one with higher than normal binding and the other with lower than normal binding to IgG - can both produce similar clinical manifestations (Wu et al., 1997) (Salmon et al., 1996) (Kyogoku et al., 2002).

The Toll-like receptor and type I interferon signaling pathways

The role of Toll-like receptors (TLRs) in SLE is extensively studied and will be discussed in depth in a later section of the introduction. TLRs are a set of receptors that recognize pathogen-associated molecular patterns (PAMPs) and lead to the induction of a signaling pathway that results in pro-inflammatory cytokine production. In SLE, TLR signaling leads to the generation of type I interferon (IFN-I) (Rahman and Eisenberg, 2006).

Genetic analysis of TLR pathway genes identified an association with SLE for *interferon regulatory factors 5, 7,* and *8* (*Irf5, Irf7 and Irf8*) (Sigurdsson et al., 2005). IRFs are important transcription factors that lead to the production of IFN-I, along with other pro-inflammatory cytokines: TNFα, IL-12 and IL-6. Polymorphisms in the promoter region of *IRF5* may lead to an increase in its expression thereby promoting an increase in pro-inflammatory cytokine secretion (Sigurdsson et al., 2005). The role of IFN-I in SLE will be discussed in depth in a later section.

UBE2L3 is an enzyme expressed in most lymphocytes that is important for degradation of TLRs (Cui et al., 2013) In GWAS, genetic variations in the *UBE2L3* gene, causing decreased function of UBE2L3, were associated with increased risk for SLE (Cui et al., 2013). Presumably, the genetic variation of UBE2L3 allows for longer lifespans for the TLRs and thereby increases the likelihood of SLE development.

B-cell signaling

Many genes that encode for proteins involved in B-cell receptor (BCR) signaling and B-cell development have been implicated by GWAS for SLE patients. For example, *BLK* encodes for a tyrosine kinase in the src family. *LYN* encodes for a tyrosine kinase involved in BCR signaling. *BANK1* encodes for a scaffold protein involved in BCR signaling. Polymorphisms of all three genes (*BLK, LYN, BANK1*) have been identified as factors that increase the

susceptibility for SLE (Cui et al., 2013; International Consortium for Systemic Lupus Erythematosus et al., 2008) (Kozyrev et al., 2008b).

PRDM1 is involved in the differentiation of antibody producing plasma B cells. Variants of PRDM1 that are associated with SLE may increase the likelihood of autoreactive B cells to differentiate into plasma B cells (Gateva et al., 2009).

Immune complex and self-antigen clearance

There are many proposed models of how the immune system is dysregulated in SLE. Abnormalities can lead to an increased presence of autoantigen in microenvironments. This overabundance of autoantigen may be a root cause in the development of SLE. There are multiple mechanisms that could lead to an increase in antigen availability including dysregulated apoptosis/cell death and deficiencies in normal auto-antigen clearance. Many SLE patients have an accumulation of apoptotic cells in the lymph node, and in the skin after sun exposure (Gaipl et al., 2007). Additionally, neutrophils in some SLE patients have a reduced ability to uptake debris (Gaipl et al., 2007). Genetic factors that decrease the ability of the immune system to clear debris and apoptotic cells can contribute to SLE etiology.

Mice deficient in *Dnase1* lose the ability to clear extra-cellular DNA. These mice also develop SLE-like symptoms. SLE patients with nonsense mutations in DNASE1 have also been described (Yasutomo et al., 2001). This implies that an

overabundance of DNA antigen due to inefficient DNA clearance can contribute to SLE pathogenesis.

TREX1 is an intracellular DNase. Loss of TREX1 function increases DNA auto-antigen availability. Mutated TREX1 genes have been implicated in Aicardi-Goutieres syndrome (AGS) another autoimmune disorder. In addition, a subset of SLE patients has frameshift or missense mutations in the TREX1 gene. (Lee-Kirsch et al., 2007). Hypermorphic mutations of RNAse H2 also result in AGS (Chon et al., 2013). This indicates that lack of RNA clearance may also be responsible for the induction of autoimmunity.

SLE-like symptoms were observed in mice deficient for the scavenger receptor SCARF1. DCs, macrophages, and endothelial cells use SCARF1 in order to engulf debris from apoptotic cells. In SCARF1 knockout mice, dying cells accumulate in the bone marrow microenvironments. This leads to the induction of inflammation, spontaneously activating the immune system to produce autoreactive antibodies, and thus, initiating lupus-like pathology (Ramirez-Ortiz et al., 2013).

Patients with deficiencies in the classical complement pathway components C1q, C4 and C2 have been shown to develop severe early onset SLE symptoms (Meyer et al., 1985; Pickering et al., 2000). It has been proposed that this is due to the role of complement in the clearance of both ICs and apoptotic cells. Human patients with deficient complement responses accumulate ICs and self-antigen that can lead to SLE pathology (Truedsson et al., 2007).

Environmental risks and triggers

Along with genetic factors, many environmental factors have been identified that increase the risk of developing SLE. Smoking cigarettes, high body mass index (BMI >21kg/m^2), and over-exposure to the sun in fair-skinned women have all been associated with a greater risk of developing SLE (Bengtsson et al., 2002). Epstein–Barr Virus (EBV) infection can trigger SLE pathology. There are higher levels of antibodies specific for EBV proteins in SLE patients compared with healthy individuals. Also, more cells are infected with EBV in SLE patients (James et al., 1997; James et al., 2001; Riemekasten and Hahn, 2005). It is important to keep in mind that there is no single cause of SLE. In fact, many factors play a role in its development, and these factors vary between individual patients.

SLE mouse models

Mouse models of SLE have provided an excellent source for the study of genetic factors and molecular mechanisms that lead to pathology. Many mouse models spontaneously develop key symptoms and signs of human SLE disease. Advantages and disadvantages of a select set of mouse models will be discussed in this section.

MRL/*lpr* is a mouse model of SLE. The MRL congenic mouse is an autoimmune strain, which develops late onset immune complex driven glomerulonephritis (Andrews et al., 1978). A much stronger autoimmune phenotype is observed when MRL mice are homozygotic for a mutation of *lpr*.

The *lpr* mutation leads to an underexpression of the Fas antigen. Since Fas signaling leads to apoptosis of peripheral autoreactive T cells, the *lpr* mutation leads to more autoreactive T cells on the MRL background. MRL/*lpr* mice have high levels of autoimmunity and enlarged lymph nodes (Gillette-Ferguson and Sidman, 1994).

New Zealand Black x Swiss Webster ([NZBxSWR] or SNF1) mice are an F1 strain that develop SLE-like disease, in contrast to either parental strain, NZB or SWR. Lupus nephritis and deposition of ICs in the kidney occur at about 5-8 months in SNF1 mice (Datta et al., 1982). SNF1 mice produce IgG autoantibody reactive to nuclear antigens. The parental strain, NZB, has many abnormalities. NZB mice have high B cell hyperactivity and high levels of retrovirus in their sera (Alexander et al., 2002; Klinman, 1990). Finally, SWR mice have no known phenotype, but contribute an unknown genetic component for the severe SLE–like glomerulonephritis in SNF1 (Alexander et al., 2002).

NZB x New Zealand White (NZW) (NZBxNZW) mice are another important mouse model. The complex genetics of this SLE mouse model began to be uncovered with this F1 mouse. The result of the inbreeding was called the New Zealand Mixed (NZM) mouse (Rudofsky et al., 1993). Using this mouse, model three loci were identified that increase SLE susceptibility: *Sle1*, *Sle2, and Sle3* (Morel et al., 1994). Similar to humans, the inheritance of SLE was multi-genetic in mice. It is clear that no single gene is sufficient to produce the early onset SLE-like disease observed in NZM mice (Wakeland et al., 1997). Further dissection of the contribution of the individual loci, Sle1, Sle2, and Sle3,

demonstrated that each locus contributed to aspects of SLE pathogenesis. For example, Sle2, located on chromosome 4, lowers the threshold for the activation of B cells. However, autoreactive IgG antibodies were not produced. Lupus nephritis was also not found in mice with the Sle2 locus (Mohan et al., 1997; Mohan et al., 1999).

The work presented in this dissertation uses a mouse model of SLE developed in the Imanishi-Kari laboratory, termed 564Igi. 564Igi mice have a dual knock-in of two antibody genes into the antibody gene loci. 564Igi knock-in genes are two pre-rearranged genes: an immunoglobulin (Ig) heavy (H) and an Ig light (L) chain. Both genes were derived from a hybridoma obtained from the fusion of SNF1 splenic cells with myeloma cells. The 564 genes were chosen to create 564Igi because, when injected into young SNF1 females, IgG encoded by 564 Ig genes was shown to accelerate lupus nephritis (Vlahakos et al., 1992). 564Igi as a model of SLE has many advantages. On a C57BL/6 background, this simple knock-in of two antibody genes leads to the loss of B-cell tolerance, the production of IgG autoantibody, and glomerulonephritis (Berland et al., 2006). This allows our laboratory to answer questions about the progression of disease in a system based on the production of one autoantibody with a known germline genetic sequence. In contrast, the multi-genetic nature of mouse models such as NZM and MRL/lpr can complicate the study of the etiopathology of SLE.

The 564Ig H antibody gene was introduced into the endogenous joining (JH) region. This allows for 564Igi mice to switch from Cμ to every isotype. Rag-mediated V_H replacement also can occur in 564Igi from the same chromosome

as the knock-in. D or J editing from the same chromosome cannot occur as the gene was inserted in such a way as to cut out the remaining D and J segments. Translocation of D and J regions is possible in 564Igi from the 2nd chromosome (without a 564 H knock-in gene) into the chromosome with a 564 knock-in gene (Fig. I.2).

The 564Ig antibody has a characteristic idiotype (Id) defined by both its H and L chain genes. Id^{+} B cells express a B cell antigen receptor (BCR) on their surface that was encoded by the 564 H and L chain genes. Serum IgG antibodies from 564Igi mice bind to nucleoli of HEp-2 cells in a speckled pattern, suggesting specificity for RNA or RNA-associated antigens. On a C57BL/6 background, 564 Id^{+} anti-RNA class-switched pathogenic antibodies are produced. 564Igi mice develop glomerulonephritis and other symptoms of human SLE disease (Berland et al., 2006) (Fig. I.3).

Modeling the SLE in mouse models is a challenge as the human disease is multi-factorial and diverse. 564Igi has limitations as a model of human SLE. First, 564Igi does not produce a diverse set of autoantibody. SLE is characterized by a diverse set of autoreactive antibody. 564Igi produces an antibody that is transcribed from a transgene of specific sequence and reactivity.

564Igi also has a pre-rearranged antibody gene. This may have an effect on B cell development. Under normal circumstances the rearrangement of both heavy and light chanins is a highly orchestrated process that does no occur in the 564 gene loci.

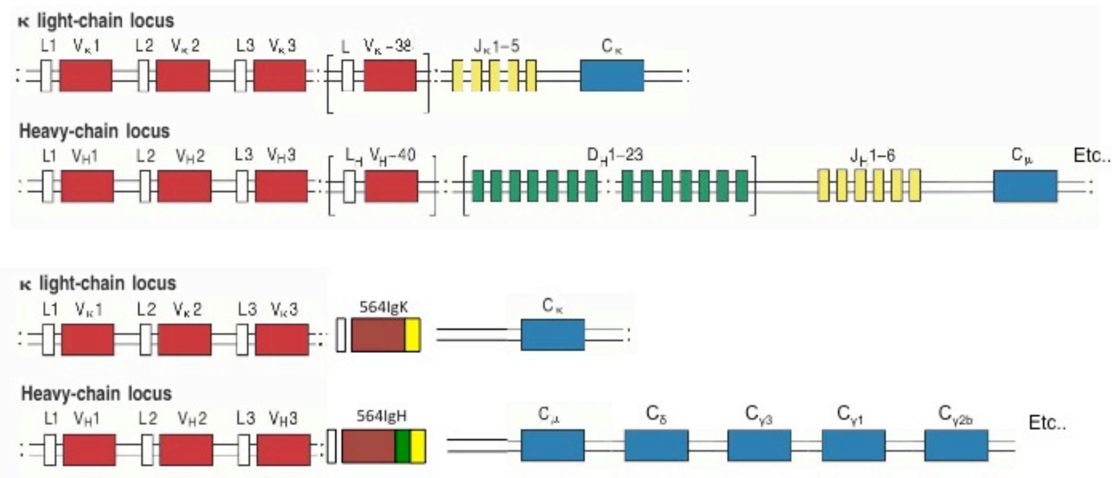


Figure I.2. The 564 transgene in the H and L chain loci. (Top) Mouse antibody genes before RAG mediated rearrangement. V_k and V_H gene segments followed by D and J regions. These are followed by C regions. (Bottom) After the pre rearranged 564 transgenes are inserted into antibody gene loci. V_H regions remain upstream of 564 genes. C regions available for CSR on H chain 564-gene locus.

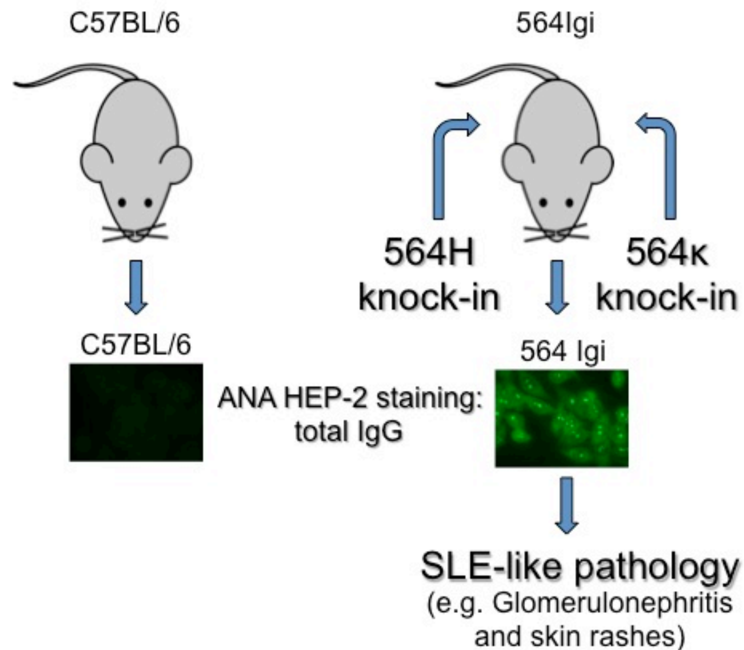


Figure I.3. **564Igi, a mouse model of SLE.** 564Igi is a knock-in of the 564 IgH gene and the 564 κ gene in the Ig loci. Anti nuclear antigen (ANA) IgG staining of HEp-2 cell stains by C57BL/6 and 564Igi sera. Autoantibody production in 564Igi leads to SLE-like pathology

I.5 Activation-Induced Deaminase

RAG-mediated recombination of the variable V and J gene segments in light chain genes, and the recombination of the V, D, and J gene segments in heavy chain genes, account for the diverse repertoire of the immunoglobulin loci. Importantly, these RAG-mediated recombinations occur in the absence of antigen (Hozumi and Tonegawa, 1976) (Alt and Baltimore, 1982; Sakano et al., 1980; Schatz et al., 1989) (Oettinger et al., 1990). Further diversification of the antibody repertoire occurs after stimulation with the cognate antigen of an antibody receptor. This secondary diversification occurs through multiple mechanisms including SHM, Ig somatic gene conversion (sGC; in certain

species), and class-switch recombination (CSR). SHM increases diversity by adding point mutations to the antibody genes. CSR alters the effector functions of an antibody by switching the constant portion of the antibody gene to new isotypes (Delker et al., 2009).

Activation Induced Deaminase (AID) was originally discovered as a critical protein for the process of CSR in a B cell line (Muramatsu et al., 1999). Results obtained with AID deficient mice led the same group to conclude that AID was critical for both SHM and CSR (Muramatsu et al., 2000). Having one enzyme responsible for both SHM and CSR was surprising at the time, because they are seemingly distinct processes. More recently, the mechanisms by which AID mediates both processes have become clearer. A brief summary of these molecular mechanisms will be provided in the next section.

Somatic Hypermutation

SHM plays an important role in shaping an immune response by introducing diversity in antibody genes through mutation. AID is critical for SHM. In order to initiate SHM, AID directs the deamination of cytidine (C) residues. This transforms cytidine into uridine (U) and, thus, C:G pairs into U:G unmatched pairs. After this initial deamination by AID, there are multiple mechanisms that lead to a diverse set of point mutations. When DNA replication occurs over the U residue this creates C to T or G to A mutations. Alternatively, before this process occurs uracil DNA glycosylase (UNG) generates an abasic site by removing the U residue. This abasic site is repaired; however, often with errors. Therefore,

after the removal of U by UNG any residue can be replaced at the original site of AID–mediated deamination. Upon recognition of the U residue mutations can also be spread to adjacent nucleotides. This occurs through a faulty, error-prone mismatch repair (MMR) pathway involving the enzymes Msh2/Msh6 and Pol η. These three mechanisms can account for all possible mutations including transition and transversions of C:G or adjacent A:T base pairs (Liu and Schatz, 2009) (Di Noia and Neuberger, 2007)).

Class switch recombination

The constant region of the antibody heavy chain (C_H) defines the isotype of an antibody. The isotype contributes to the antibody's effector function (Stavnezer et al., 2008). In a pro-B cell, a rearranged VDJ heavy chain is associated with a Cμ constant region gene. The rearranged H gene (V_H-D-J_H-Cμ) will be transcribed and translated to give rise to a μH chain. The μH chain is expressed on the surface of the pre-B cell with a surrogate light chain. Another rearrangement of V_L and J_L at the light chain loci occurs at the pre-B cell stage. The V_L-J_L will be associated with one light chain constant region (C_L). Finally, There are four C_L regions in mice: Cκ, Cλ1, Cλ2, and Cλ3. The final combination of H and L chains will form a BCR in an immature B cell (Stavnezer et al., 2008).

Initially, the BCR on an immature B cell will be IgM with a Cμ constant region. In the heavy chain locus, the Cδ constant segment is directly 3' of the Cμ constant region. The primary mRNA of a transcribed heavy chain locus has introns in between the Cμ and Cδ genes. Alternative splicing of the gene can

produce IgM heavy chains or IgD heavy chains; however, CSR is not required for IgD expression. IgM and IgD are often co-expressed and translated from one mRNA. That being said, the expression pattern and function of IgD remains enigmatic (Geisberger et al., 2006).

There are eight isotypes of Ig in the mouse: IgM, IgD, $IgG_{3,}$ IgG_1, $IgG_{2b,}$ $IgG_{2a,}$ IgE and IgA. The last six of these require AID mediated CSR in order to be produced. The C_H genes of each isotype are organized along the antibody gene in the following order: Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα. Before Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα, there are specific switch (S) regions. These S regions are genetic segment critical for CSR to occur (Stavnezer et al., 2008).

The regulation of CSR is very important in shaping the antibody repertoire as it determines the isotypes of antibodies produced in the system. The deamination of cytidine (dC) by AID initiates the process of CSR. AID targets dC nucleotides in the S regions upstream of C_H segments and converts the nucleotide to dU. The S regions contain many “hotspot” for AID, sequences that preferentially targeted (Stavnezer et al., 2008).

After the creation of mismatched U:G base pairs, the base excision repair pathway (BER) creates single stranded breaks (SSBs) in the DNA. This process isinitiated when UNG removes the dU making an abasic site in the DNA. The abasic site is targeted by apyrimidinic endonuclease (APE). APE cuts the backbone of DNA, thus creating SSBs. Cells have, however, evolved to repair SSBs. Normally, a SSB would be filled in by DNA Polymerase β (Pol β) (Beard and Wilson, 2006). It is thought that, within S regions of antibody genes, there

are so many SSBs during CSR that the Pol β mediated repair systems are overwhelmed (Wu and Stavnezer, 2007). This allows for a small number of SSBs to develop into double stranded breaks (DSBs) within the S regions. For example, if two SSBs were formed near each other on opposite strands, this would likely result in a DSB. It is also possible for DSBs to form from two SSBs that are relatively distant from each other (Stavnezer and Schrader, 2006) (Fig. I.4).

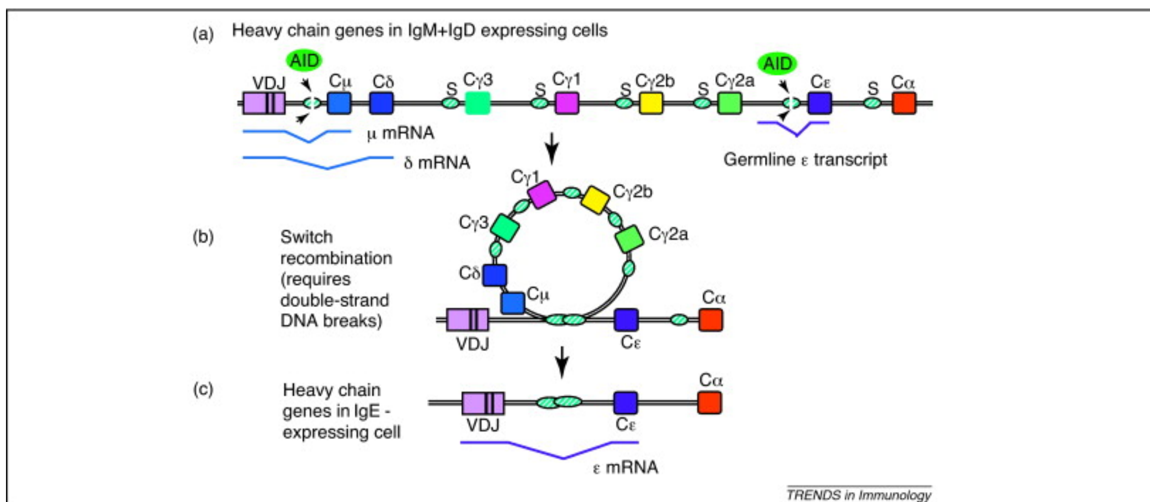


Figure. I.4. CSR uses DSBs at S regions and circle transcripts are created during the process. During CSR, AID acts on two S regions (displayed here: Sμ and Sε, but could be any S region). Eventually this results in DSBs. One DSB in each S region will lead to a deletion of a circle transcript as displayed in (b). After CSR to IgE the H chain loci would appear as depicted in (c). The remaining gene segment between the VDJ and C segments is spliced out. Figure is taken from (Stavnezer, 2011).

In order for CSR to occur, two DSBs are needed - one in both the donor and the acceptor S regions. Non-homologous end joining occurs between the two DSBs. The portion of the gene between the two S regions is excised antibody and forms a circle transcript (CT). What remains is a C_H region specific

for an isotype adjacent to the S region with CSR complete (Stavnezer and Schrader, 2006) (Fig. I.4).

Antibody isotypes in SLE

Both IgM and IgG autoantibodies circulate in the sera of SLE patients. In SLE, the IgG isotype is the most well established as being pathogenic (Ehrenstein et al., 1995; Ohnishi et al., 1994; Radic et al., 1995; Tsao et al., 1992), while the role of auto-reactive IgM is unclear (Boes et al., 2000; Vaughan, 1993). MRL/*lpr* mice, a model of SLE that are unable to secrete IgM ($sIgM^{-/-}$ mice) produce high levels of pathogenic IgG and have severe disease (Boes et al., 2000). IgG is the dominant isotype found in the kidneys during murine lupus nephritis (Madaio and Shlomchik, 1996). Specifically, $IgG_{2a,}$ IgG_{2b}, and IgG_3 have been implicated in the pathogenesis of SLE and these are the classical Th1 isotypes (Peng et al., 2002; Takahashi et al., 1996) (Greenspan et al., 2012).

AID in early developing B cells

At one point, AID was thought to be expressed only in mature B cells. However, AID can be expressed in a pre-B cell transformed by murine leukemia virus Ab-MLV (Yancopoulos et al., 1986). Additionally, upon inoculation with Ab-MLV pro/pre-B cells in the BM of mice express high levels of AID (Gourzi et al., 2007). AID induced in this model is fully functional with CSR and SHM in early developing B cells (Gourzi et al., 2006, 2007). Indeed, AID is also expressed in the early developing B cells of normal mice. CSR spontaneously transpires

throughout B cell development, including pro-, pre- and immature B cells (Edry et al., 2007; Han et al., 2007; Mao et al., 2004; Ueda et al., 2007).

As described earlier, in normal mice, pre-B cells express a μ heavy chain and a “surrogate” light chain to constitute the pre-BCR and the rearrangement of the light chain occurs as the B cell develops further. In μMT mice, an exon of the gene encoding the transmembrane portion of the μ-chain constant region has a stop codon (Kitamura et al., 1991). This provides a developmental block at the pro-B cell stage. μMT mice are considered to be mature B cell deficient; however, μMT mice have circulating class-switched antibodies (Hasan et al., 2002; Macpherson et al., 2001). This suggests that, in the absence of mature B cells, IgG can still be produced. Furthermore, pro-B cells can survive without a μH chain and eventually undergo CSR to produce class switched Ig (Han et al., 2007; Edry et al., 2007). This implies that AID can be expressed without signaling through a fully mature BCR.

I.6 Toll-like Receptors

The discovery of the Toll-like receptor family

In *Drosophila*, the protein Toll is critical for the dorsal-ventral configuration during development (Anderson et al., 1985a) (Anderson et al., 1985b). Loss of function mutations in the *Toll* gene produced dorsalized embryos, while gain of function mutations in the *Toll* gene results in ventralized embryos (Anderson et al., 1985a) (Anderson et al., 1985b). Later, it was discovered that Toll had a

function in innate immune response in insects. Toll is an important component in the pathway responsible for a potent antifungal immune responses in *Drosophila* (Lemaitre et al., 1996). Specific mutations in the Toll signaling pathway dramatically reduced survival after fungal infection of *Drosophila* (Hoffmann, 1995).

A human homologue of the Toll protein was identified by sequence and pattern database searches (Medzhitov et al., 1997). The human Toll protein was described as a transmembrane molecule with a leucine-rich repeat (LRR) domain in the extracellular portion and a cytoplasmic region. Charles Janeway's group demonstrated that human Toll induces the activation of NF-kB. Upon NF-kB activation upregulated expression of the inflammatory cytokines IL-1, IL-6, and IL-8 was observed (Medzhitov et al., 1997).

At that point, the natural ligands for human Toll had not been discovered. Presently, a set of Toll like receptors (TLRs) have been identified that are all related to the human Toll. TLRs use pattern-recognition to detect a limited set of conserved molecular patterns. These patterns are often found on pathogens and are known as pathogen-associated molecular patterns (PAMPs). Most mammalian species have 10 to 15 TLRs. The specific role of TLRs in SLE will be discussed in the next section.

TLRs in SLE

In SLE, TLRs play a role in the activation of autoreactive B cells and subsequent pathology directed by ICs (Rahman and Eisenberg, 2006). A set of

TLRs recognizes nucleic acids, designed to identify foreign pathogens. However, self-nucleic acids could potentially signal through the same TLR, leading to the activation of autoreactive B cells or other immune cell types. The TLRs that recognize nucleic acids are TLR3 (double stranded (ds) RNA), TLR7 (single stranded (ss) RNA), TLR8 (ssRNA) and TLR9 (un-methylated CpG and dsDNA). Polymorphisms in TLR7, TLR8 and TLR9, are all associated with SLE disease in humans (Enevold et al., 2014) (Lee et al., 2012). (Fig. I.5)

Of note, the TLRs that recognize nucleic acids are primarily found on the membranes of endosomes. This allows compartmentalization of the innate immune response. Whereas, self-nucleic acids do not enter the endosome under normal cirumstances, endo-lysomal packaging of pathogens will deliver foreign nucleic acids to TLRs, thus alerting the immune system in the presence of viral and bacterial infections. Nevertheless, there are exceptions to endosomal expression of this set of TLRs. TLR9 is found on B cell plasma membranes (Guerrier et al., 2014), while TLR3 is present on the plasma membrane (Pohar et al., 2014).

TLR adaptor molecules transmit signals after the engagement of ligand and receptor. Adaptor molecules for the TLRs, MyD88, TIRAP, TRIF, and TRAM, all contain a homologous Toll-IL-IR (TIR) domain, which mediates signaling. MyD88 is used as the primary adaptor molecule for all of the mammalian TLRs with the exception of TLR3 (Chi and Flavell, 2008). Differences in signaling events between endosomal TLRs may account for different phenotypes observed when stimulating with various TLR agonists.

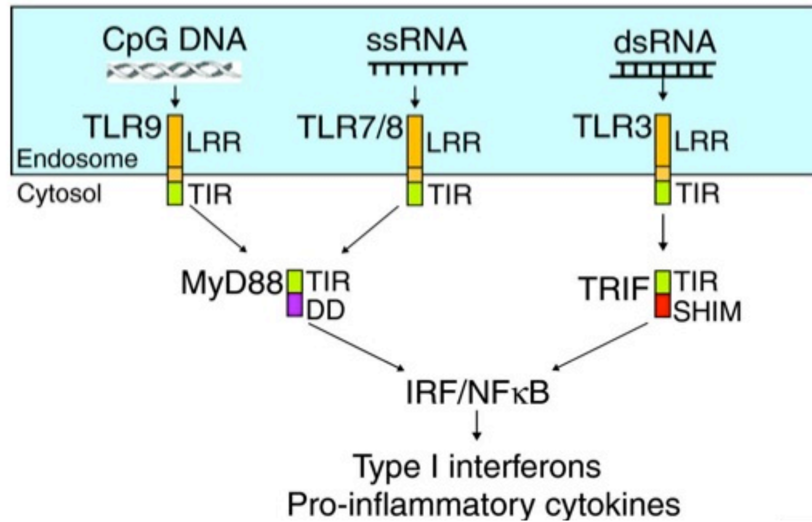


Figure I.5. The endosomal TLRs. TLR9 recognizes DNA with CPG sequences. TIR-TIR interactions with MyD88 begin signaling, which leads to IFN-I via IRF and other pro-inflammatory cytokines via NF-κB. TLR7 and TLR8 recognize ssRNA and use similar downstream signaling to TLR9. TLR3 recognizes dsRNA. TLR3's adaptor molecule is TRIF. Adapted from (Chi and Flavell, 2008)

Increases in TLR expression drive autoimmune symptoms in various mouse models of SLE. Mice transgenic for multiple copies of *Tlr7 (Tlr7^{tg}*) developed severe autoimmunity (Deane et al., 2007). *Tlr7^{tg}* mice have high levels of anti-RNA antibody. A mouse model that over expresses a human TLR8 transgene also produces anti-RNA antibody and develops autoimmune symptoms (Guiducci et al., 2013).

Additionally, *Y-chromosome linked autoimmune-accelerator (Yaa)* has provided insight into the contribution of *Tlr* genes on the X-chromosome to autoimmunity. *Yaa* is a translocation of the telomeric end of the X-chromosome onto the Y chromosome. Notably, the *Yaa* segment of the chromosome contains both of the genes *Tlr7* and *Tlr8*. *Fcγrllb*-deficient C57BL/6 mice and Sle-1

congenic mice were crossed to mice bearing the *Yaa* segment. The male offspring, unlike the Sle-1 congenic parental strain, produce autoantibody to RNA and RNA-associated molecules associated with severe disease symptoms (Pisitkun et al., 2006) (Subramanian et al., 2006).

In male *Yaa* mice, there is one *Tlr7* gene on the X- and Y-chromosomes. Initially, the *Yaa* phenotype was attributed solely to the transcription of *Tlr7* from the two chromosomes in male mice. (Pisitkun et al., 2006) (Subramanian et al., 2006). However, this was put into question by a report that the *Yaa* phenotype is not completely abrogated by the deletion of *Tlr7* from the translocated gene. Therefore, another genetic factor on the *Yaa* segment of the X-chromosome can drive disease when doubled in male mice (Fairhurst et al., 2008). *Tlr8* is a likely candidate.

Endosomal TLRs can induce IFN-I in multiple cell types (Kopp and Medzhitov, 1999). Cytoplasmic receptors for both endogenous and microbial DNA and RNA may play a role in the induction of IFN-I (Unterholzner, 2013). The next section will discuss the role of IFN-I in SLE pathogenesis.

I.7 IFN-I in SLE

The type I IFN (IFN-I) family of cytokines has more than 10 IFN-α and 1 IFN-β sub-family members. Circulating IFN-I is recognized by the IFN-α/β receptor (IFNAR). IFNAR is composed of two peptide chains encoded by the genes *Ifnar1* and *Ifnar2.* Initially, IFN-I was shown to be important for protection

from viral infection. IFN-I can also modulate innate and adaptive immunite responses to microbial infections (Pietras et al., 2006).

The binding of the IFNAR by IFN-I leads to the induction of the JAK-STAT signaling pathway. Tyk2 is constitutively associated with IFNAR-I, and JAK1 with IFNAR-2. Signaling through the IFNAR leads to the phosphorylation of TYK2 and JAK1 (Schindler et al., 2007). At the same time, cross-phosphorylation of the cytoplasmic tails of IFNAR subunits occurs via JAKs. The IFNAR-phosphorylated sites are docking sites for STAT family members. The activated JAKs will in turn phosphorylate docked STAT proteins. Activated STATs then form dimers, which translocate into the nucleus. In the nucleus, the STATs bind promoter regions of IFN-sensitive genes (Schindler et al., 2007). The products of these genes eventually direct anti-viral immune responses.

IFN-I is an important cytokine in immune defense; of particular interest, IFN-I has been shown to be an important factor in human SLE pathogenesis (Baechler et al., 2003; Bennett et al., 2003; Bronson et al., 2012a; Crow, 2014; Kono et al., 2013). IFN-α subclasses have been shown to be the dominant mediator of SLE pathology compared with IFN-β (Crow, 2014). One proposed mechanism for IFN-I mediated SLE involves autoreactive B cell and plasma cell differentiation (Bekeredjian-Ding et al., 2005). Interestingly, increased IFN-I seems to be more important for the induction of disease in murine SLE than in the exacerbation of symptoms after SLE has been established (Baccala et al., 2012).

Evidence for the importance of IFN-I in SLE is found in studies from both humans and mice. First, many components of the IFNAR pathway are implicated in GWAS associations with SLE (Cui et al., 2013). Signaling by IFN-I is clearly important for SLE development in mice. For examples, deficiency of IFNAR, or targeting IFNAR with monoclonal antibodies, reduces SLE symptoms in mouse models (Baccala et al., 2012; Santiago-Raber et al., 2003).

There is also evidence to support the idea that IFN-I can induce SLE. Recombinant IFN-I, when used as a therapy for cancer or chromic hepatitis C has side effects similar to symptoms of SLE (Ronnblom et al., 1991) (Niewold, 2008). Patients with a positive anti-nuclear antibody (ANA) test before treatment with IFN-I are especially susceptible to SLE induction (Bell et al., 1999). This indicates that IFN-I can exasperate disease symptoms and may have the potential to initiate pathology.

High serum levels of IFN-*α* in SLE are associated with kidney disease and autoantibody titers (Kirou et al., 2005). However, the exact source of increased sera IFN-I needs further study. There are many potential cell types responsible for IFN-I production in SLE. Future studies defining the cellular sources of IFN-I production are critical for our understanding etiopathology of the disease.

Cellular source of IFN-I

Plasmacytoid dendritic cells (pDCs) are thought to be the major IFN-I-producing cells in SLE. Human pDCs in circulation produce IFN-I in SLE patients. Additionally, sorted pDCs from peripheral blood stimulated with IgG from SLE

serum produce of IFN-I *in vitro* (Bave et al., 2000; Blomberg et al., 2003; Means et al., 2005). However, in human blood only 0.5% of PBMCs are pDCs, and the number of pDCs in the blood of SLE patients is even less (Scheinecker et al., 2001; Zhuang et al., 2005). pDCs found in the blood produce IFN-I; still, the small number of pDCs in the blood makes it hard to imagine that pDCs the primary source for the increased circulating IFN-I in SLE. Other cellular sources of IFN-I have begun to be proposed as major contributors to SLE pathogenesis.

Expansion of neutrophils in SLE

In many human SLE patients, neutrophils comprise high percentage of PBMCs. Also, granulopoiesis signature genes are upregulated in SLE patients (Bennett et al., 2003; Hacbarth and Kajdacsy-Balla, 1986). In the study by Bennett et al., 2003, granulopoiesis signature genes had the highest level of overexpression of all genes studied. This indicates that an increase in the granulocyte development is an important aspect of SLE disease.

Neutrophil activation has also been implicated in SLE. Neutrophils from SLE patients express high levels of CD11b, a characteristic marker indicating that the neutrophils have been activated (Molad et al., 1994) Neutrophils that up-regulate of CD66b and CD11b have enhanced phagocytic activity and increased IL-8 production after *in vitro* stimulation by nucleosomes (Ronnefarth et al., 2006).

The role of neutrophils in SLE is complicated by the fact that many SLE patients actually have fewer neutrophils in their blood - a condition called

neutropenia. This may be due to autoreactive IgG that specifically targets neutrophils in certain SLE patients. Supporting this is the fact that neutrophils in most SLE patients have IgG bound to their membrane leading to opsonization (Hadley et al., 1987). Also, there are more apoptotic neutrophils in SLE than healthy people, indicating that an increase in apoptosis may decrease the total number of circulating neutrophils (Courtney et al., 1999). Neutropenia in the blood of some patients does not discount a possible increase in granulopoiesis in the BM if neutrophils are being targeted by IgG and undergoing apoptosis at a high rate.

1.8. Questions addressed in this dissertation

There are many unanswered questions concerning the etiology of SLE. Using 564Igi, this study addresses several important questions pertaining to the molecular and cellular requirements for disease progression in SLE. Many of the results have been published in three articles, (Han et al., 2014; Umiker et al., 2014) and Umiker, 2014b. In the following results chapters, I will describe in detail the results obtained. Here I will pose the questions that our group addressed in these publications and are addressed in this dissertation.

In sum, 564Igi allows our laboratory to study SLE pathology using a simple model. All resulting pathology in 564Igi began with the expression of an anti-RNA autoantibody encoded by the 564 transgenes. We were able to ask the question, what aspects of pathology do the expression of anti-RNA antibodies generate? Is there an effect on the populations of myeloid cells, granulocytes, and monocytes as has been observed in human SLE patients? Also, is there an

up-regulation of cytokines, such as IFN-I in 564Igi and what is the cellular source of an increased cytokine response?

IgM$^+$ Id$^+$ B cells in the periphery of 564Igi mice have all the classic signs of anergic B cells (Berland et al., 2006). Despite the anergic profile of peripheral B cells, there is circulating pathogenic IgG anti-RNA antibody in 564Igi. There are two possible sources of IgG autoantibody in 564Igi. One is that anergic mature IgM$^+$ B cells are activated through TLR/BCR signaling. This could allow B cells to break anergy, undergo CSR to IgG and differentiate into antibody secreting cells (ASCs). A second possibility is that non-anergic immature B cells may be able to differentiate into IgG producing ASCs. Immature B cells are a non-canonical source of plasma cell differentiation. In this thesis, I will address the question: what is the cellular source of IgG autoantibody in 564Igi? Do antibody genes class switch to IgG in anergic IgM$^+$ B cells or in earlier stages of B-cell development?

Using 564Igi, we asked how the expression of specific TLRs in B cells and neutrophils affects pathogenesis? The expression of TLR7, TLR8, and TLR9 has been previously shown to have an effect on the progression of disease in various mouse models of SLE. Using a model that expresses an anti-RNA antibody, I was able to ask questions pertaining to the specific role of each of these three TLRs. The cell type-specificity of TLR expression and function is also addressed in this dissertation.

RESULTS

CHAPTER 1

A dual role for AID in early developing B cells in the production of autoantibody in 564Igi

1.1 Rationale

AID is expressed in germinal centers (Muramatsu et al., 1999). AID-mediated SHM and CSR are critical for the process of affinity maturation. Affinity maturation leads to a highly specific adaptive immune response (Jacob et al., 1991) (Neuberger and Milstein, 1995) (Meffre et al., 2001). Since then, AID expression has been reported in early developing B cells—including in pre-B cells and immature B cells (Mao et al. 2004) (Han et al., 2007). This led us to question what (if any) role AID plays in early developing B cells. Specifically we asked, if AID expressed in early developing B cells affects the autoantibody repertoire in 564Igi?

1.2 Results

AID expressed in early developing B cells prevents production of anti-RNA IgM autoantibodies.

Conditional expression of AID does not affect B-cell viability in 564Igi.

Mice on a 564Igi background were bred that expressed AID at specific stages of B-cell development. These mice were bred to address questions about how AID expression at different points in B-cell development affects autoantibody production. In 564Igi *Aicda*$^{-/-}$ mice, AID was expressed by an AID transgene (*Aicda*^{tg}) described in (Muto et al., 2006) and Fig. 1A. The *Aicda*^{tg} contains a GFP

gene flanked by *flox* sites. This GFP gene prevents the transcription of the *Aicda* gene. Upon Cre-recombination, the GFP gene is floxed out and AID expression is driven by the upstream *pCAG* actin promoter. In order to achieve B-cell development stage-specific expression of AID the 564Igi $Aicda^{-/-}$ $Aicda^{tg}$ mice were bred to three Cre mice: mb1-cre (Hobeika et al., 2006), CD19-cre (Rickert et al., 1997) and CD21-cre (Kraus et al., 2004). Mb1-cre mice have Cre recombinase expression in all stages of B-cell development. CD19-cre mice have inefficient Cre recombination throughout B-cell development. CD21-cre mice have Cre recombinase expression only as a B cells develop into the late transitional to mature B cell stages (Fig. 1B and Fig. 2).

Throughout this report, 564Igi $Aicda^{-/-}$ $Aicda^{tg}$ CD21-cre will be referred to as "564Igi CD21-cre", 564Igi $Aicda^{-/-}$ $Aicda^{tg}$ CD19-cre will be referred to as "564Igi CD19-cre" and 564Igi $Aicda^{-/-}$ $Aicda^{tg}$ mb1-cre will be referred to as "564Igi mb1-cre." When referring to all three strains, "564Igi-cre mice" will be used.

AID expression has been shown to increase the likelihood for B cells to undergo apoptosis (Zaheen et al., 2009). On the other hand, AID can promote cell survival signals, preventing a loss in cell viability (Wu et al., 2005). Considering that AID expression affects viability, it was important to see if the expression of AID from the $Aicda^{tg}$ affects B-cell populations in 564Igi-cre mice, and thereby influence the outcome of experiments.

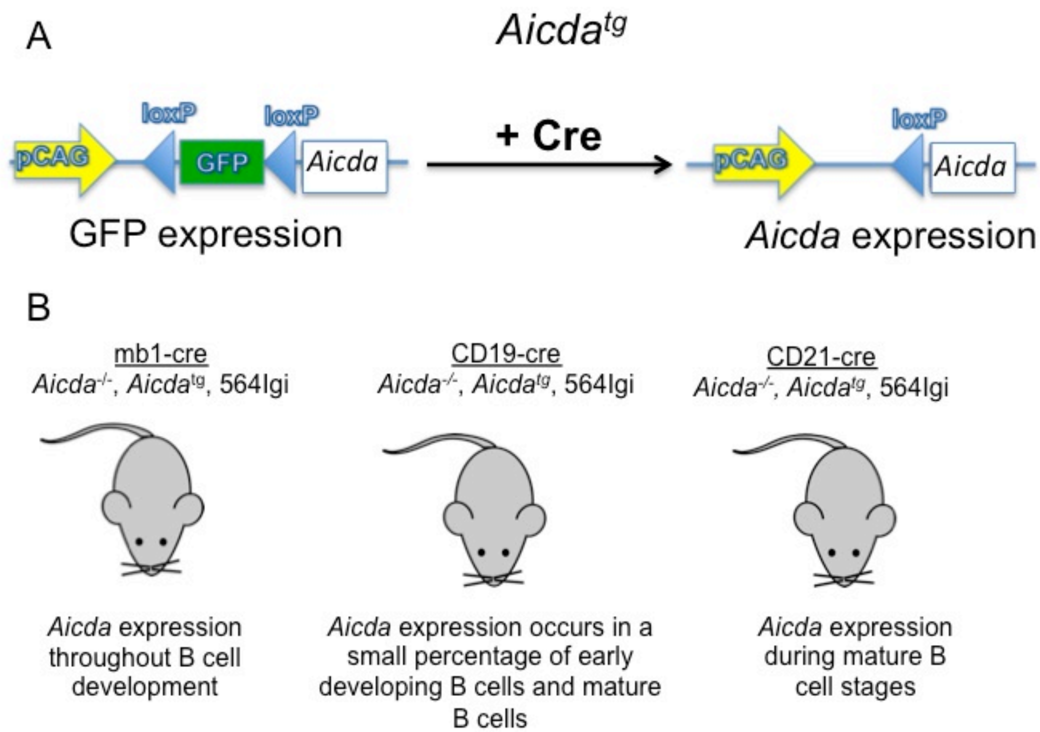


Figure 1. The *Aicda*^{tg} and 564Igi-cre mice. A. The construct for *Aicda*^{tg}: GFP is excised after Cre-recombination. *Aicda*^{tg} is then expressed through the pCAG promoter. **B.** *Aicda*^{tg} and 564IgH and IgL chain genes were bred to CD19-cre, mb1-cre and CD21-cre mice on an *Aicda*$^{-/-}$ background. These strains have B cell developmental stage-specific expression of the *Aicda*^{tg}.

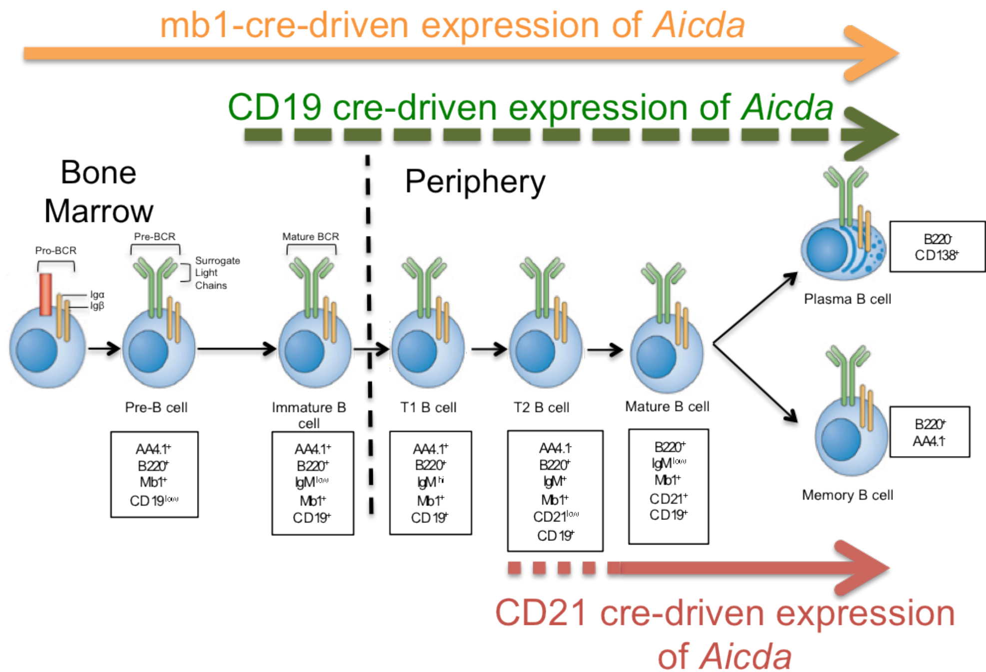


Figure 2. B-cell developmental stage-specific expression of the *Aicda*^{tg} in 564Igi-cre mice. 564Igi mb1-cre mice produced AID efficiently throughout B cell development. 564Igi CD19-cre inefficiently produced AID throughout B-cell development. 564Igi CD21-cre mice produced AID in transitional and mature B cell stages.

As shown in Fig. 3A, 564Igi, C57BL/6, and the 564Igi-cre mice have similar numbers of nucleated cells in their BM. 564Igi has fewer B cells (B220^{+}) in the BM than C57BL/6 mice (Fig. 3B). Similar numbers of B cells were found in the 564Igi mice BM compared to the 564Igi-cre mice BM (Fig. 3B). There is no significant difference between 564Igi mice and 564Igi-cre mice in terms of AA4.1^{+} B220^{+} immature B cells (Fig. 3B).

In the spleen there are fewer total mononucleated cells in 564Igi than in C57BL/6 (Fig. 4A). This is also true of splenic B cells (Fig. 4B). Fewer splenic B cells in 564Igi than C57Bl/6 is most likely due to the fact that IgM^+ auto-reactive B cells in the periphery are anergic and do not proliferate (Berland et al., 2006). There are a similar number of B cells in 564Igi, 564Igi CD19-cre, and mb1-cre mice. In the spleens of 564Igi CD21-cre mice, there are slightly more B cells than in 564Igi spleens, but less than in C57BL/6 spleens (Fig. 4B). A similar trend is observed in splenic $CD21^+$ mature B cells, and $CD138^+$ antibody producing cells (Fig. 4B). In conclusion, we find that the high levels of AID expressed by *Aicda*^{tg} mice are not affecting the viability of B cells or normal B-cell development, confirming results that have been reported previously (Muto et al., 2006).

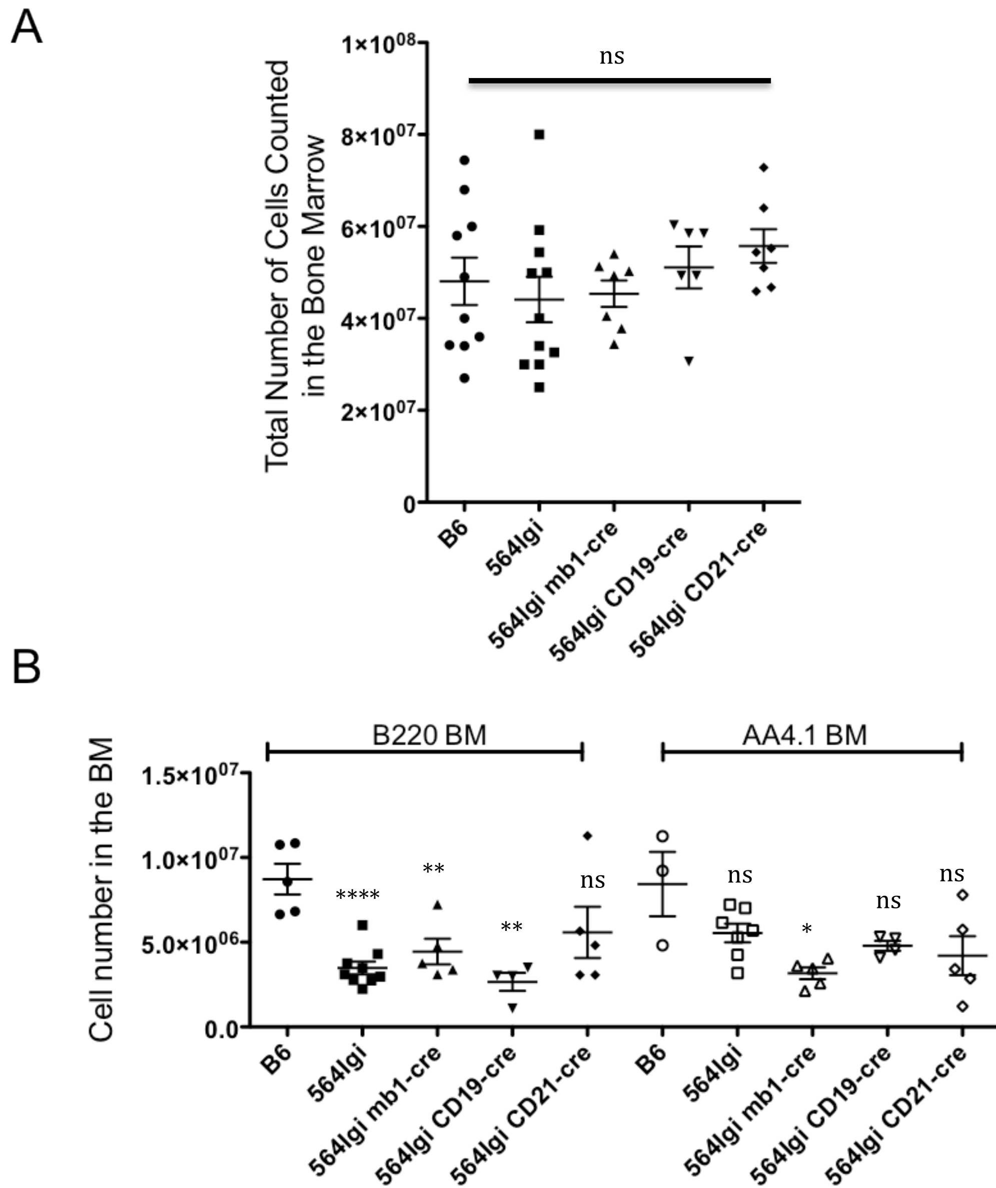


Figure 3. Absolute numbers of BM B cells is similar in 564Igi and 564Igi-cre mice. A. Total number of lymphocytes in the bone marrow as counted on a hemocytometer. **B.** $B220^+$ and $AA4.1^+$ populations analyzed by FACs. The percentage of live lymphocytes (PI^-) is displayed. Error bars are SEM. Statistical analysis is based on a two tailed Student's t-test compared to B6; significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001.

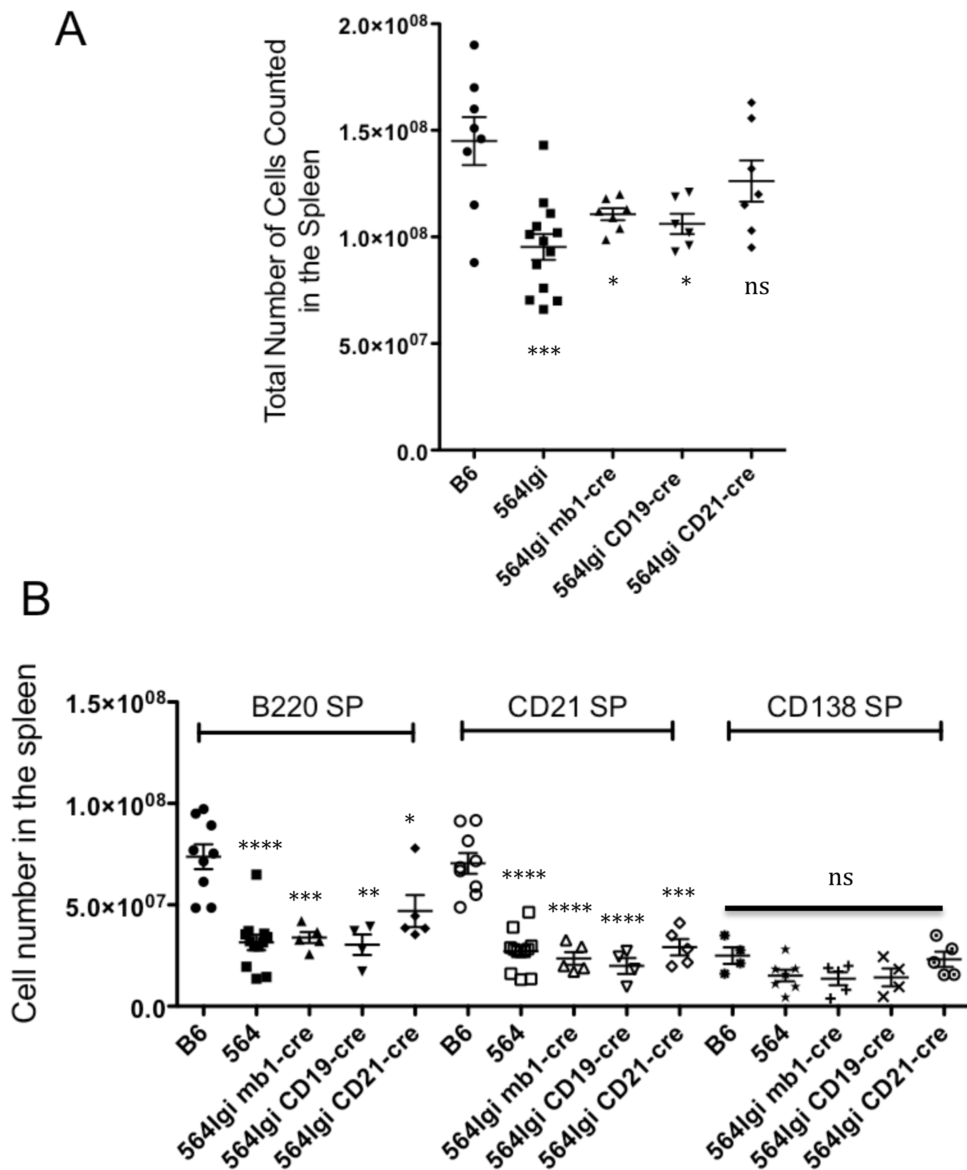


Figure 4. Absolute numbers of SP B cells is similar in 564Igi and 564Igi-cre mice. A. Total number of lymphocytes in the spleen as counted on a hemocytometer. **B.** $B220^{+}$ $CD21^{+}$ and $CD138^{+}$ populations analyzed by FACs. The percentage of live lymphocytes (PI^{-}) is displayed. Error bars are SEM. Statistical Statistical analysis is based on a two tailed Student's t-test compared to B6; significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001.

Cre-mediated recombination in 564Igi-cre mice is stage-specific, but has varied efficiencies in 564Igi mb1-cre and 564Igi CD19-cre mice

Confirmation that Cre-recombination occurs in a B-cell developmental stage specific manner in the 564Igi-cre mice was needed. As shown in Fig. 1A, prior to Cre-recombination a B cell with the *Aicda*^{tg} will express GFP. Cre-recombination was easy to trace with FACs analysis: GFP^{+} cells have not undergone Cre-recombination while GFP^{-} cells have. Therefore, GFP^{-} cells are expressing AID in 564Igi-cre mice.

Viable mono-nucleated cells from the BM of C57BL/6, 564Igi, and 564Igi-cre mice were gated into three populations B220^{+} IgM^{-} (pro/pre B cells), B220^{+} IgM^{+} (immature B cells and mature recirculating B cells), and B220^{+} AA4.1^{+} (immature and pre-B cells) (Fig. 5A-C). GFP expression was analyzed in B-cell populations from 564Igi-cre mice by FACs.

The 564Igi mb1-cre and 564Igi CD19-cre mice were designed to express the *Aicda*^{tg} throughout B-cell development. Therefore, one would expect that all B cells are GFP^{-} in both 564Igi mb1-cre and 564Igi CD19-cre. However, in CD19-cre mice, both B220^{+} IgM^{-} and B220^{+} IgM^{+} cells are around 75% GFP+ (Fig. 5B). This low frequency of *Aicda*^{tg} expression in very early developing B cells (B220^{+} IgM^{-}) could be due to a lower Cre-recombination efficiency. Inefficient Cre-recombination in CD19-cre mice has been reported previously (Hobeika et al., 2006). 564Igi mb1-cre mice have very efficient Cre-recombination at early stages of B-cell development in the BM (Fig. 5B). 564Igi CD21-cre mice should not express the *Aicda*^{tg} during early stages of B-cell development. Accordingly, the majority of immature and pro/pre B cells (B220^{+} AA4.1^{+} and B220^{+} IgM^{+}

respectively) in the 564Igi CD21-cre BM were GFP+ (Fig. 5B and 5C).

In both 564Igi mb1-cre and 564Igi CD21-cre mice, *Aicda*^{tg} expression is evident in splenic B cells. The majority of splenic B cells (B220^{+}) and mature splenic B cells (CD21^{+}) in 564Igi mb1-cre and 564Igi CD21-cre mice are GFP^{-} (Fig. 6). In both antibody-secreting plasma cell precursors (CD138^{low}) and terminally differentiated antibody-secreting cells (CD138^{high}) there is a high percentage of *Aicda*^{tg} expression indicated by a lack of GFP expression in 564Igi mb1-cre and 564Igi CD21-cre mice (Fig. 6). Cre-recombination in splenic 564Igi CD19-cre B cells is less efficient than in the other 564Igi-cre mice, indicated by high percentages of GFP^{+} in all splenic B cells (B220^{+}, CD21^{+}, CD138^{low}, and CD138^{high}) (Fig. 6).

CD21 is expressed highly on follicular dendritic cells (FDCs) (Liu et al., 1997). Therefore, in 564Igi CD21-cre mice, FDCs will be GFP- and express AID. FDCs most likely constitute a large proportion of the GFP- B220- cell population observed in 564Igi CD21-cre spleens (68% Fig. 6). In 564Igi mb1-cre mice, FDCs do not express AID. Therefore, the expression of AID in FDCs in 564Igi CD21-cre mice must be considered in the interpretation of results using 564Igi-cre mice.

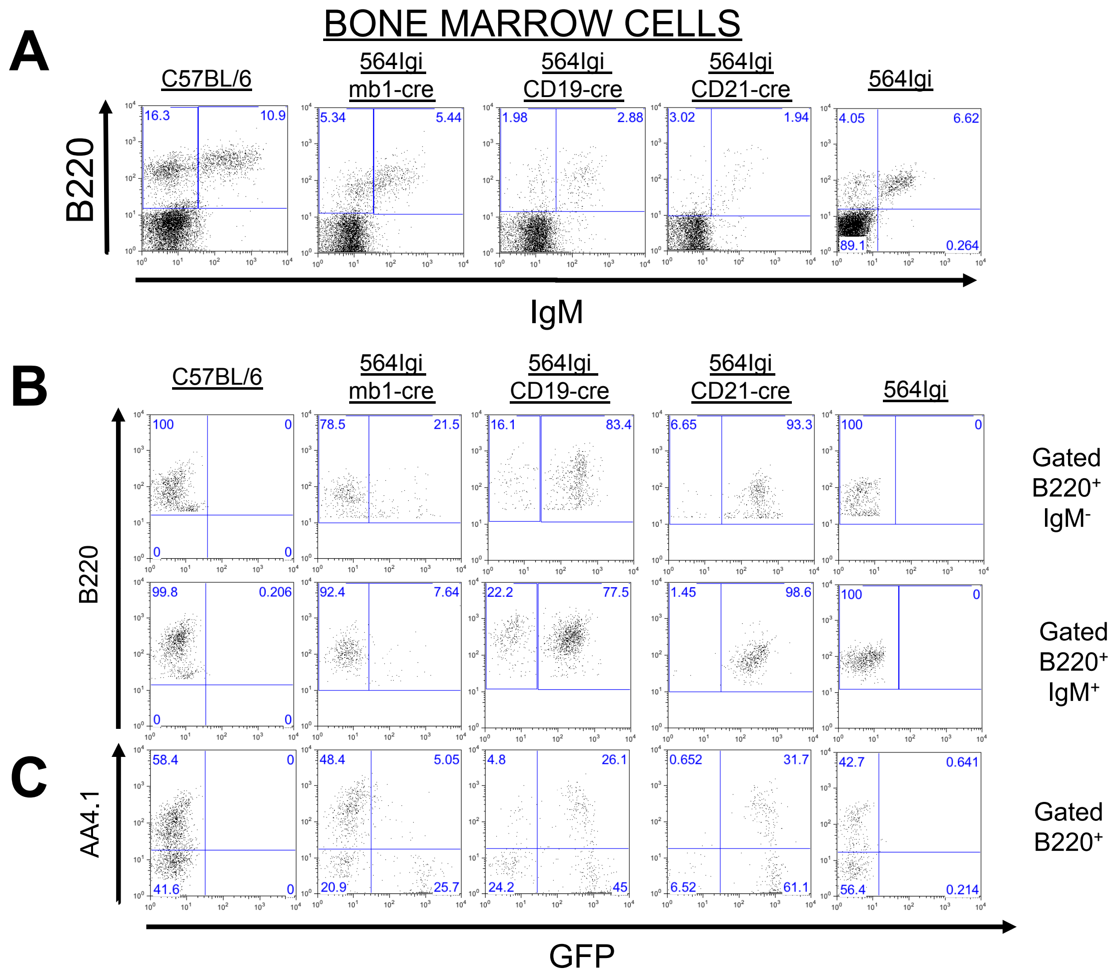


Figure 5. Mb1, CD19 and CD21 promoters allow for varied efficiencies of Cre-recombination in the BM. A. C57BL/6, 564Igi mb1-cre/*Aicda*^{tg}, 564Igi CD19-cre/*Aicda*^{tg}, 564Igi CD21-cre and 564Igi mice BM cells were analyzed by flow cytometry for B220 and IgM surface expression. **B,C.** GFP expression in BM cells gated on B220^{+} and IgM$^{+/-}$ (**B**) or AA4.1^{+} (**C**). Populations measured for GFP expression represent the efficiency of Cre-mediated recombination. Representative of at least 3 mice.

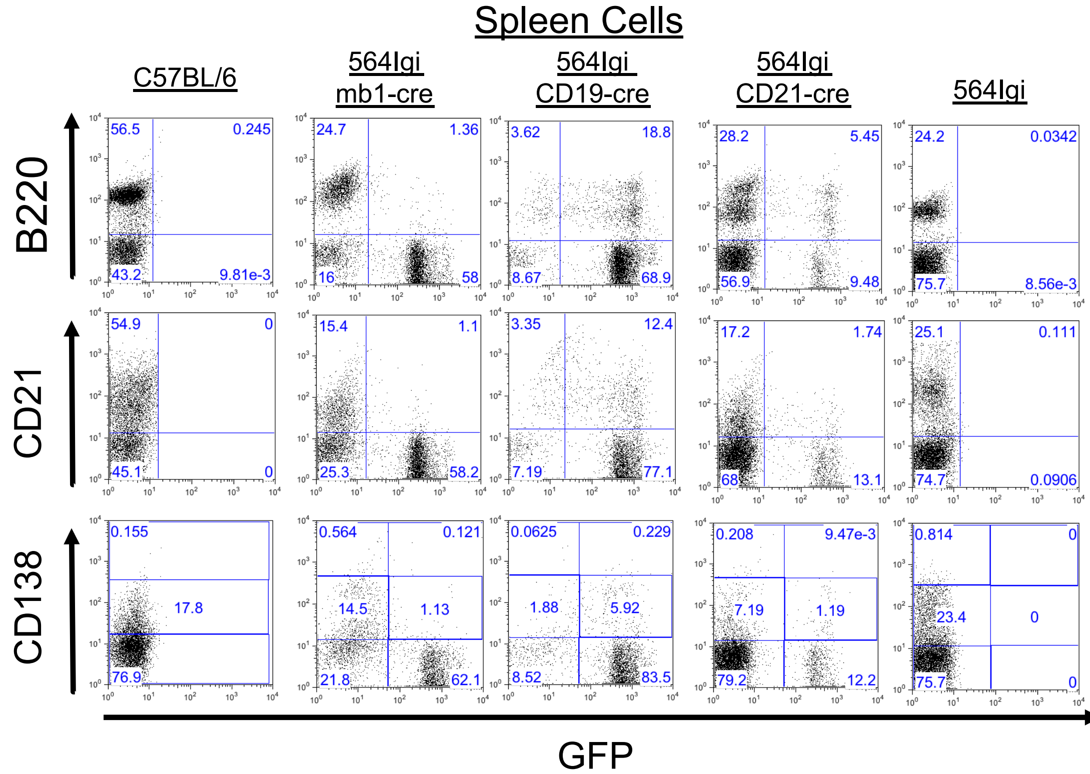


Figure 6. Mb1, CD19 and CD21 promoters allow for varied efficiencies of Cre-recombination in the SP. A. C57BL/6, 564Igi mb1-cre/*Aicda*^{tg}, 564Igi CD19-cre/*Aicda*^{tg}, 564Igi CD21-cre, and 564Igi spleen cells were analyzed by flow cytometry for B220^{+}, CD21^{+}, and CD138^{+} cells. Each cell population was analyzed for GFP expression representing the efficiency of Cre-mediated recombination. Representative of at least 3 mice.

The Aicda^{tg} is expressed at high levels in 564Igi-cre mice.

In order to confirm that the *Aicda*^{tg} is expressed in the B cells of 564Igi-cre mice, $GFP^{-}B220^{+}$ cells were sorted from both the BM and the spleen of 564Igi-cre mice. An example of a sort from the BM of 564Igi mb1-cre is displayed in Figure 7A. Transcript levels of *Aicda* were measured by qRT-PCR for each population of sorted B cells.

There are significantly higher transcript levels in 564Igi-cre B220+ GFP- compared with endogenous *Aicda* transcript levels in either 564Igi or C57BL/6 B220+ B cells in both the BM and spleen (Fig. 7B). 564Igi mb1-cre mice have higher *Aicda* transcript levels in GFP^{-} BM and SP B cells compared to 564Igi CD19-cre and 564Igi CD21-cre mice.

BM B cells in 564Igi have higher levels of *Aicda* transcripts than BM B cells in C57BL/6 (Fig. 7B). This may due to more early developing B cells being activated in 564Igi mice. Splenic B cells express similar levels of *Aicda* transcripts in 564Igi and C57BL/6 (Fig. 7B). A more thorough investigation of *Aicda* expression in different B cell stages may provide further valuable information.

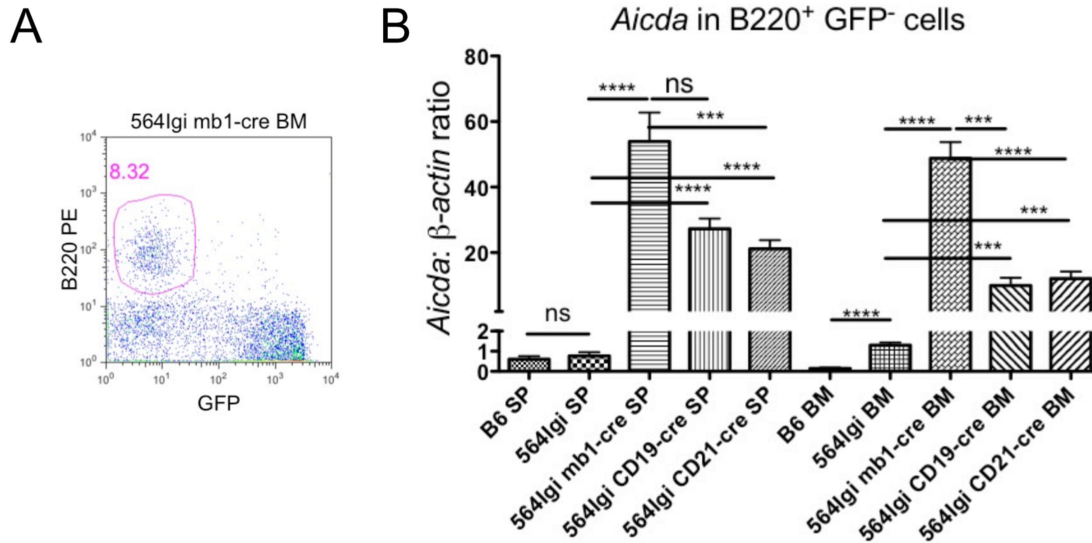


Figure 7. *Aicda* is expressed in B220$^+$ GFP$^-$ B cells from the bone marrow and spleen of 564Igi-cre mice. A. An example of flow cytometry sort. B220 is on the y-axis and GFP is on the x-axis. This is from the BM of a 564Igi mb1-cre. Boxed cells were sorted as B220$^+$ GFP$^-$ B cells. **B.** *Aicda* mRNA transcript levels relative to *β-actin* from B220$^+$GFP$^-$ cells were measured by real-time PCR (qPCR) from C57BL/6, 564Igi, 564Igi mb1-cre, 564Igi CD19-cre, and 564Igi CD21-cre mice. Three mice were tested for each strain. Three dilutions of cDNA were tested from each mouse, and each dilution was tested in triplicate. Data are shown as the mean +/- SEM of n=3 samples and are pooled from 3 independent experiments. Mice were tested at 3 to 4 months of age. Statistical analysis is based on a two tailed Student's t-test; significance at $p<.05$. * $p< .05$, ** $p< .01$, *** $p< .001$ and **** $p< .0001$.

AID-mediated CSR occurs in 564Igi-cre mice.

There is high expression of the *Aicda*^{tg} in 564Igi-cre mice. However, confirmation was required that the expression leads to CSR in the 564Igi-cre mouse strains. Total serum IgG antibody titers were measured in C57BL/6, 564Igi, 564Igi *Aicda*$^{-/-}$, and 564Igi-cre mice by ELISA in order to investigate

whether CSR occurred in all strains. Three isotypes were measured: IgG_1, IgG_{2a} and IgG_{2b}. The knock-in heavy and light chain antibody genes in 564Igi encode for the “a” allotype of IgG_{2a}. Wild-type C57BL/6 does not express the “a” allotype of IgG_{2a}; however, Balb/c does.

The titers of total serum IgG_1 in 564Igi-cre mice are comparable to those of 564Igi and C57BL/6 mice (Fig. 8). In 564Igi mice, endogenous *Aicda* is expressed at low levels compared to very high levels in 564Igi-cre mice from the *Aicda*^{tg}. Taken together, the data suggest that AID was mediating CSR in B cells from both 564Igi and 564Igi-cre mice with vastly different levels of *Aicda* transcripts. High levels of sera IgG_1 can be produced from small numbers of activated B cells. Therefore, a more thorough investigation of the ability for B cells to undergo CSR in vitro from each of the 564 mb1-cre mouse strains is needed.

There are lower antibody titers of IgG_{2a} antibody in 564Igi CD21-cre mice compared to 564Igi mice, but similar antibody titers of IgG_{2b} and IgG_1 (Fig. 8). In 564Igi CD21-cre mice, *Aicda* is not expressed in early developing B cells. Therefore, the production of serum IgG in 564Igi CD21-cre mice suggests that *Aicda* expression in mature B cells ($CD21^+$) is sufficient for CSR (Fig. 8). 564Igi CD19-cre mice also have high titers sera IgG (Fig. 8) even though these mice have fewer B cells expressing *Aicda* due to less efficient Cre-recombination (Fig. 5 and 6). The titers for total IgM are comparable in C57BL/6, 564Igi, and 564Igi-cre mice (Fig. 9).

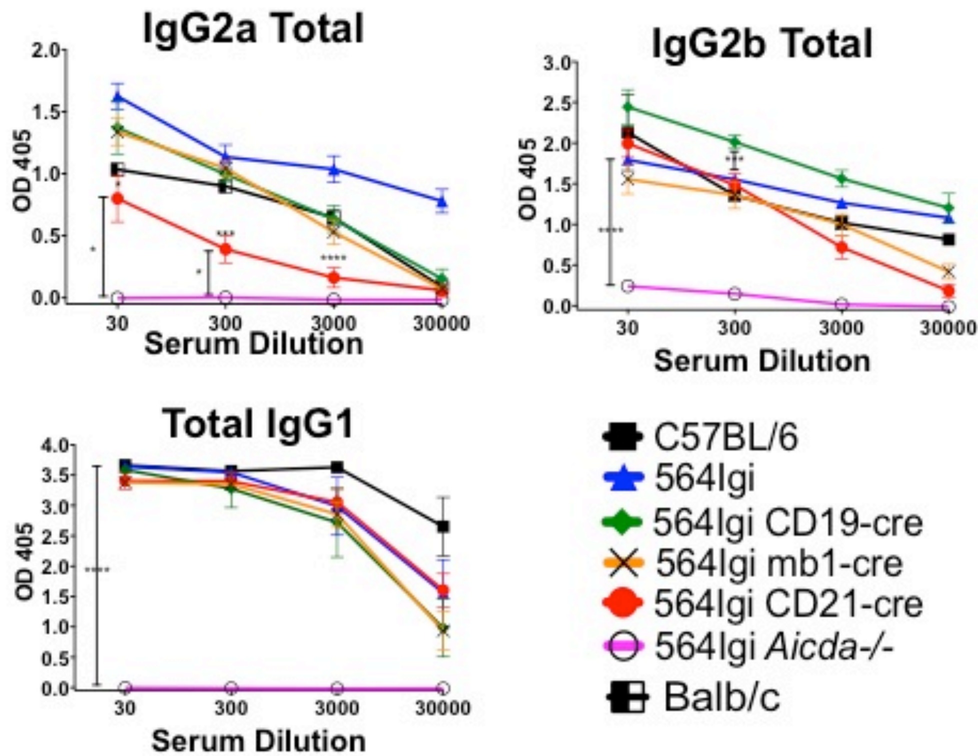


Figure 8. CSR occurs in 564Igi-cre mice. Serum titers measured by isotype-specific ELISAs (IgG_1, IgG_{2a}, and IgG_{2b}) for total antibody. Balb/c n=2 (IgG_{2b} only) C57BL/6 n=4, 564Igi n=7, 564Igi mb1-cre n=9, 564Igi CD19-cre n=7, 564Igi CD21-cre n=25, 564Igi *Aicda*$^{-/-}$ n=8. Statistical analysis is based on a two tailed Student's t-test; significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001. Data collected by Ben Umiker and Amma Larbi.

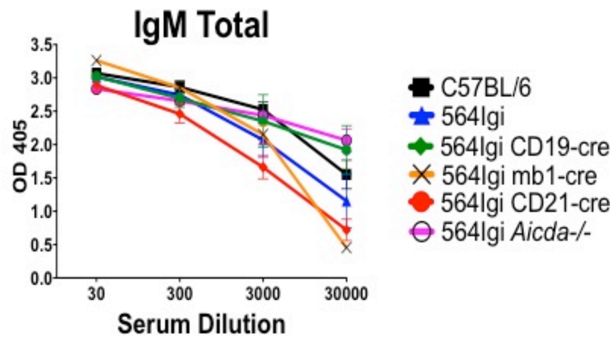


Figure 9. Similar levels of total IgM in 564Igi-cre mice. Serum titers measured by IgM ELISAs for total antibody. C57BL/6 n=8, 564Igi n=15, 564Igi mb1-cre n=10, 564Igi CD19-cre n=21, 564Igi CD21-cre n=26, 564Igi *Aicda*$^{-/-}$ n=11. Data collected by Ben Umiker and Amma Larbi.

AID expression in developing B cells prevents anti-RNA IgM autoantibody production.

564Igi mice have relatively low anti-RNA and Id$^+$ IgM antibody serum titers (Fig. 10A and 10B). 564Igi IgM$^+$ B cells in the periphery are anergic and therefore do not produce anti-RNA IgM antibody (Berland et al., 2006).

Interestingly, in 564Igi *Aicda*$^{-/-}$ mice, there are significantly higher serum titers of anti-RNA IgM than in 564Igi mice (Fig. 10A). The serum titers are also high for IgM Id$^+$ antibodies in 564Igi *Aicda*$^{-/-}$ mice (Fig. 10B), but not in C57BL/6 *Aicda*$^{-/-}$ mice. This demonstrates that anti-RNA IgM antibodies in 564Igi *Aicda*$^{-/-}$ mice are the product of the 564 knock-in IgH and Igκ genes.

In 564Igi CD21-cre mice, there are elevated levels of serum anti-RNA and Id$^+$ IgM autoantibodies compared to 564Igi mice. On the other hand, 564Igi mb1-cre mice have similar levels of serum anti-RNA and Id$^+$ IgM autoantibodies compared to 564Igi mice (Fig. 10A and 10B). The levels of total IgM in the sera

are similar amongst all 564Igi-cre mice (Fig. 9). The ratio of O.D.405 values obtained from the anti-RNA IgM and total IgM assays (anti-RNA:total IgM) gives an indication of the relative amount of anti-RNA binding per IgM molecule in the sera of 564Igi-cre mice. The anti-RNA:total IgM ratio calculated from 564Igi CD21-cre sera is similar to that of 564Igi *Aicda*$^{-/-}$, while the anti-RNA:total IgM ratio calculated for 564Igi mb1-cre sera is similar to that of 564Igi (Fig. 11). Higher levels of IgM RNA binding in 564Igi CD21-cre compared to 564Igi mb1-cre may suggest that AID expression in developing B cells is critical to prevent high titer of serum IgM autoantibody.

564Igi CD19-cre mice have intermediate anti-RNA:total IgM ratio compared with 564Igi CD21-cre and 564Igi mb1-cre mice. 564Igi CD19-cre lack functional AID in the majority of early developing B cells (Fig. 5A-C). The inefficient expression of AID during these B-cell stages contribute to the increased anti-RNA IgM antibody.

Multiple mechanisms could contribute to low titers of serum IgM autoantibody in the absence of AID expression in early developing b cells. One is that AID decreases the viability of auto-reactive B cells. Another is that AID may be required to establish B-cell tolerance, via RAG–mediated receptor editing. Thirdly, SHM of autoreactive autoantibody genes in early developing B cells could drive the genes away from self-reactivity.

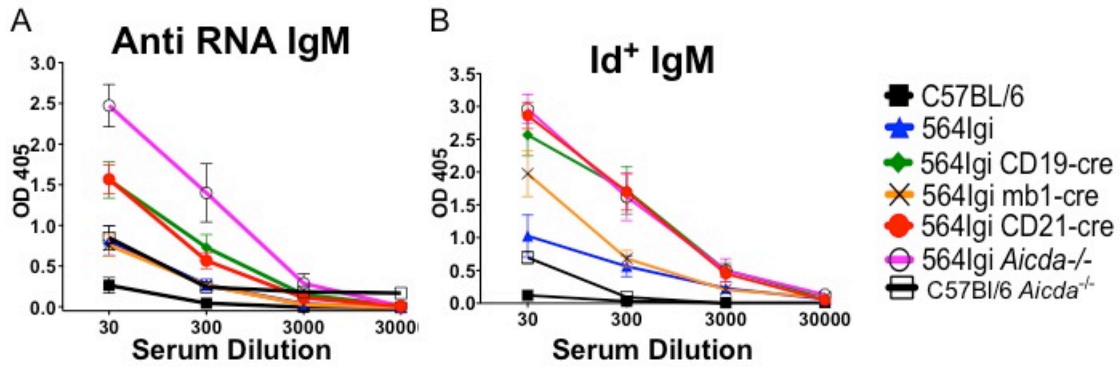


Figure 10. Serum titers of anti-RNA Id$^+$ IgM are higher in mice lacking AID during very early stages B cell development. A. Serum anti-RNA IgM antibody titers measured by ELISA. C57BL/6 n=4, 564Igi n=13, C57BL/6 *Aicda*$^{-/-}$ n=6, 564Igi *Aicda*$^{-/-}$ n=15. **B.** Serum Id$^+$ IgM antibody titers measured by ELISA. C57BL/6 n=5, 564Igi n=10, C57BL/6 *Aicda*$^{-/-}$ n=10, 564Igi *Aicda*$^{-/-}$ n=10.
Data collected by Ben Umiker and Amma Larbi.

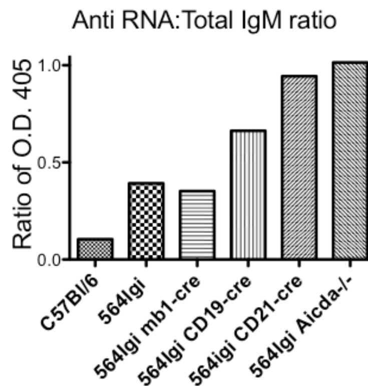


Figure 11. RNA reactivity of IgM compared to total IgM in the sera in 564Igi-cre mice. Ratio of the average optical densities (O.D.) from IgM anti-RNA ELISA and total IgM ELISA for the indicated mouse strain. Ratio of O.D. 405nm for anti-RNA IgM ELISA at 1:30 serum dilution to the O.D. 405nm from total IgM ELISA at 1:3000 serum dilution.

AID expression in early developing B cells results in somatically mutated autoantibody genes that no longer encode IgM anti-RNA autoantibodies.

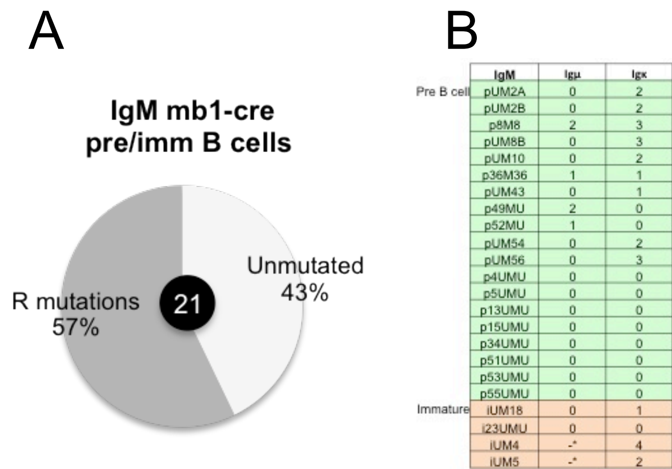
SHM of heavy and light chain genes in early developing B cells driven by the Aicda^{tg}.

To test the hypothesis that the lower level of anti-RNA IgM serum antibody titers in 564Igi mb1-cre mice compared to 564Igi CD21-cre mice is due to SHM, the variable regions of the 564 Igμ and Igκ chain genes were cloned and sequenced from single B cells sorted from the BM and the spleen. This allowed us to detect mutations that occurred in the knock-in genes at various stages of B-cell development in 564Igi-cre mice in individual B cells.

In 564Igi mb1-cre pre-B cells and immature B cells, there are mutations in the 564 Igμ and Igκ chain genes. 57% of immature and pre-B cells have at least

one replacement mutation (R) in either 564 Igμ and Igκ chain genes (Fig. 12). In 564Igi mb1-cre pre-B cells and immature B cells, the rate of R mutations for 564 Igμ and Igκ is 2.1 mutations per one thousand base pairs (Fig. 13B). In 564Igi *Aicda*$^{-/-}$ B cells, the rate of R mutation for 564 Igμ and Igκ is 0.43 mutations per one thousand base pairs (Fig. 13A). The mutations in the 564Igi *Aicda*$^{-/-}$ B cells represent background mutations acquired during the process of cloning and sequencing. Another possibility is that these background accumulate in the absence of AID *in vivo*.

564Igi Igμ and Igκ in early developing B cells from mb1-cre mice have significantly higher rates of mutations than the 564Igi *Aicda*$^{-/-}$ B cells. Therefore SHM is occurring in early developing B cells in 564Igi mb1-cre mice. The replacement mutation to silent mutation ratio (R/S) of early developing B cell Igμ and Igκ genes in 564Igi mb1-cre mice is 6.13. This is higher than what would be expected for random mutations (> 2.9) (Shlomchik et al., 1987). The majority of R mutations in early developing B cell Igμ and Igκ genes in 564Igi mb1-cre mice were in the CDR region (32/49) (Fig. 13).



	IgM	Igμ	Igκ
Pre B cell	pUM2A	0	2
	pUM2B	0	2
	p8M8	2	3
	pUM8B	0	3
	pUM10	0	2
	p36M36	1	1
	pUM43	0	1
	p49MU	2	0
	p52MU	1	0
	pUM54	0	2
	pUM56	0	3
	p4UMU	0	0
	p5UMU	0	0
	p13UMU	0	0
	p15UMU	0	0
	p34UMU	0	0
	p51UMU	0	0
	p53UMU	0	0
	p55UMU	0	0
Immature	iUM18	0	1
	i23UMU	0	0
	iUM4	-*	4
	iUM5	-*	2

Figure 12. Replacement mutations in early developing B cells in 564Igi mb1-cre mice. A. Percentage of B cells from 564Igi mb1-cre BM pre-B and immature B cells that have at least one R mutation in either the Igμ or Igκ. **B.** Number of R mutations in IgH and IgL genes from single cells.

A

		564 *Aicda*-/- BM B cells		
		Igμ	Igκ	Total
Total	Clones Sequenced	20	21	41
	Clones with R mutations	3	3	6
	Percentage of R mutated clones	15.00%	14.29%	14.63%
	R Mutations	3	3	6
	R Mutations per 10^3 base pairs	0.44	0.45	0.46
	S Mutations	4	2	6
	R/S Ratio	0.75	1.5	1
CDR	R Mutations	1	1	2
FWR	R Mutations	2	2	4

B

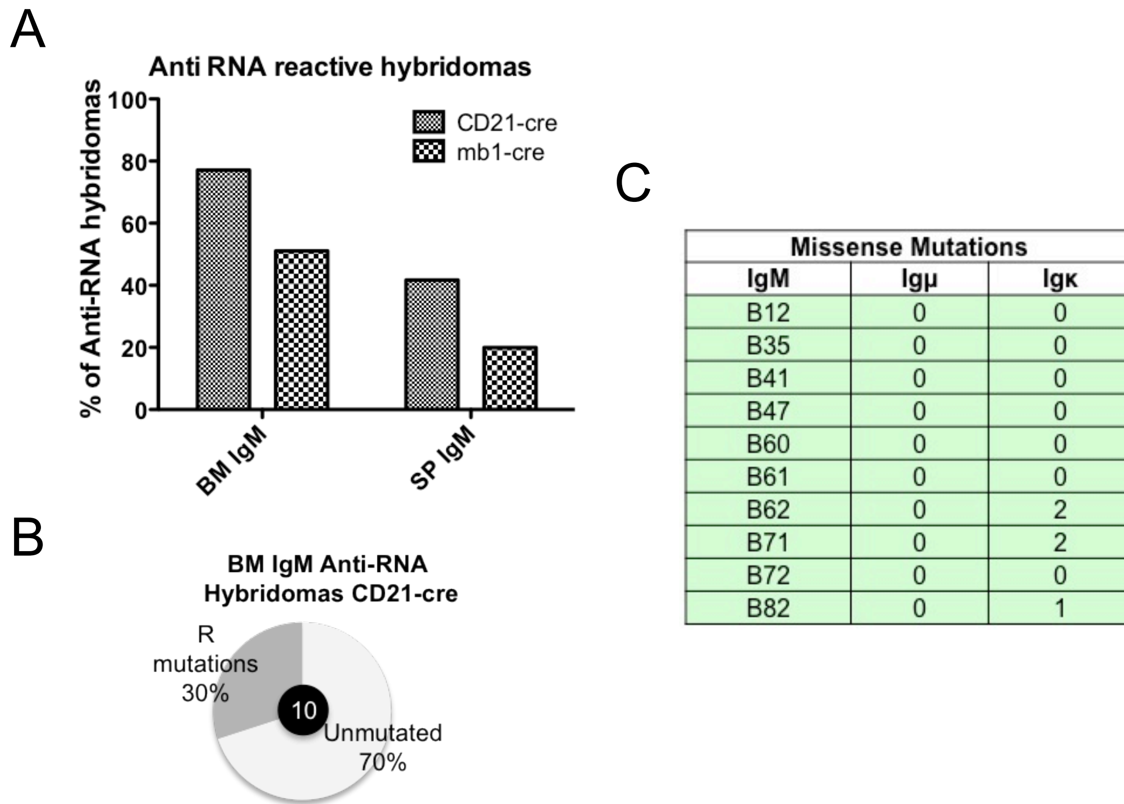
		564Igi mb1-cre BM pre B		564Igi mb1-cre BM imm. B cell		Total
		Igμ	Igκ*	Igμ	Igκ*	
Total	Clones Sequenced	19	25	2	23	69
	Clones with R mutations	4	12	0	14	30
	Percentage of R mutated clones	21%	48%	0%	61%	43%
	R Mutations	6	20	0	23	49
	R Mutations per 10^3 base pairs	0.93	2.52	0.00	3.14	2.10
	S Mutations	1	4	0	3	8
	R/S Ratio	6	5	n/a	7.67	6.13
CDR	R Mutations	3	12	0	17	32
FWR	R Mutations	3	8	0	6	17

Figure 13. High rate of mutations in early developing B cells in 564Igi mb1-cre mice. Mutations in IgH and Igκ cDNA clones from the **A.** BM of 564Igi *Aicda*$^{-/-}$ and **B.** spleen (SP) and bone marrow (BM) of 564Igi mb1-cre. The total number of mutations, mutations in the complementary determining regions (CDR) and framework regions (FWR) are displayed. Replacement (R) and silent (S) mutations are displayed along with replacement:silent mutation ratios (R/S). **A.** Mutations in IgH and IgL genes from BM B cells in 564Igi *Aicda*$^{-/-}$ mice **B.** Mutations in IgH and IgL genes of pre-B and immature B cells of 564Igi mb1-cre. Data in A. was collected by Gabrielle McDonald.

To validate the correlation between AID expression in early developing B cells and a loss of anti-RNA IgM antibody serum titers, hybridomas were fused from the BM and SP cells in both 564Igi mb1-cre and 564Igi CD21-cre mice. The hybridoma antibody products were screened for RNA reactivity with an IgM specific ELISA. A higher percentage of hybridomas produce anti-RNA IgM

antibody from the BM and spleen of 564Igi CD21-cre than those from mb1-cre mice (Fig. 14A).

Antibody genes were sequenced from 564Igi CD21-cre IgM anti-RNA hybridomas. A high percentage (70%) of anti-RNA IgM^+ hybridomas had no replacement mutations (Fig. 14B). The rate of mutations in 564Igi CD21-cre anti-RNA IgM^+ hybridomas was 0.74, only slightly higher than background (0.46). Background was determined by sequencing antibody genes from B cells in the 564Igi *Aicda*$^{-/-}$ (Fig. 13A). The absence of SHM in 564Ig genes of early developing B cells in 564Igi CD21-cre contributes to high titers of anti-RNA IgM antibodies in their sera.



Missense Mutations		
IgM	Igμ	Igκ
B12	0	0
B35	0	0
B41	0	0
B47	0	0
B60	0	0
B61	0	0
B62	0	2
B71	0	2
B72	0	0
B82	0	1

Figure 14. There were fewer RNA reactive antibody producing B cells in 564Igi mb1-cre than 564Igi CD21-cre. A. BM and SP derived hybridomas that were positive for growth were screened for anti-RNA IgM antibody production by ELISA. The percentage of RNA reactive hybridomas per the total positive for growth is displayed. **B.** Ig μ and Ig κ antibody genes were cloned and sequenced from selected anti-RNA IgM antibody-producing hybridomas from 564Igi CD21-cre BM. Shown is the percentage of hybridomas with replacement (R) mutations in either IgH or IgL genes. **C.** Number of missense mutations in antibody genes cloned from individual 564Igi CD21-cre hybridomas. Data collected by Ben Umiker and Thereza Imanishi-Kari.

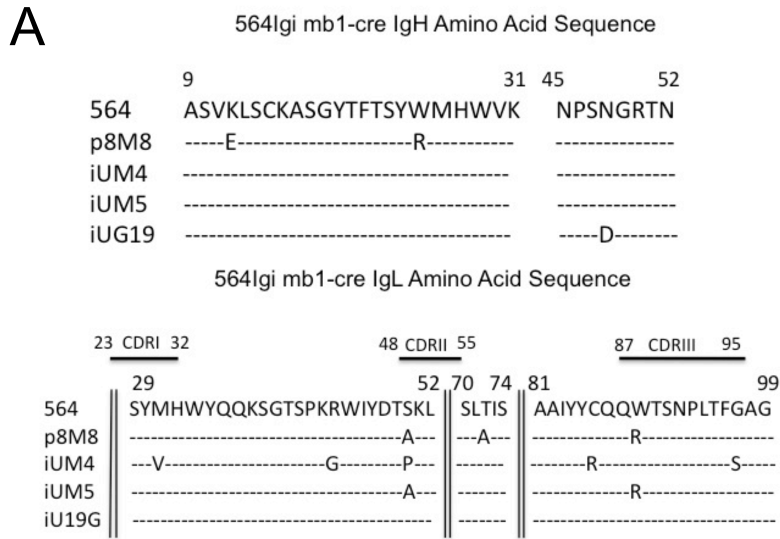
SHM of heavy and light chain genes in early developing B cells results in low titers of auto-reactive IgM.

Next it was determined whether the AID-mediated SHM of 564Igi Ig genes in early developing B cells from 564Igi mb1-cre mice could lead to a loss of self-reactivity. This was done by cloning the variable regions of mutated Igμ and Igκ

genes from single cells of 564Igi mb1-cre into expression vectors. When transfected into HEK293 cells, the expression vectors produce recombinant antibodies. Recombinant antibodies have a human IgG_1 Fc and murine variable region. The variable region is comprised of an IgH and an Igκ cloned and sequenced from an individual B cell. Three recombinant antibodies were produced in HEK293 cells from Igμ and Igκ sequences cloned from early developing B cells of 564Igi mb1-cre : p8M8, iUM4, and iUM5. The variable regions of these three recombinant antibodies have R mutations, which are shown in Fig. 15A.

A recombinant antibody with an unmutated 564IgH and Igκ variable regions binds RNA. This was measured using ELISA that detected human IgG_1 anti-RNA antibody. Another ELISA was run to determine the concentration of total human IgG_1. This ELISA used a purified human IgG_1 of known concentration to make a standard curve. Dilutions of the HEK293T cell transfection supernatants were diluted and run on the ELISA. Using the standard curve an estimate for the concentration of human IgG_1 in the supernatant was determined. At a concentration around 10 μg/mL, the unmutated recombinant antibody has an OD405 of over 2.0 (Fig. 15B). However, the mutated antibodies tested all has OD405 values at less than 0.5 at similar concentrations (Fig. 15B). These three mutated IgG_1 antibodies do not bind RNA at high concentration.

A



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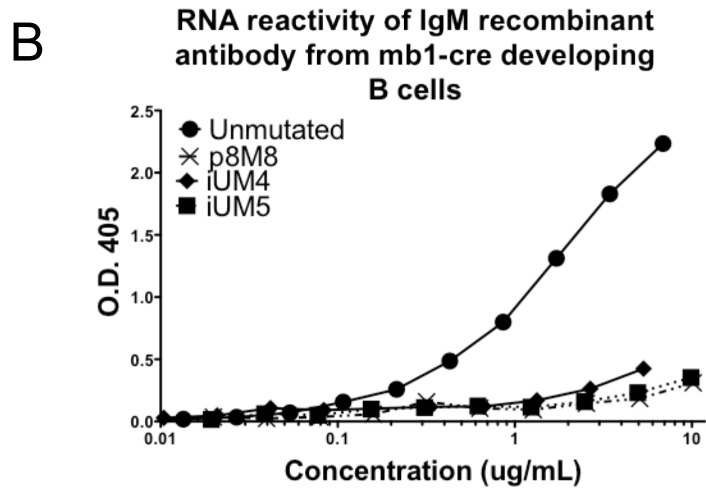

Figure 15. RNA reactivity is lost during B cell development after SHM of 564 Igμ and Igκ genes. A. Amino acid sequences of antibody genes from single cells. Amino acid changes compared to the 564 sequences are indicated with letters. No changes in sequence are indicated with dashes. Vertical double lines indicate spaces in the protein sequence. Complementarity determining regions (CDR) locations are shown above the sequences. **B.** Igμ and Igκ genes were amplified from developing B cells and RNA-reactivity was compared to the unmutated 564 IgH and Ig κ genes by ELISA.

The lack of RNA binding indicates that SHM in the VH and VL regions is responsible for a loss of RNA specificity. This suggests that SHM occurring early in B cell development may contribute to a loss of self-reactivity, and could contribute to the low levels of anti-RNA IgM in the sera of 564Igi mb1-cre compared to that in 564Igi CD21-cre.

cDNA from single B cells was PCR amplified with 564 specific Igμ VH and Igκ VL primers. Depending on the developmental B-cell stage tested, the number of amplified PCR products varied. Igκ genes were readily amplified with 564-Vκ primers in early developing B cells from 564Igi mb1-cre. Over 80% of pre-B cells and immature B cells tested from 564Igi mb1-cre mice had 564 light chain genes that amplified (Fig. 16B). However, 564 Igμ heavy chain genes were amplified in less than 50% of pre B cells and less than 5% of immature B cells from 564Igi mb1-cre mice (Fig. 16A). Assuming that the PCR was effective, this indicated that H-chain knock-in genes in a high percentage of pre-B and immature B cells were edited and cannot be amplified with 564-VH specific primers. RAG-mediated secondary rearrangements of H-chain genes could be responsible for gene alterations. It is possible that the cryptic sites critical for H-chain secondary rearrangements may be more effective in the 564 transgene compared with other rearrangements.

564 Igκ genes from 564Igi CD21-cre immature B cells amplified in 100% of the cells tested (Fig. 16C). Unlike those from 564Igi mb1-cre mice, 564 Igμ genes from 564Igi CD21-cre immature B cells surprisingly amplified at a high rate (80% Fig. 16C). If RAG-mediated secondary rearrangements are responsible for

IgH gene editing in immature B cells in 564Igi mb1-cre, then the rearrangements are occurring at a much lower rate in immature B cells of 564Igi CD21-cre.

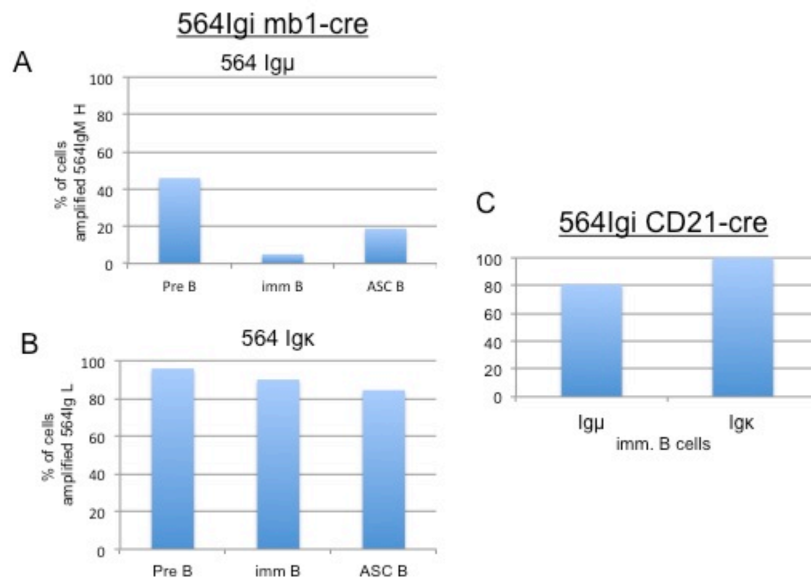

Figure 16. Few B cells express un-edited 564 IgH genes in 564Igi-cre mice. Percentage of single cells expressing 564 IgH and IgL genes in 564Igi mb1-cre and CD21-cre mice. Single bone marrow (BM) pre-B cells ($B220^{low}$ κ^{-}) (n=24), BM immature B cells (imm B, $B220^{low}$ κ^{+}) (n=40) and splenic (SP) antibody secreting cells (ASC B, $CD138^{hi}$) (n=32) were sorted into 96 wells and RNA was reverse transcribed and cDNA amplified with **A.** 564 IgVH forward and Cμ reverse primers or **B.** 564 IgL forward and Cκ reverse primers from 564Igi mb1-cre sorted B cells. **C.** As in **A** and **B** except with sequences from 564Igi CD21-cre B cells. Data in **C** collected by Gabrielle McDonald.

1.3. Results. Class Switch Recombination in autoreactive early developing B cells

CSR in early developing B cells is necessary for the production of pathogenic anti-RNA IgG in 564Igi mice.

Sera from 564Igi mice stain the nucleoli and cytoplasm of HEp-2 cells in an IgG anti nuclear antibody (ANA) assay (Fig. 17A and 17B). This “speckled” pattern of staining indicated the presence of IgG autoantibody that is reactive to RNA or RNA-associated molecules (Berland et al., 2006). Anti-RNA Id+ IgG_{2a} and IgG_{2b} antibody are detected in the sera of 564Igi mice by ELISAs (Fig. 18 and 19A-B).

Serological analysis of the 564Igi-cre mice was used to determine the stages of B-cell development where expression of *Aicda* is necessary for CSR of 564IgH genes and for the production of IgG autoantibody production in 564Igi mice. IgG autoantibodies are detected at high levels in the sera of 564Igi mb1-cre and CD19-cre mice by ANA HEp-2 staining (Fig. 17A and B), anti-RNA ELISA (Fig. 18), and Id^+ ELISA (Fig. 19). *Aicda* is expressed throughout B cell development in both 564Igi mb1-cre and CD19-cre mice; however, it is expressed less efficiently in CD19-cre mice (Fig. 5 and 6). In 564Igi CD21-cre mice, where *Aicda* is only expressed in $CD21^+$ mature B cells, anti-RNA IgG autoantibodies are rarely detected (Fig. 17A, B and 18). Id^+ IgG antibody is detected in the sera of many 564Igi CD21-cre mice (Fig. 19), but in most cases this is in the absence of RNA binding. Therefore, these antibodies retain the epitope detected by the anti-Id antibody but lose auto-reactivity through receptor alterations.

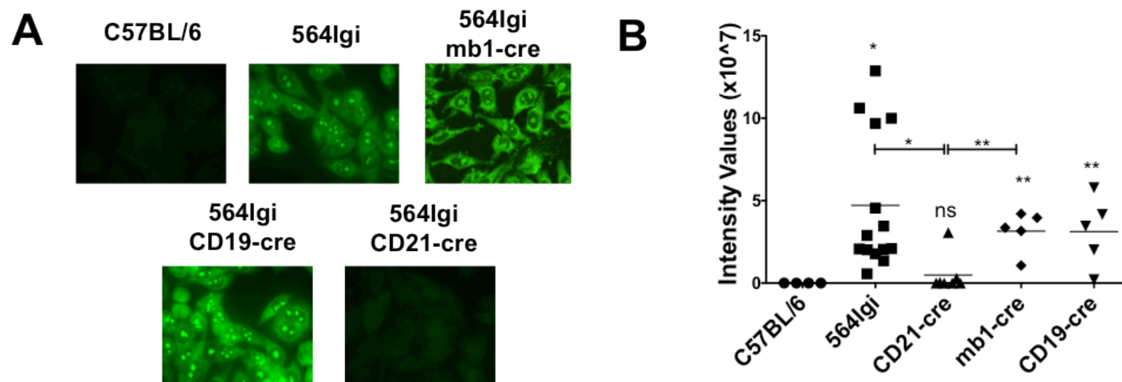

Figure 17. Generation of IgG anti-RNA antibody requires *Aicda* expression at early developing B cells stages. A. IgG anti-nuclear antibody (ANA) stains of Hep-2 cells using sera from the indicated mouse strains. **B.** Quantification of the intensity values from ANA staining in **A**. Asterisks above points indicate significant differences compared to C57BL/6. Statistical analysis is based on a two tailed Student's t-test; significance at p<.05. * p< .05, ** p< .01. Data collected by Ben Umiker and Amma Larbi.

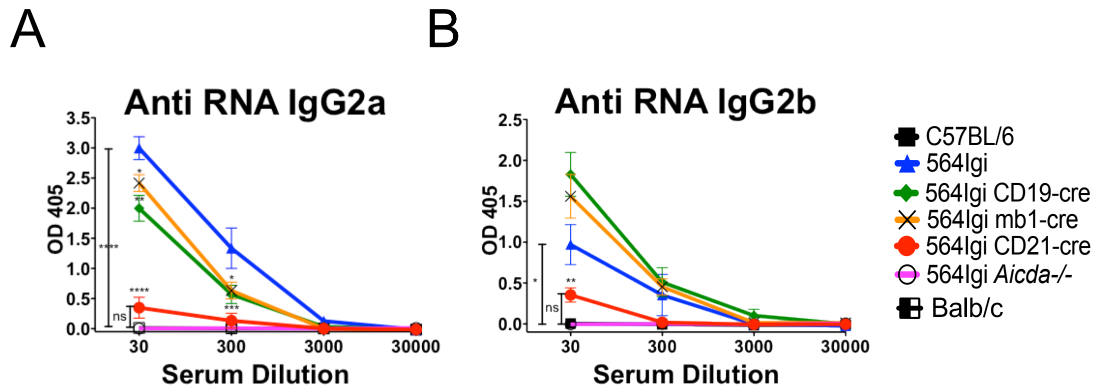

Figure 18. *Aicda* expression exclusively in mature B cell stages is insufficient for the production of IgG anti-RNA autoantibody. Sera tested for anti-RNA **A.** IgG2a and **B.** IgG2b antibodies by ELISAC57BL/6 n=8, 564Igi n=8, 564Igi mb1-cre n=10, 564Igi CD19-cre n=18, 564Igi CD21-cre n=25, 564Igi *Aicda*$^{-/-}$ n=8. Asterisks above points indicate significant differences compared to 564Igi. Statistical analysis is based on a two tailed Student's t-test; significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001. Data collected by Ben Umiker and Amma Larbi.

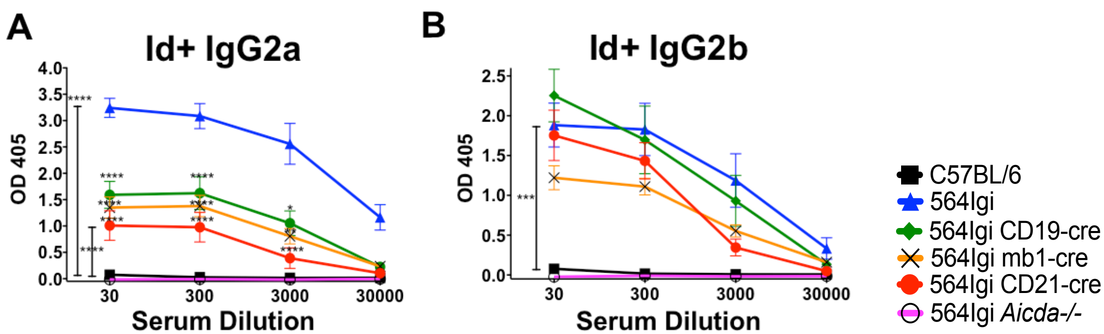

Figure 19. There were 564 Id+ antibodies in some 564Igi CD21-cre sera that were not RNA reactive. Sera tested for Id+ IgG2a **A** and IgG2b **B** antibodies by ELISA C57BL/6 n=8, 564Igi n=8, 564Igi mb1-cre n=10, 564Igi CD19-cre n=18, 564Igi CD21-cre n=25, 564Igi *Aicda*$^{-/-}$ n=8. Asterisks above points indicate significant differences compared to 564Igi. Statistical analysis is based on a two tailed Student's t-test; significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001. Data collected by Ben Umiker and Amma Larbi.

The sera from 67% of 564Igi CD19-cre mice and 53% 564Igi mb1-cre mice have anti-RNA titers as high as the sera from 564Igi mice. The sera from the remaining 564Igi CD19-cre (43%) and mb1-cre (47%) have anti-RNA titers, but the titers are significantly lower compared with the titers from the sera of 564Igi mice (Fig. 20A-C).

Sera from 564Igi CD21-cre mice rarely have IgG2a or IgG2b anti-RNA autoantibody**.** 14% of the 564Igi CD21-cre mice have detectable IgG serum RNA reactive antibody. Only 3% of 564Igi CD21-cre mice have IgG anti-RNA antibody titers comparable to the sera of 564Igi mice (Fig. 20A-C). Sera from 72% of 564Igi CD21-cre mice have either Id^+ IgG antibodies with no anti-RNA titers or neither Id^+ nor IgG anti-RNA titers (Fig. 20A-C). Therefore, the vast majority of 564Igi CD21-cre mice do not produce IgG anti-RNA autoantibodies. This is in comparison to high levels of anti-RNA IgG titers in the sera from both 564Igi mb1-cre and 564Igi CD19-cre mice. Hybridomas were obtained by fusion of the BM and the spleen cells of 564Igi CD21-cre mice to confirm that their B cells did not produce class switched IgG that binds RNA. Indeed, out of 96 growing hybridomas from the BM and 96 from the spleen, zero produce anti-RNA IgG antibody (Fig. 21).

A high percentage of mature B cells ($CD21^+$) and antibody secreting cells ($CD138^{high}$) in the spleen of 564Igi CD21-cre mice are GFP^- (*Aicda*$^+$) (Fig. 6D). 564Igi CD21-cre mice are fully capable of CSR, as indicated by the production of IgG_{2a}, IgG_{2b}, and IgG_1 antibodies (Fig. 8). However, 564Igi CD21-cre mice do not have auto-reactive IgG in their sera.

A small percentage of hybridomas from the BM and the spleen of 564Igi mb1-cre mice produce IgG anti-RNA antibody (Fig. 21). This demonstrates that a small number of auto-reactive B cells can lead to significant serum anti-RNA IgG antibody titers.

In conclusion, AID has the ability to mediate CSR of pathogenic autoantibody genes in early stages of B-cell development. Not only that but AID expressed in early B-cell development is critical for expression of IgG autoantibody.

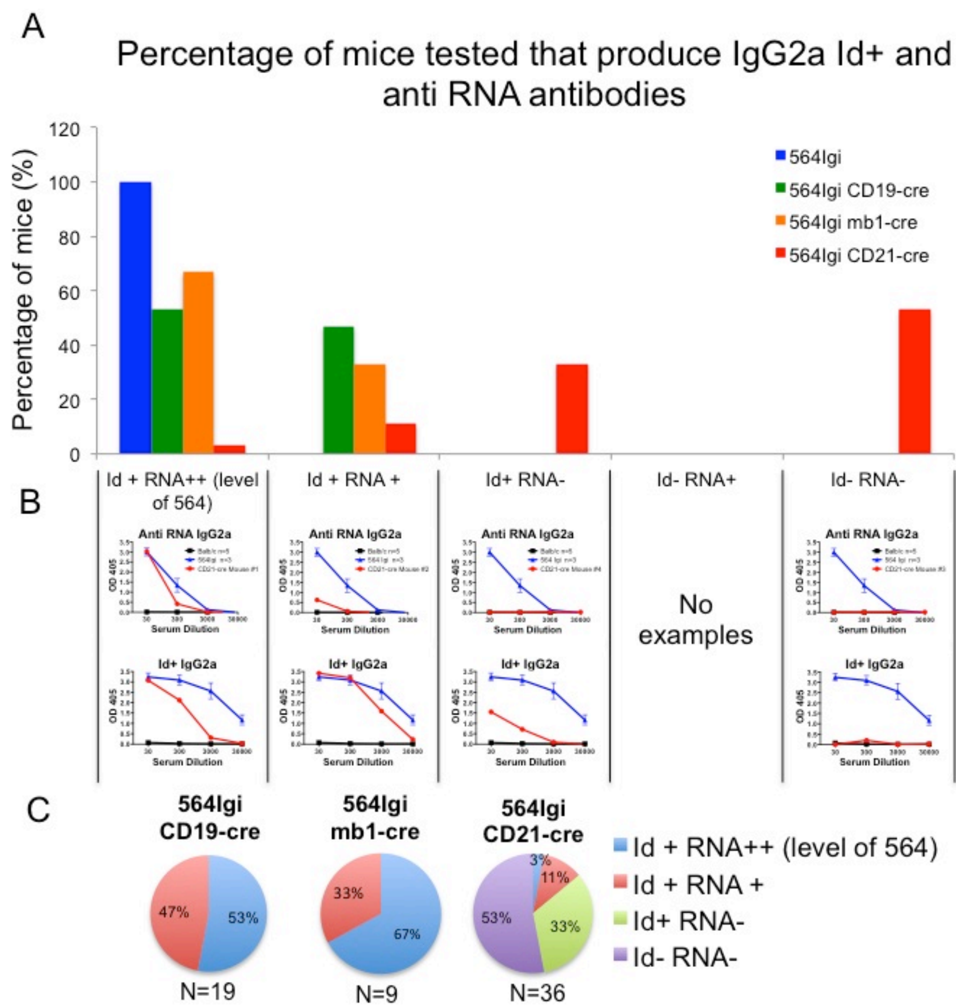

Figure 20. Majority of 564Igi CD21-cre mice have no detectable anti-RNA antibody. A. Percentage of mice from the indicated strain that produce serum IgG2a Id^+ and anti-RNA antibodies. Mice that are Id^+ RNA++ have high levels of anti-RNA antibodies, similar to 564Igi mice, and have Id^+ antibodies. Mice that are Id^+ RNA+ have lower levels of anti-RNA antibodies and are Id^+. Mice that are Id^+ RNA- have no detectable anti-RNA IgG2a antibodies but have Id^+ antibodies. Mice that are Id^- RNA^- have neither anti-RNA antibodies nor Id^+ antibodies. **B.** ELISA analysis of serum IgG2a antibodies from individual examples of 564Igi CD21-cre compared to sera from 564Igi as described in **A**. **C.** Percentage of mice from the indicated strain with specific antibody reactivity. Data collected by Ben Umiker and Amma Larbi.

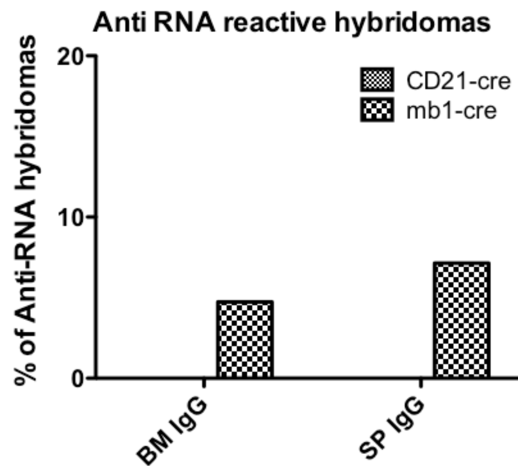

Figure 21. RNA reactive antibody producing B cells in 564igi mb1-cre mice. BM and SP derived hybridomas that are positive for growth were screened for anti-RNA IgG antibody production by ELISA. The percentage of RNA reactive hybridomas per the total is displayed. Data collected by Ben Umiker and Thereza Imanishi-Kari.

CHAPTER 2

TLR7 and TLR8 are critical for SLE-like pathology in 564Igi mice

2.1 Rationale

564Igi have IgG_{2a} and IgG_{2b} autoantibodies in their sera. These autoantibodies bind RNA or RNA associated molecules. Deletion of *Tlr7* in 564Igi significantly reduces autoantibody titers (Berland et al., 2006). The partial dependency on TLR7 for anti-RNA autoantibody production raised the following question: what if any other factors are critical for mediating autoantibody production in 564Igi *Tlr7*$^{-/-}$ mice? TLR9 has been found to mediate auto-reactive B-cell activation and proliferation (Leadbetter et al., 2002). Therefore, TLR9 may be involved in the activation of B cells in 564Igi. TLR8 is an excellent candidate as a mediator of anti-RNA autoantibody production. TLR8 senses ssRNA similarly to TLR7. In 564Igi mice, the BCR of auto-reactive B cells will recognize RNA or RNA associated molecules and deliver the autoantigen to the endosome. It is possible that TLR8 plays a redundant role with TLR7. Both receptors may recognize RNA in the endosome leading to the activation of auto-reactive B cells along with possibly other cell types. It is also possible that a non-TLR factor is critical to produce low levels of autoantibody in 564igi *Tlr7*$^{-/-}$ mice. This chapter will discuss the roles of TLR7, TLR8, and TLR9 in the production of autoantibody in 564Igi mice.

2.2 Results: The expression of either TLR7 or TLR8 is critical for anti-RNA autoantibody production in 564igi

564Igi mice produce IgG antibodies that recognize self-antigens (Berland et al., 2006). IgG_{2a} and IgG_{2b} antibodies in the sera of 564Igi bind RNA detected by ELISA (Fig. 22A and B). 564Igi *Myd88*$^{-/-}$ mice have no circulating RNA reactive IgG (Fig. 22A and B); therefore, the production of class-switched RNA reactive autoantibody is dependent on MyD88 signaling. It is as of yet not clear in which cell types MyD88 expression is critical for IgG autoantibody production.

MyD88 is a critical adaptor protein for most TLR signaling; however, TLR3 and TLR4 can utilize a MyD88-independent TRIF pathway in the induction of IFN-I production (Yamamoto et al., 2003). TLR7, TLR8 and TLR9 signaling is dependent on MyD88 (Janeway and Medzhitov, 2002) (Akira et al., 2001). The IL-1 receptor also utilizes MyD88 leading to the production of *Ifn-*γ (Takeda and Akira, 2004). There are high levels of 564 Id^{+} IgG antibody in 564Igi *Ilr*$^{-/-}$ (Fig. 24). IL-1R signaling therefore does not play a role in 564Igi autoantibody production. And it can be concluded that TLR signaling was critical for the production of class-switched IgG RNA reactive autoantibody in 564Igi.

It was previously shown that 564Igi mice deficient in *Tlr7* do not have high levels of IgG autoantibody in their sera (Berland et al., 2006). Similarly, 564Igi *Tlr7*$^{-/-}$ mice have an intermediate amount of serum anti-RNA IgG_{2a} and IgG_{2b} compared with 564Igi and C57BL/6 mice by anti-RNA ELISA (Fig. 23A and B). This led us to conclude that another MyD88 dependent TLR besides TLR7 may be mediating the production of IgG autoantibody in 564Igi mice.

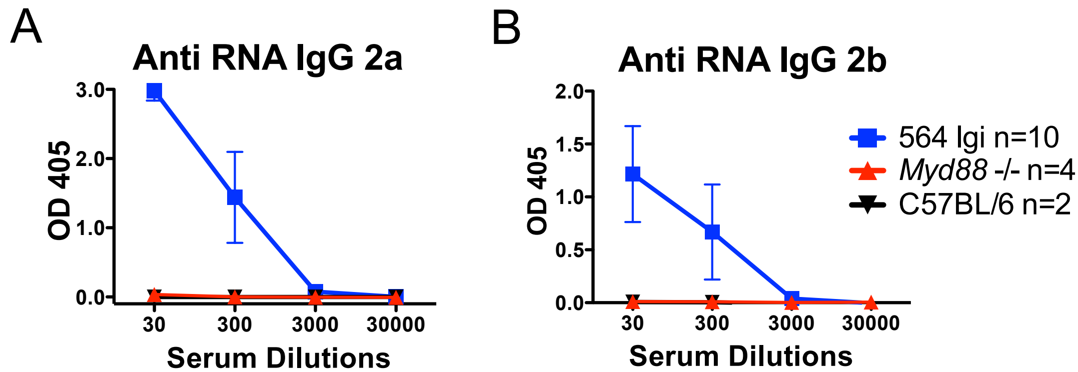

Figure 22. IgG anti-RNA antibody production in 564Igi is MyD88 dependent. Dilutions of sera from 564Igi, 564Igi *Myd88*$^{-/-}$ and C57BL/6 mice were tested for RNA reactivity. Anti-IgG$_{2a}$ **(A)** and IgG$_{2b}$ **(B)** alkaline phosphatase (AP) labeled secondary antibodies were used to detect isotype specificity. Data collected by Ben Umiker and Amma Larbi.

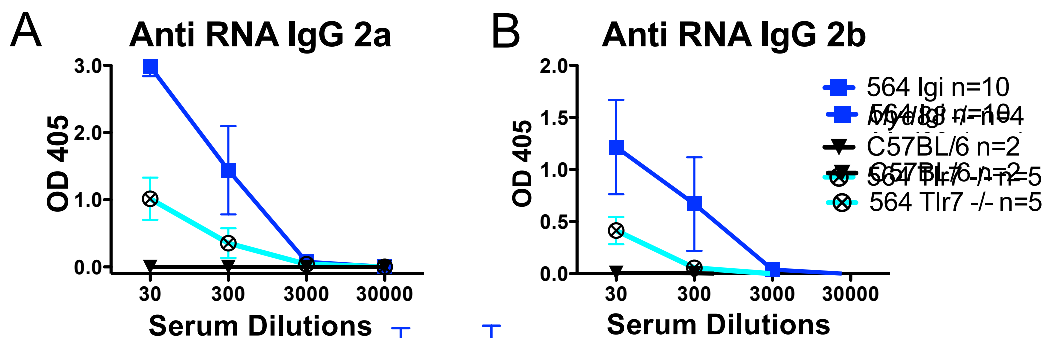

Figure 23. IgG anti-RNA antibody production in 564igi is partially dependent on TLR7. Dilutions of sera from 564Igi, 564Igi *Tlr7*$^{-/-}$ and C57BL/6 mice were tested for RNA reactivity. Anti-IgG$_{2a}$ **(A)** and IgG$_{2b}$ **(B)** alkaline phosphatase (AP) labeled secondary antibodies were used to detect isotype specificity. Data collected by Ben Umiker and Amma Larbi.

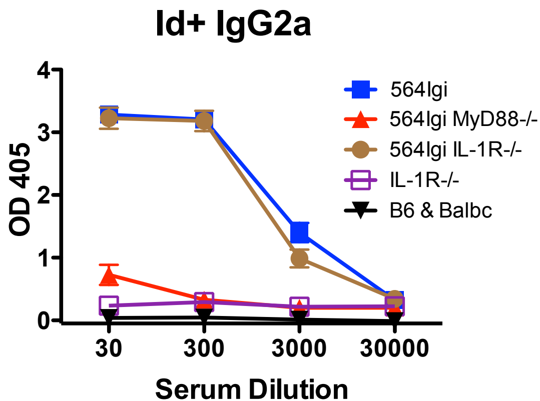

Figure 24: Production of autoantibody in 564Igi is not dependent on IL1-receptor. Serum Id^+ IgG_{2a} antibody titers measured by ELISA. 564Igi n=7, 564Igi *Myd88*$^{-/-}$ n=4, 564Igi *Il1r*$^{-/-}$ n=3, *Il1r*$^{-/-}$ n=3, B6/Balb/c n=5. Performed by Shauna Andersson.

TLR9 has been implicated as a mediator of pathology in SLE (Ehlers et al., 2006; Ng et al., 2005) (Means et al., 2005). On the other hand, mouse models of SLE deficient in *Tlr9* have an increase in disease pathology, suggesting a negative regulatory role for TLR9 (Christensen et al., 2006) (Fukui et al., 2009a; Wu and Peng, 2006). These contradictory studies made it important to try and elucidate the role of TLR9 in 564Igi pathogenesis. Thus, a 564Igi *Tlr9*$^{-/-}$ strain was bred. In 564Igi *Tlr9*$^{-/-}$ mice IgG_{2a} and IgG_{2b} anti-RNA antibody production is similar to 564Igi by ELISA (Fig. 25A and 25B). *Tlr8* deficient 564Igi were also bred. The 564Igi *Tlr8*$^{-/-}$ produce class-switched IgG anti-RNA antibody (Fig. 25C and 25D). Therefore the absence of either *Tlr8* or *Tlr9* has no effect on the serum anti-RNA IgG_{2a} and IgG_{2b} levels in 564Igi.

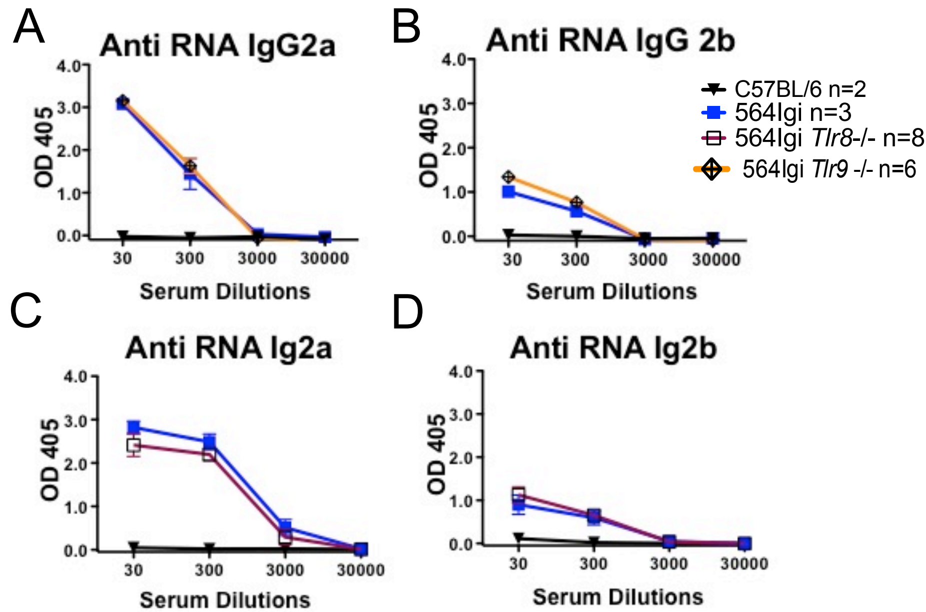

Figure 25. The absence of TLR8 or TLR9 has no effect on IgG anti-RNA antibody production. Dilutions of sera from 564Igi, 564Igi *Tlr9*$^{-/-}$ **(A, B)**, 564Igi and C57BL/6 mice were tested for RNA reactivity. Anti-IgG$_{2a}$ **(A, C)** and IgG$_{2b}$ **(B, D)** alkaline phosphatase (AP) labeled secondary antibodies were used to detect isotype specificity. Data collected by Ben Umiker and Amma Larbi.

In 564Igi *Tlr7/8*$^{-/-}$ there are no detectable anti-RNA IgG$_{2a}$ or IgG$_{2b}$ (Fig. 26A and 26B). However, non-RNA binding IgG$_{2a}$ and IgG$_{2b}$ are found in the sera of 564Igi *Tlr7/8*$^{-/-}$ mice by an ELISA, which detects antibody regardless of specificity (Fig. 27A and 27B). Therefore, the absence of TLR7 and TLR8 do not affect the production of non-self reactive IgG antibody-encoding genes in 564Igi mice.

564Igi *Tlr7*$^{-/-}$ have low levels of anti-RNA IgG autoantibody in their sera; however, the combined deficiency of *Tlr7* and *Tlr8* in 564Igi leads to a complete loss of serum anti-RNA IgG antibody. Therefore, TLR8 can mediate the production of IgG autoantibody in the absence of TLR7. However, the absence of *Tlr8* has no effect on IgG autoantibody production in 564Igi. TLR7 can therefore

compensate completely for the absence of TLR8 in mediating IgG autoantibody production.

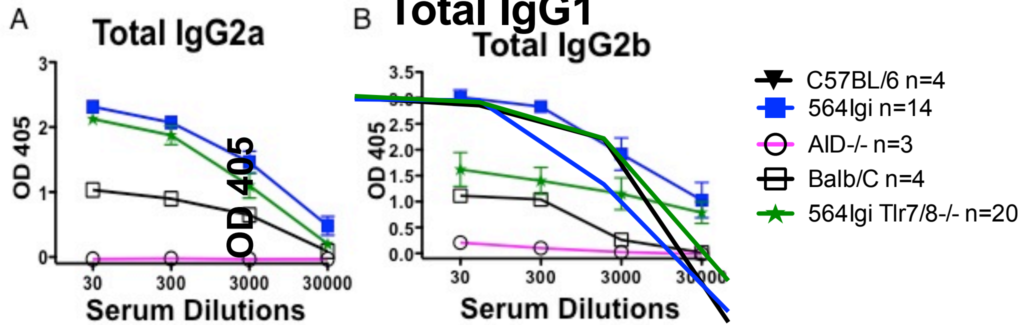

Figure 26. The expression of either TLR7 or TLR8 is critical for IgG anti-RNA antibody production. Dilutions of sera from 564Igi, 564Igi *Tlr7/8*$^{-/-}$ and C57BL/6 mice were tested for RNA reactivity. Anti-IgG$_{2a}$ **(A)** and IgG$_{2b}$ **(B)** alkaline phosphatase (AP) labeled secondary antibodies were used to detect isotype specificity. Data collected by Ben Umiker and Amma Larbi.

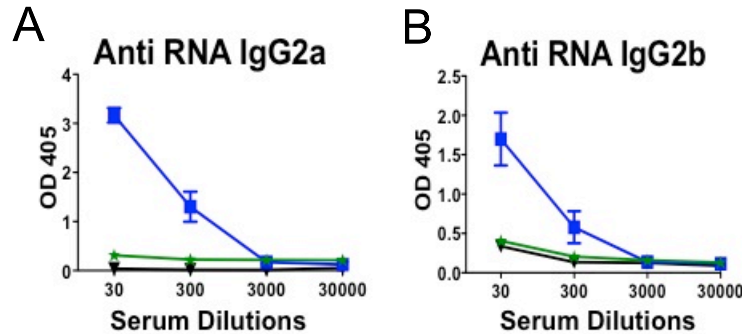

Figure 27. 564Igi *Tlr7/8-/-* produce class switched serum IgG antibody. Total IgG$_{2a}$ and IgG$_{2b}$ detected in the sera of mice by ELISA using anti-IgG$_{2a}$ or anti-IgG$_{2b}$ capture antibodies and detecting binding with AP conjugated anti-IgG$_{2a}$ or anti-IgG$_{2b}$ antibodies. Data collected by Ben Umiker and Amma Larbi.

IgG specific ANA staining of HEp-2 cells by sera from 564Igi has nucleoli-specific “speckled” staining, indicative of anti-RNA IgG antibody. ANA staining confirmed that IgG autoantibody production is dependent on the expression of either TLR7 or TLR8 in 564Igi (Fig. 28). Sera from 564Igi *Tlr9*$^{-/-}$ and *Tlr8*$^{-/-}$ have

similar levels of ANA staining as 564Igi sera. 564Igi *Tlr7*$^{-/-}$ sera also stain HEp-2 cells in the ANA. However, 564Igi *Tlr7/8*$^{-/-}$ sera did not stain HEp-2 cells. Taken together the ANA results confirm the findings from serological analysis by anti-RNA ELISA. The 564Igi *Tlr7/8*$^{-/-}$ mice have the entire portion of the chromosome between the two genes knocked out. There are no known open reading frames between *Tlr7* and *Tlr8.* However, it can not be ruled out that the absence of other genetic elements affect the phenotype of 564Igi *Tlr7/8*$^{-/-}$ mice

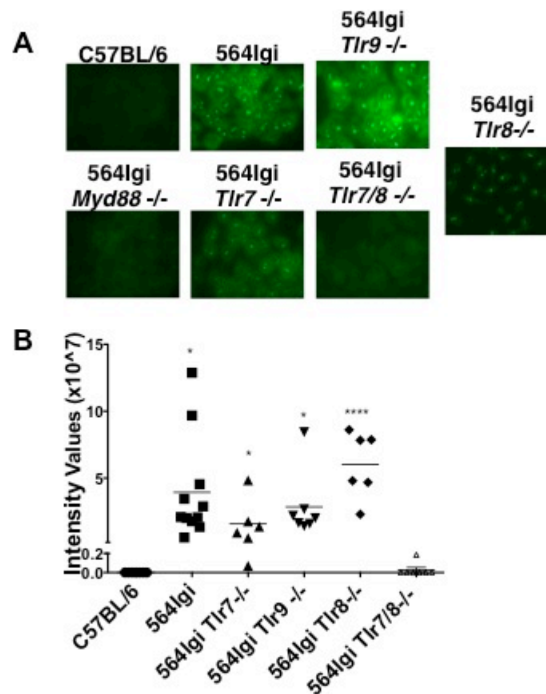

Figure 28. IgG autoantibody production is dependent on the expression of either TLR7 or TLR8. A. IgG ANA staining of HEp-2 cells. Sera were tested from C57BL/6, 564 Igi, 564Igi *Tlr7*$^{-/-}$, 564Igi *Tlr9*$^{-/-}$, 564Igi *Tlr8*$^{-/-}$, 564Igi *Tlr7/8*$^{-/-}$, and 564Igi *MyD88*$^{-/-}$ mice. **B.** Intensity of fluorescent signal from pixels in ANA staining of Hep-2 cells. Significance is compared to C57BL/6. A Student's t-test was run, statistically significance at $p<.05$. * $p< .05$, and **** $p< .0001$. Data collected by Ben Umiker, Amma Larbi, and Shauna Andersson.

We then investigated the expression profiles of endosomal TLRs in B cells in 564Igi. B cells from 564Igi mice have higher levels of *Tlr7* and *Tlr8* transcripts than B cells from C57BL/6 mice (Fig. 29A). *Tlr9* is not up-regulated in 564Igi B cells (Fig. 29A). Western Blots of cell lysates from B cells show an increase in the protein levels of TLR7 and TLR8 (Fig. 29B and C).

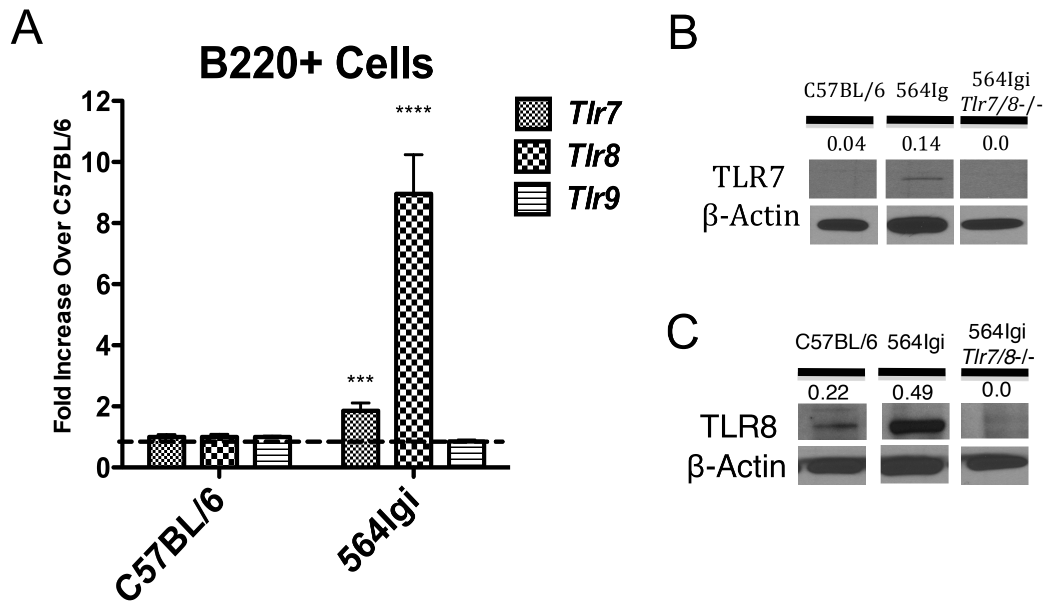

Figure 29. TLR7 and TLR8 are up-regulated in B cells from 564Igi compared to B cells from C57BL/6. A. Relative mRNA transcript levels of *Tlr7, Tlr8* and *Tlr9* to *β-actin* in BM B220^{+} B cells were measured by qPCR. 564Igi n=3, C57Bl/6 n=3. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. A Student's t-test was run, statistically significance at p<.05. *** p< .001 and **** p< .0001. **B.** Western blot for TLR7 from B220^{+} B cells, TLR7/β-ACTIN densitometry ratios included. **C.** Western blot for TLR8 from B220^{+} B cells, TLR8/β-ACTIN densitometry ratios included. Bands shown for TLR7 and TLR8 run between the molecular weights of 64 and 97. Actin runs between 39K and 51K MD. Western Blots performed by Ben Umiker and Luis Fernandez.

2.4 The expression of either TLR7 or TLR8 is critical for an increase in the number of neutrophils in the BM of 564Igi.

The BM of C57BL/6, 564Igi and all 564Igi *Tlr*-deficient mice have similar numbers of mono-nucleated cells (Fig. 3). Therefore, the percentage of total mono-nucleated cells for a particular cell type reflects the absolute number of that cell type in the BM. The percentage of neutrophils amongst live (PI^-) mono-nucleated cells in the BM was measured by Flouresence-activated cell sorting (FACs). Neutrophils are defined as $CD11b^+$ $Ly6C^{med}$ by FACs (Zhu et al., 2007).

The percentage of neutrophils in the BM of 564Igi is higher compared to C57BL/6 (Han et al., 2013) (Fig. 30). The increase of neutrophils in the BM is MyD88 dependent and therefore dependent on TLR or IL1R signaling (Fig. 30B). In 564Igi *Tlr7/8*$^{-/-}$ there are similarly low levels of neutrophils in the BM as C57BL/6 (Fig. 30B). The increase in neutrophils may be due to an increase in granulopoiesis. One mechanism by which granulopoiesis has been shown to be increased is through the cytokine GM-CSF (Panopoulos and Watowich, 2008) (Kimura et al., 2009). Interestingly, BM B cells in 564Igi have increased GM-CSF mRNA levels compared to C57BL/6 B cells (Fig. 30C).

There are circulating IgG autoantibodies in 564Igi *Tlr7*$^{-/-}$, *Tlr8*$^{-/-}$, *Tlr9*$^{-/-}$ (Fig. 23 and 25). Therefore, we used these strains to test the role of individual TLRs in mediating an increase of BM neutrophils in the presence of IgG autoantibody in the circulation. The 564Igi *Tlr9*$^{-/-}$ mice have an increase of BM neutrophils, which is significantly higher than in 564Igi mice (Fig. 31). Similarly, high levels of BM neutrophils are found in 564Igi *Tlr7*$^{-/-}$ mice (Fig. 31). 564igi *Tlr8*$^{-/-}$ mice, on the other hand, have significantly fewer BM neutrophils than 564Igi. 564igi *Tlr8*$^{-/-}$

mice have more BM neutrophils than C57BL/6 mice (Fig. 31). Therefore, an increase of neutrophils in the BM is partially dependent on TLR8 in the presence of circulating autoantibody.

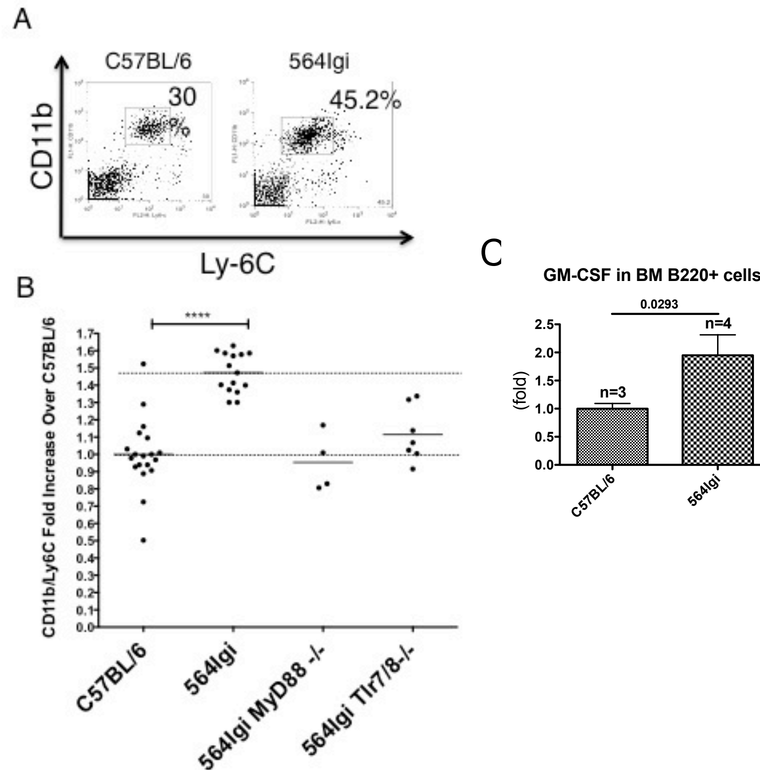

Figure 30. An increase in the number of BM neutrophils is dependent on TLR signaling. A. Flow cytometric profile of neutrophils (CD11b hi Ly-$6C^{med}$ (Zhu et al., 2007) in the BM of C57BL/6 and 564Igi. **B.** The percentage of viable (PI^{-}) $CD11b^{+}$Ly-$6C^{med}$ cells from the BM was determined by flow cytometry. The fold increases of neutrophils over C57Bl/6 BM neutrophils in each experiment the percentage of BM neutrophils in C57BL/6 BM was determined and set as 1.0. Each symbol represents the fold increase over C57BL/6 BM cells from one mouse. A Student's t-test was run, statistically significance at p<.05. **** p< .0001. Data collected by Ben Umiker, Shauna Andersson, and Jin Hwan-Han. **C.** GM-CSF mRNA transcript levels as measured by qPCR

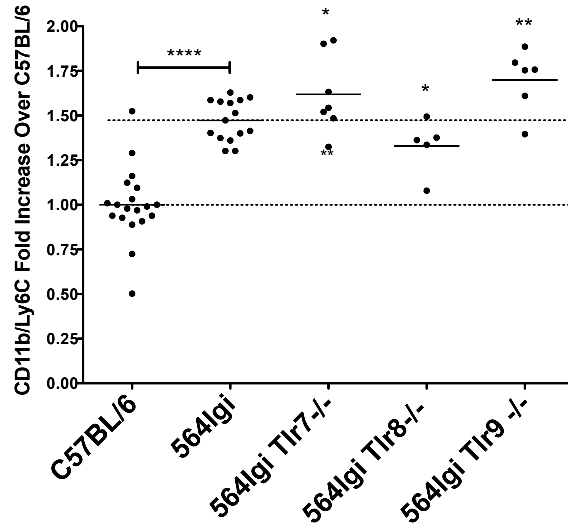

Figure 31. Increased number of neutrophils in 564Igi is partially dependent on TLR8. Flow Cytometry was performed as described in Fig. 30 for 564Igi *Tlr7*$^{-/-}$, 564Igi *Tlr8*$^{-/-}$, and 564Igi *Tlr9*$^{-/-}$ mice. A Student's t-test was run, statistically significance at p<.05. * p< .05, ** p< .01, and **** p< .0001. Significances above data points indicate differences compared to 564Igi. Data collected by Ben Umiker, Shauna Andersson, and Jin Hwan-Han.

2.4. Female 564Igi *Tlr7/9*$^{-/-}$ mice, but not male mice have SLE-like disease.

Tlr7/9 double knockout mice were crossed to 564Igi mice. Female 564Igi mice lacking *Tlr7* and *Tlr9* produce IgG anti-RNA antibodies detected through ANA staining and ELISA (Fig. 32 and 33). On the other hand, male 564Igi *Tlr7/9*$^{-/-}$ mice have no serum anti-RNA IgG (Fig. 32 and 33). A difference between males and females in autoantibody production is not observed in other strain tested besides 564Igi *Tlr7/9*$^{-/-}$. Figure 34 demonstrates that there is no difference between male and female for 564Igi and 564Igi *Tlr7*$^{-/-}$ in serum anti RNA $IgG_{2a.}$

Female 564Igi *Tlr7/9*$^{-/-}$ has IgG autoantibody, while female 564Igi *Tlr7/8*$^{-/-}$ has no IgG autoantibody. Therefore, TLR8 is sufficient to mediate autoantibody production in female 564Igi *Tlr7/9*$^{-/-}$. However, TLR8 in male 564Igi *Tlr7/9*$^{-/-}$ is insufficient for autoantibody production.

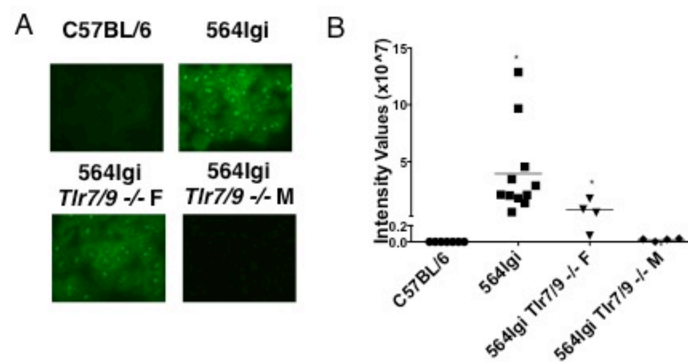

Figure 32. In female mice TLR8 is sufficient for IgG autoantibody production. A. IgG ANA staining of HEp-2 cells. Sera were tested from C57BL/6, 564 Igi, 564Igi *Tlr7/9*$^{-/-}$ Female and male mice. **B.** Intensity of fluorescent signal from pixels in ANA staining of Hep-2 cells. Significance is compared to C57BL/6. A Student's t-test was run, statistically significance at p<.05. * p< .05. Data collected by Ben Umiker, Shauna Andersson, and Amma Larbi.

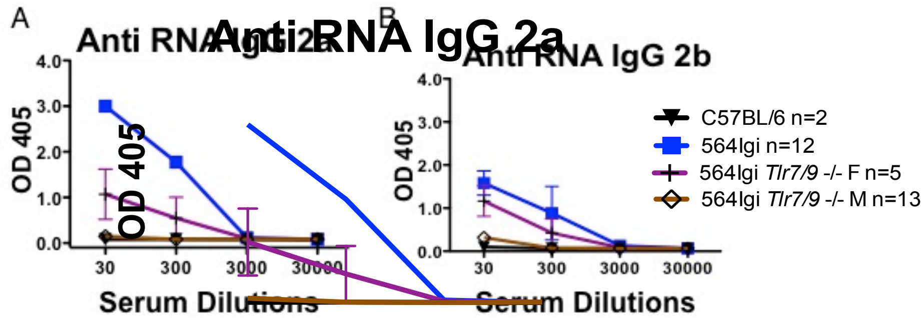

Figure 33. TLR8 mediates anti-RNA IgG antibody production in female 564Igi *Tlr7/9*$^{-/-}$ mice. Dilutions of sera from 564Igi, 564Igi *Tlr7/9*$^{-/-}$ male, 564Igi *Tlr7/9*$^{-/-}$ female and C57BL/6 mice were tested for RNA reactivity. Anti-IgG_{2a} **(A)** and IgG_{2b} **(B)** alkaline phosphatase (AP) labeled secondary antibodies were used to detect isotype specificity. Data collected by Ben Umiker and Amma Larbi.

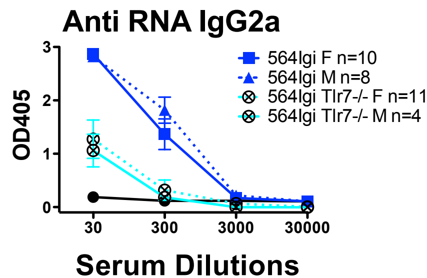

Figure 34. No difference in IgG_{2a} autoantibody production between male and female 564Igi. Dilutions of sera from 564Igi male and female, and 564Igi *Tlr7*$^{-/-}$ male and female mice were tested for RNA reactive antibody. Anti-IgG_{2a} alkaline phosphatase (AP) labeled secondary antibodies was used to detect isotype specificity.

In female 564Igi *Tlr7/9*$^{-/-}$ mice, there is an increase in BM neutrophils compared to 564Igi mice (Fig. 35). Male 564Igi *Tlr7/9*$^{-/-}$ mice have fewer BM neutrophils than females and 564igi, but slightly more than C57BL/6 mice (Fig. 36). This demonstrated that in females TLR8 is sufficient for a MyD88 dependent increase in BM neutrophils, but not in males.

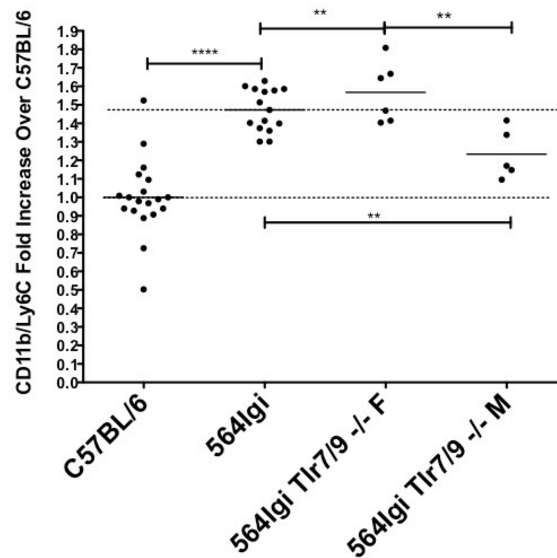

Figure 35. Increased numbers of neutrophils are observed in the BM of female, but not male 564Igi *Tlr7/9*$^{-/-}$ mice. Flow cytometry analysis was performed as described in Fig. 30 for 564Igi *Tlr7/9*$^{-/-}$ female and 564Igi *Tlr7/9*$^{-/-}$ male. Data collected by Ben Umiker, Shauna Anderson, Jin Hwan-Han, and Parimal Korgoankor.

In BM B cells from female 564Igi *Tlr7/9*$^{-/-}$ mice, *Tlr8* is highly up-regulated (Fig. 36) Transcript levels of *Tlr8* in male 564Igi *Tlr7/9*$^{-/-}$ B cells are much lower than in females (Fig. 36). This difference in *Tlr8* mRNA levels in B cells may be responsible for the difference in autoantibody observed between male and female 564Igi *Tlr7/9*$^{-/-}$ mice. Furthermore, it suggests that auto-reactive B cells in

the absence of TLR7 or TLR9 would need a large increase in TLR8 expression in order to produce autoantibody. In B cells from 564Igi *Tlr7*$^{-/-}$ mice, there is a modest increase in *Tlr8* transcript levels. There is also only modest levels of IgG autoantibody in the sera.

On the other hand, in BM B cells from 564Igi *Tlr8*$^{-/-}$ there are low levels of *Tlr7* transcripts (Fig. 36) and 564Igi *Tlr8*$^{-/-}$ produce high levels of IgG anti-RNA antibody. Presumably, this autoantibody production is mediated by TLR7. However, since *Tlr7* transcripts are not up regulated, these results suggest that in the absence of TLR8high levels of TLR7 are not required for auto-reactive B cells to produce IgG autoantibody.

In BM B cells from 564Igi *Tlr9*$^{-/-}$ mice, there are low levels of *Tlr8* and *Tlr7* transcripts compared with 564Igi. However, the 564Igi *Tlr9*$^{-/-}$ mice have high levels of serum auto-reactive IgG. This demonstrates that in the absence of TLR9 low levels of *Tlr7* and *Tlr8* can be sufficient for autoantibody production in 564Igi. The correlations between *Tlr* transcript levels and autoantibody production are summarized in Table 1 (pg. 107).

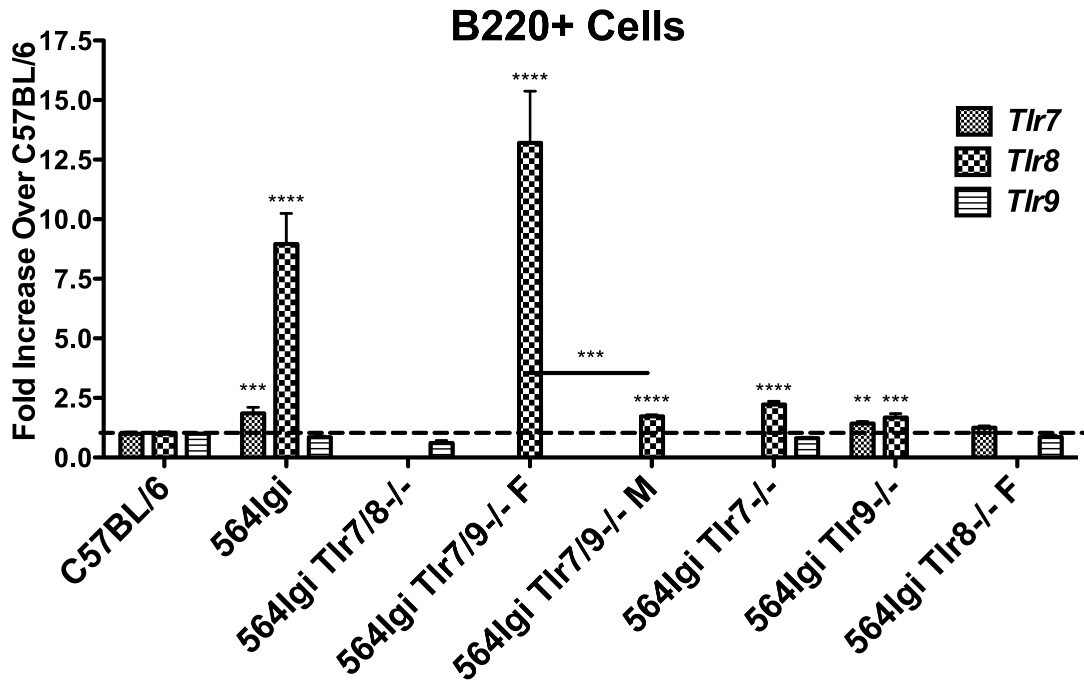

Figure 36. B-cell expression of *Tlr7*, *Tlr8* and *Tlr9*. Relative mRNA transcript levels of *Tlr7, Tlr8* and *Tlr9* to *β-actin* in BM B220$^+$ B cells were measured by qPCR. n=3 for all mice strains. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. A Student's t-test was run, statistically significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001. Unless otherwise indicated significance is compared to transcript level of C57BL/6.

2.5. The phenotypic difference between the sexes in 564Igi *Tlr7/9*$^{-/-}$ was due to the dosage of the *Tlr8* gene.

The *Tlr7* and *Tlr8* genes are located on the X-chromosome. Therefore males have one copy of each gene, while females have two. The difference between male and female 564Igi *Tlr7/9-/-* in autoantibody production and number of BM neutrophils could be due to the hormonal differences between females and males. Alternatively, these observations could be caused by the difference in *Tlr8* copy number in males and females. 564Igi female mice with a single copy of the *Tlr8* gene on a *Tlr7/9* deficient background were bred to distinguish between these two possibilities. The breeding strategy for the development of this mouse strain is in Figure. 37.

Female 564Igi *Tlr7/9*$^{-/-}$ mice with a single copy of *Tlr8* have no detectable IgG anti-RNA by ELISA (Fig. 38A) or by ANA (Fig. 38B) similar to that found in male 564Igi *Tlr7/9*$^{-/-}$ mice, which also only have one copy of *Tlr8* (Fig. 33). The B cells from female 564Igi *Tlr7/9*$^{-/-}$ mice with a single copy of *Tlr8* have lower transcript levels of *Tlr8* than female 564Igi *Tlr7/9*$^{-/-}$ B cells (Fig. 39). B cells from female 564Igi *Tlr7/9*$^{-/-}$ mice with a single copy of *Tlr8* have higher transcript levels of *Tlr8* than male 564Igi *Tlr7/9*$^{-/-}$ (Fig. 39). Similarly, females 564Igi *Tlr7/9*$^{-/-}$ with the single copy of *Tlr8* have lower percentages of BM neutrophils similar to that found in male 564Igi *Tlr7/9*$^{-/-}$, but not to that found in females 564Igi *Tlr7/9*$^{-/-}$ (Fig 40). These results indicate that the dosage of the *Tlr8* gene determines the gender difference observed in 564Igi *Tlr7/9*$^{-/-}$.

There is an increased expression of TLR8 on B cells in females compared with male 564Igi *Tlr7/9*$^{-/-}$ mice (Fig. 36). This increase of *Tlr8* transcription could

lead to a lower activation threshold for auto-reactive B cells when stimulated by RNA/antibody ICs or RNA associated molecules. The resulting activation would lead to autoantibody production in female, but not male 564Igi *Tlr7/9*$^{-/-}$ mice.

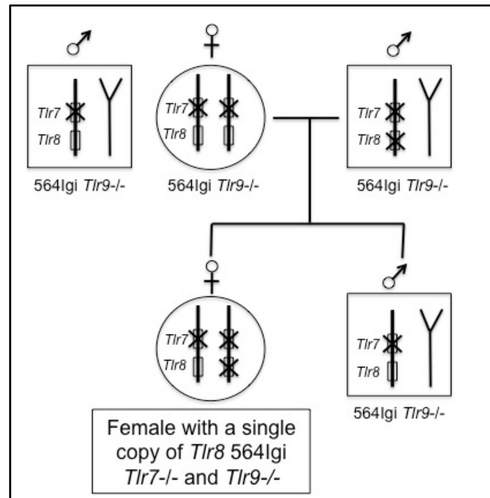

Figure 37. Breeding strategy for 564Igi *Tlr7/9*$^{-/-}$ *Tlr8*$^{+/-}$ mice. Female 564Igi *Tlr7/9*$^{-/-}$ were crossed with male 564Igi *Tlr7/8/9*$^{-/-}$. Resulting 564Igi females had a single copy of *Tlr8* and were *Tlr7/9*$^{-/-}$.

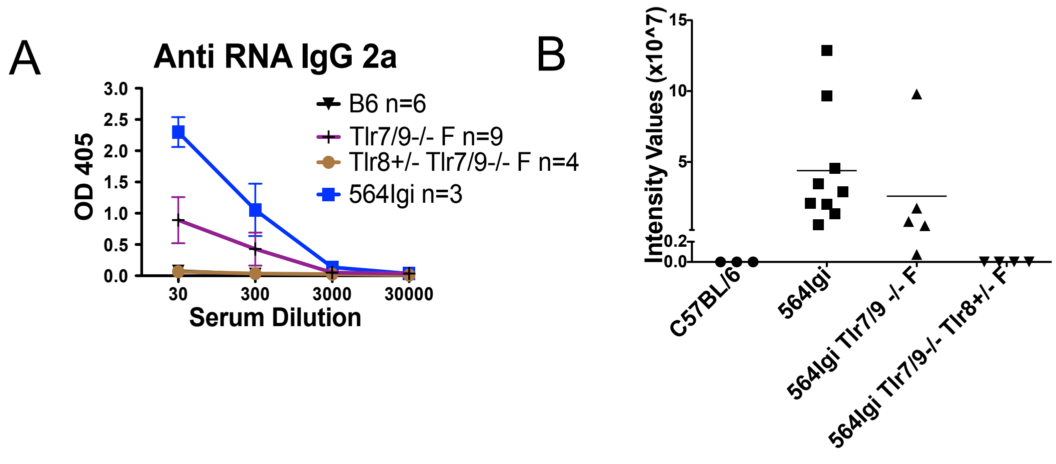

Figure 38. A single copy of the *Tlr8* gene in female 564Igi *Tlr7/9*$^{-/-}$ is insufficient for the production of anti-RNA IgG antibody. A. Dilutions of sera from 564Igi, 564Igi, 564Igi *Tlr7/9*$^{-/-}$ female, 564Igi *Tlr7/9*$^{-/-}$ *Tlr8*$^{+/-}$ female and C57BL/6 mice were tested for RNA reactivity. Anti-IgG_{2a} alkaline phosphatase (AP) labeled secondary antibodies was used to detect isotype specificity. **B.** IgG ANA staining of HEp-2 cells. The Intensity of fluorescent signal from pixels in ANA staining of Hep-2 cells was measured.

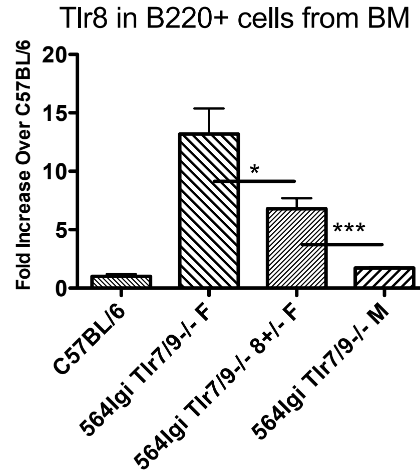

Figure 39. *Tlr8* mRNA levels are less in male than in female mice with only one copy of the *Tlr8* gene. Relative mRNA transcript levels of *Tlr8* to *β-actin* in BM B220$^+$ B cells were measured by qPCR. n=3 for all mice strains. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. A Student's t-test was run, statistically significance at p<.05. * p< .05, ** p< .01, *** p< .001 and **** p< .0001.

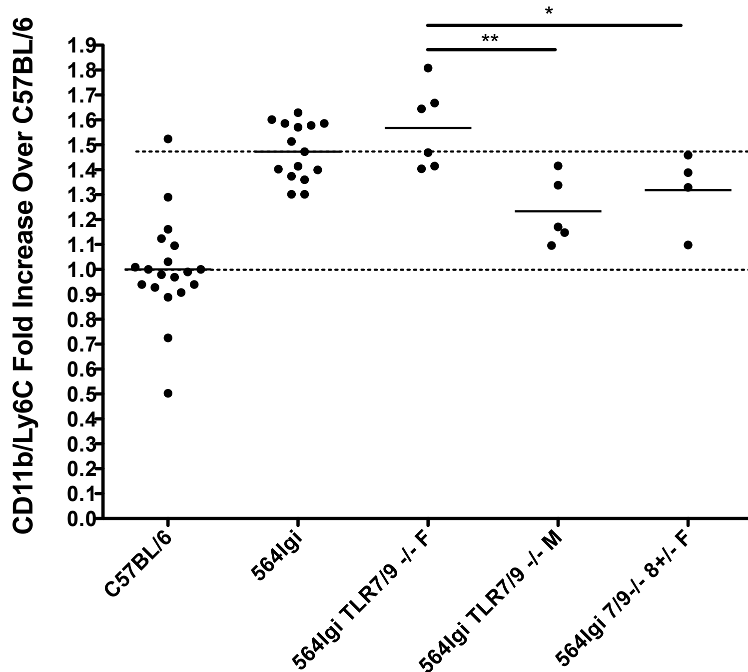

Figure 40. A single copy of the *Tlr8* gene in female 564Igi *Tlr7/9*$^{-/-}$ is insufficient for an increase in the number of BM neutrophils. Flow cytometry analysis was performed as described in Fig. 30 for 564Igi *Tlr7/9*$^{-/-}$ female and 564Igi *Tlr7/9*$^{-/-}$ male and *Tlr7/9*$^{-/-}$ *Tlr8*$^{+/-}$ female mice. A Student's t-test was run, statistically significance at p<.05. * p< .05, ** p< .01

Table 1: *Tlr* mRNA levels in B cells from various 564Igi *Tlr* knockout mice

B cells	C57BL/6	564Igi	564Igi Tlr7$^{-/-}$	564Igi Tlr8$^{-/-}$	564Igi Tlr9$^{-/-}$	564Igi Tlr7/9$^{-/-}$		564Igi Tlr7/8$^{-/-}$
	Female	Female	Female	Female	Female	Female	Male	Female
Anti-RNA IgG antibody	-	+++	+	+++	+++	+	-	-
Tlr7 transcripts in B cells	+	+++	-	+	++	-	-	-
Tlr8 transcripts in B cells	+	+++	++	-	++	++++	++	-
Tlr9 transcripts in B cells	++	+	+	+	-	-	nd	+

- No antibody or detectable mRNA transcript
+ Low levels of antibody or mRNA transcript
++ Moderate level of antibody or mRNA transcript
+++ High level of antibody or mRNA transcript
++++ Very high level of antibody or mRNA transcript

CHAPTER 3

TLR7, TLR8, TLR9 expression regulates IFN-I production by neutrophils

3.1 Rationale

Type-I interferon (IFN-I), especially IFN-α, is a central mediator of pathology in SLE (Crow, 2014). Genome-wide association studies strongly indicate that IFN-I response genes are important SLE risk factors (Bronson et al., 2012b). SLE patients have increased circulating IFN-I. (Hooks et al., 1979) (Bengtsson et al., 2000). In some cases, treatment with recombinant IFN-I in cancer patients led to the development of SLE symptoms (Ronnblom et al., 1991) These findings suggest that increased IFN-I may be a main factor in the etiopathology of disease. For years, research into SLE has focused on the adaptive autoimmune response, in particular the production of class-switched autoantibodies. However, it is recognized that autoantibodies are not sufficient for pathology. The innate immune response leading to IFN-I production is important for the induction of much of the pathology induced in SLE patients (Crow and Kirou, 2004) (Ronnblom et al., 2011). The cellular source of IFN-I in lupus has been long thought to be plasmacytoid dendritic cells (pDCs). There is strong evidence that pDCs produce IFN-I that stimulates adjacent cells through the IFN-alpha receptor (Farkas et al., 2001). However, the small number of pDCs in humans make it hard to imagine how they could produce all the IFN-I found circulating in SLE patients (Hooks et al., 1979) (Bengtsson et al., 2000). There has been a recent surge in the study of neutrophils as producers of IFN- I (Denny et al., 2010; Kaplan, 2013; Lindau et al., 2013), especially from neutrophils in the

BM (Palanichamy et al., 2014). Since an increase in BM neutrophils was observed in 564Igi (Chapter 2), it is possible that they are significant producers of IFN-I. This chapter will investigate the role of specific TLRs in the production of IFN-I by neutrophils in 564Igi.

Results 3.2. Circulating IgG immune complexes in 564Igi.

The formation of immune complexes (ICs) is important for SLE pathology. ICs can be comprised of autoantibody and auto-antigen. They are deposited in the kidney of SLE patients (Trouw et al., 2004) and can contribute to renal pathology.

The complement pathway component C1q can efficiently bind ICs, but is unable to bind either the anti-nuclear antibody or the nucleic acid antigen alone (Lambert et al., 1974). An ELISA was performed that detected ICs with purified C1q. A purified IgG_{2a} anti-RNA antibody named S7.1 did not bind C1q (Fig. 41). RNA isolated from the BM of a C57BL/6 mouse also did not bind C1q by ELISA (Fig. 41). S7.1 and BM RNA were incubated together for 1 hour at 37°C in order to form ICs. These RNA/IgG_{2a} ICs did bind C1q and are detected by C1q ELISA (Fig. 41). There are no IgG_{2a} ICs detected in C57BL/6 sera. However, IgG_{2a} IC are circulating in the sera of 564Igi mice (Fig. 41).

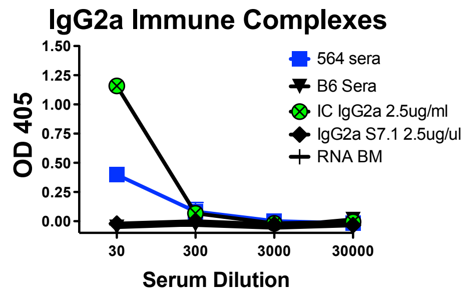

Figure 41. IgG_{2a} ICs circulate in the sera of 564igi mice. Immune complexes containing IgG_{2a} antibody detected in the sera of mice by ELISA with C1q as a capture molecule. Sera from 564igi mice n=3, sera from C57Bl/6 n=3. ICs were formed with IgG_{2a} (clone #S7.1) purified anti-RNA antibody and mouse BM RNA. IgG_{2a} S7.1 and RNA from the BM were also run on the ELISA.

ICs have been shown to induce IFN-I responses. Specifically, pDCs can endocytose ICs via FcγRs, thereby inducing TLR7- and TLR9-mediated IFN-I production (Lovgren et al., 2004) (Martin and Elkon, 2006). We have proposed that ICs activate neutrophils and monocytes to produce IFN-I through similar mechanisms as pDCs. There is strong evidence that neutrophils and monocytes play an important role in the pathology of 564Igi mice, especially as producers of IFN-I (Han et al., 2013). Neutrophils in 564Igi express high levels of TLR8 and high levels of activating FcγRIV (Han et al., 2013). Neutrophils, through the expression of TLR8 and FcγRIV, have the potential to recognize ICs composed of RNA and anti-RNA antibody. In this chapter, the role of individual TLRs on IFN-I expression by neutrophils will be examined.

Results 3.3. TLR9 negatively regulated IFN-I production by neutrophils *in vivo* and *in vitro*.

The increase of neutrophils in the BM of 564Igi mice is dependent on TLR expression (Fig. 30). An increase in TLR expression by neutrophils could contribute to their activation by ICs. Therefore, the expression of *Tlr7, Tlr8,* and *Tlr9* were investigated in 564Igi neutrophils. Levels of *Tlr7*, *Tlr8* and *Tlr9* mRNA transcripts are higher in neutrophils from the BM of 564Igi mice compared with those from C57BL/6 (Fig. 42A). Western blots reveal that neutrophils have more TLR7 in 564Igi compared with C57BL/6 neutrophils at a protein level (Fig. 42B). TLR8 is expressed at similar levels in neutrophils in both 564Igi and C57BL/6 mice (Fig. 42B).

The next question was whether the increase in TLR expression in 564Igi neutrophils leads to an increase of IFN-I production. 564Igi and C57BL/6 neutrophils have similar transcript levels of *Ifn-α6* and *Ifn-β1* (Fig. 43). It has been shown that neutrophils and monocytes are the main producers of IFN-I in 564Igi (Han et al., 2013). There are more neutrophils in the BM, spleen, and blood of 564Igi compared with C57BL/6. This increased number of neutrophils could be responsible for an increase in IFN-I production by neutrophils, even without an increase in *Ifn-I* mRNA transcripts on a per cell basis in neutrophils.

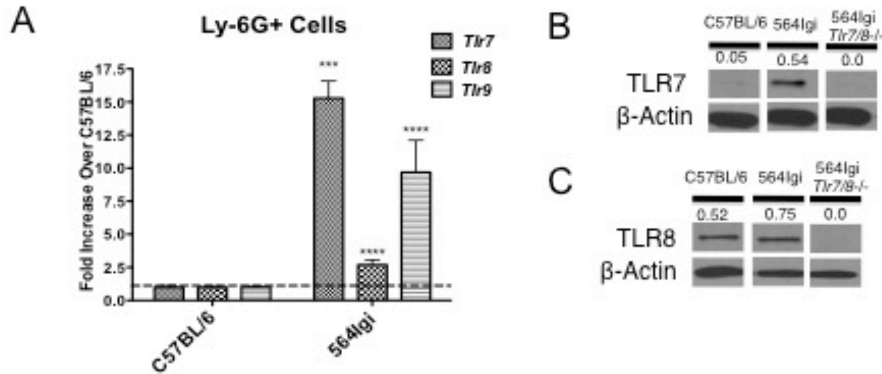

Figure 42. Higher mRNA transcript levels of *Tlr7, 8,* and *9* in 564Igi neutrophils compared with C57BL/6. mRNA transcript levels of *Tlr7, Tlr8,* and *Tlr9* relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G+ cells (neutrophils). 3 mice tested for each strain. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. *** p< .001 and **** p< .0001. **B,C** Band shown for TLR7 and TLR8 runs between the molecular weights of 64000 and 97. Actin runs between 39K and 51K. Western Blots were performed with the help of Luis Fernandez.

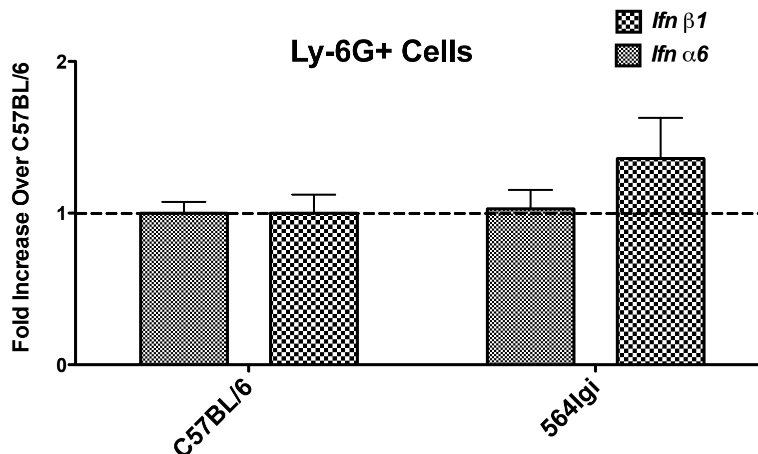

Figure 43. No significant increase in *Ifn-I* transcript levels in 564Igi neutrophils compared to C57BL/6. mRNA transcript levels of *Ifn-β1,* and *Ifn-α6* relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G+ cells (neutrophils). 3 mice tested for each strain. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. Arithmetic means +/- SEM.

564Igi *Tlr9*$^{-/-}$ mice had increased numbers of BM neutrophils (Fig. 31) and produce IgG autoantibody (Fig. 25A and B). Furthermore, 564Igi *Tlr9*$^{-/-}$ neutrophils had an increase in *Ifn-I* mRNA transcripts compared with 564Igi or C57BL/6 (Fig. 44). There is, however, no increase in either *Tlr7* or *Tlr8* transcripts in the 564Igi *Tlr9*$^{-/-}$ neutrophils (Fig. 46). Together this demonstrates that *Ifn-I* is up regulated in 564Igi *Tlr9*$^{-/-}$ neutrophils without an increase of either *Tlr7* or *Tlr8*.

Tlr9 is highly up regulated in 564Igi neutrophils. However, on a per cell basis, neutrophils in 564Igi do not have increased *Ifn-I* transcripts. Knocking out *Tlr9* increases *Ifn-I* mRNA transcripts significantly. Therefore, the high expression of TLR9 in 564Igi neutrophils leads to the suppression of the neutrophil IFN-I response.

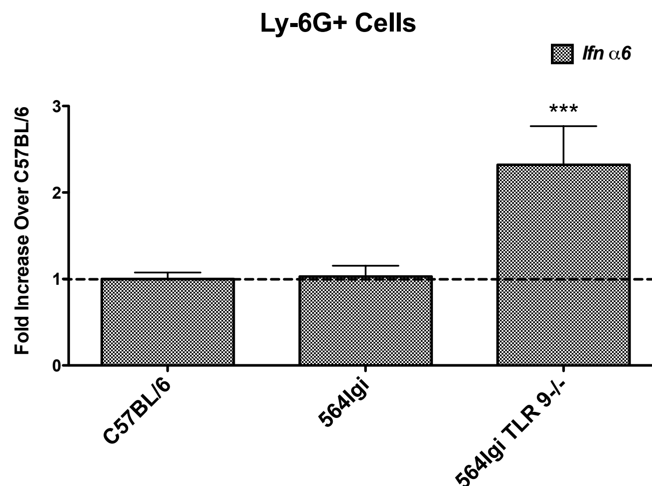

Figure 44. TLR9 suppresses *Ifn-I* expression in 564Igi neutrophils. mRNA transcript levels of *Ifn-α6* relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G+ cells (neutrophils). 3 mice tested for each strain. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. *** p< .001.

Female 564Igi mice deficient in *Tlr7* and *Tlr9* have high levels of *Ifn-α6* mRNA transcripts (Fig. 45). The male 564Igi *Tlr7/9*$^{-/-}$ neutrophils have very low levels of *Ifn-α6* mRNA transcripts (Fig. 45). The female 564Igi *Tlr7/9*$^{-/-}$ mice have IgG autoantibody in their sera, while the male 564Igi *Tlr7/9*$^{-/-}$ did not (Fig. 32 and 33). According to our hypothesis, without circulating IgG autoantibody ICs that could stimulate neutrophils in male 564Igi *Tlr7/9*$^{-/-}$, there would be no opportunity for IFN-I induction in neutrophils.

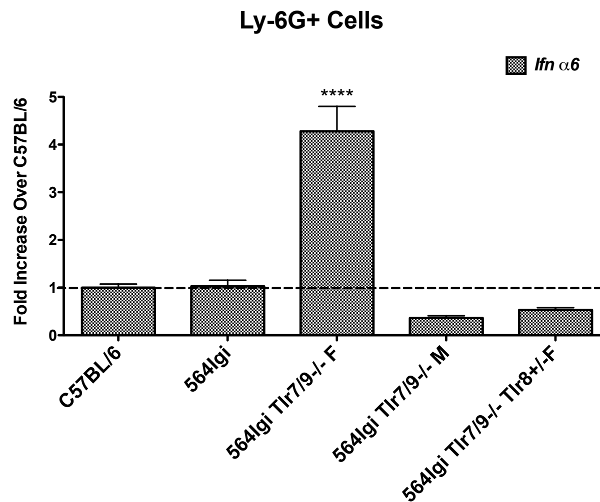

Figure 45. Increased *Ifn-I* transcript levels in female 564Igi *Tlr7/9*$^{-/-}$ neutrophils, but not in male neutrophils. mRNA transcript levels of *Ifn-α6* relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G^{+} cells (neutrophils). 3 mice tested for each strain. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. **** p< .0001.

Interestingly, in female 564Igi *Tlr7/9*$^{-/-}$ mice there is no increase in *Tlr8* mRNA transcript levels compared with C57BL/6 (Fig. 46). The same is true for 564Igi *Tlr9*$^{-/-}$ mice. In both female 564Igi *Tlr7/9*$^{-/-}$ and *Tlr9*$^{-/-}$ mice, there is significant induction of IFN-I without an increase in *Tlr8* (or *Tlr7* in the case of 564Igi *Tlr9*$^{-/-}$)*.* This implies that in the absence of TLR9, no increase of other endosomal TLRs is critical for neutrophil IFN-I induction. However, in the absence of circulating IgG autoantibody in male 564Igi *Tlr7/9*$^{-/-}$, there are similar low levels of *Tlr8* transcripts (Fig. 46). In male 564Igi *Tlr7/9*$^{-/-}$ neutrophils, however, there are low levels of *Ifn-I* transcripts. Together these data imply that the absence of TLR9 and circulating IgG autoantibody is required for an increase in *Ifn-I* in neutrophils of 564Igi.

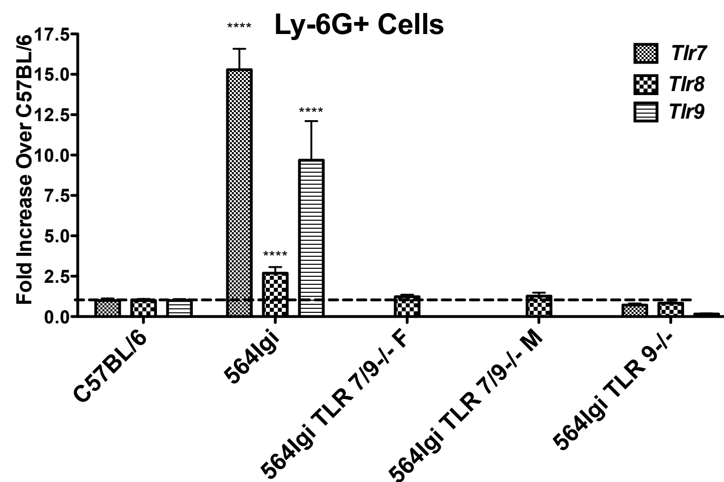

Figure 46. *Tlr7* and *Tlr8* are not up regulated in the absence of *Tlr9*. mRNA transcript levels of *Tlr7, Tlr8,* and *Tlr9* relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G+ cells (neutrophils). 3 mice tested for each strain. 3 dilutions of cDNA tested from each mouse, each dilution was tested in triplicate. Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. **** p< .0001.

Experiments using *in vitro* stimulation of neutrophils by ICs were performed in order to further demonstrate that negative regulation by various receptors was critical in suppressing IFN-I responses by neutrophils. $CD11b^+$ $Ly6G^+$ neutrophils were isolated from the bone marrow using FACs sorting, as seen in Figure 46A. These cells were then treated with ICs containing either IgG_{2a} or IgG_{2b} anti-RNA antibodies complexed with RNA. The transcript levels of *Ifna6* were measured after 4 hours in culture with IC treatments. IgG_{2b}/RNA ICs stimulate C57BL/6 neutrophils to produce modest increases in *Ifna6* transcript levels compared with untreated C57BL/6 neutrophils; however, no increase of *Ifna6* was observed with IgG_{2a}/RNA IC treatment (Fig. 47B).

There are two distinct functional classes of FcγRs: activating and inhibitory. Both recognize the Fc portion of an antibody in an IC. FcγR2B is the only inhibitory IgG receptor in mice and is expressed on many immune cells, including B cells and neutrophils (Nimmerjahn and Ravetch, 2005).

On the surface of neutrophils, the inhibitory FcγR2B has the potential to recognize IgG ICs. Theoretically, this recognition by FcγR2B on neutrophils could dampen the IFN-I response by neutrophils in a similar manner as was observed in by FcγR2B in splenocytes (Panchanathan et al., 2011). Therefore, neutrophils from *Fcγr2b*$^{-/-}$ mice were tested for *Ifn-I* responses to IC stimulation. Neutrophils deficient in *Fcγr2b* can be stimulated to produce high levels of *Ifna6* transcripts after treatment with either IgG_{2a} or IgG_{2b} ICs with RNA (Fig. 48A). Ifna6 transcripts were induced by IgG2b ICs at a higher rate than with IgG2a ICs (Fig. 48A).

MRL/*lpr* mice lacking TLR9 have higher serum IFN-I levels than wild type MRL/*lpr* (Christensen et al., 2006). *Ex vivo* neutrophils from 564 *Tlr9*$^{-/-}$ have high levels of *Ifn-I* transcripts (Fig. 45). Therefore, it was tested whether intrinsic TLR9 expression suppressed IFN-I response by neutrophils. *Tlr9*$^{-/-}$ neutrophils produce higher levels of *Ifn-a6* transcripts when treated with either IgG_{2a} and IgG_{2b} ICs compared with untreated *Tlr9*$^{-/-}$ neutrophils (Fig. 48B). After treatment, with either IgG_{2a} or IgG_{2b} ICs, *Tlr9*$^{-/-}$ neutrophils have higher levels of *Ifna6* mRNA transcripts compared with untreated *Tlr9*$^{-/-}$ neutrophils. Together, this demonstrated that both FcγR2B and TLR9 negatively regulate the expression of IFN-I in neutrophils after treatment with ICs.

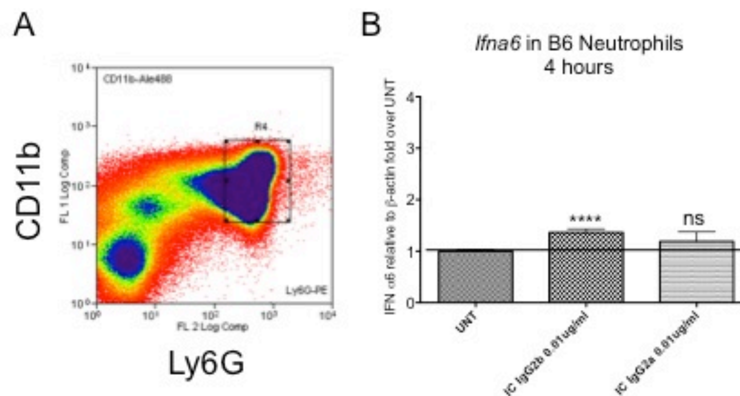

Figure 47. IFN-I in neutrophils is only modestly induced by 564 ICs. A. $CD11b^{+}$ $Ly\text{-}6G^{+}$ neutrophils were sorted as shown. **B.** *In vitro* stimulation of neutrophils with ICs was performed. ICs were formed with either IgG_{2a} or IgG_{2b} purified anti-RNA antibody. mRNA transcript levels of *Ifn-a6* relative to *Actb* was determined by qPCR with cDNA from BM $CD11b^{+}$ $Ly\text{-}6G^{+}$ cells (neutrophils). Fold increase over untreated. Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. * $p < .05$, ** $p < .01$, *** $p < .001$ and **** $p < .0001$. Data was collected by Nick Cabal.

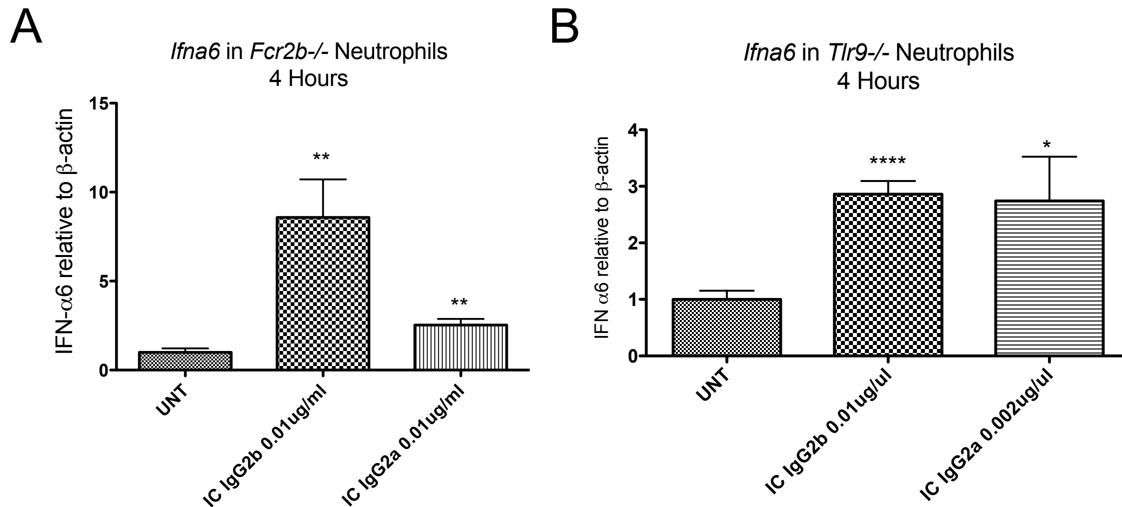

Figure 48. IFN-I induction by 564 IC is heightened in the absence of *Tlr9* or *Fcr2b* in neutrophils. A, B. *In vitro* stimulation of neutrophils with ICs was performed. ICs were formed with either IgG_{2a} or IgG_{2b} purified anti-RNA antibody. mRNA transcript levels of *Ifn-a6* relative to *Actb* was determined by qPCR with cDNA from BM $CD11b^+$ $Ly\text{-}6G^+$ cells (neutrophils). Fold increase over untreated. A. Neutrophils purified from C57BL/6 *Fcr2b*$^{-/-}$ mice. B. Neutrophils purified from C57BL/6 *Tlr9*$^{-/-}$ mice Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. * p< .05, ** p< .01, *** p< .001 and **** p< .0001. Data was collected by Nick Cabal.

564Igi *Tlr7*$^{-/-}$ mice also have high *Ifn-I* transcript levels in neutrophils (Fig. 49A). 564Igi *Tlr7*$^{-/-}$ neutrophils have low levels of *Tlr8* mRNA transcripts (Fig. 49B). Interestingly, *Tlr9* transcript levels are also low in 564Igi *Tlr7*$^{-/-}$ neutrophils (Fig. 49B). In 564Igi, neutrophils have very high levels of *Tlr9* (Fig. 46). It is possible that this difference in *Tlr9* expression contributes to the increase in *Ifn-I* in neutrophils from 564Igi *Tlr7*$^{-/-}$ compared with 564Igi.

564Igi *Tlr8*$^{-/-}$ neutrophils have low levels of *Ifn-I,* (Fig. 49A) *Tlr7,* and *Tlr9* mRNA transcript (Fig. 49B). Both 564Igi *Tlr7*$^{-/-}$ and 564Igi *Tlr8*$^{-/-}$ produce IgG

autoantibody. Neutrophils therefore could be activated in either mouse by ICs. The induction of *Ifn-I* in 564Igi *Tlr7*$^{-/-}$ mice suggests that TLR7 is not crucial for neutrophil activation. In 564Igi *Tlr8*$^{-/-}$, high levels of autoantibody but low levels of neutrophil *Ifn-I* mRNA transcripts suggest that TLR8 might play an important role in the induction of IFN-I in neutrophils.

564Igi *Tlr7/8*$^{-/-}$ have low levels of *Ifn-I* (Fig. 49A) and *Tlr9* mRNA transcript (Fig. 49B). These mice however, have no IgG autoantibody production. The lack of autoantibody may contribute to the lack of activation of neutrophils to produce IFN-I.

The correlations between autoantibody production, and increase in BM neutrophils and neutrophil IFN-I in various 564Igi *Tlr* knockout strains provide insight into putative and differential roles of individual TLRs in SLE pathology. The phenotype of each mouse strain is summarized in this results section and Table 3 (pg. 128). I will elaborate on the implications of these correlations in the discussion section of this dissertation.

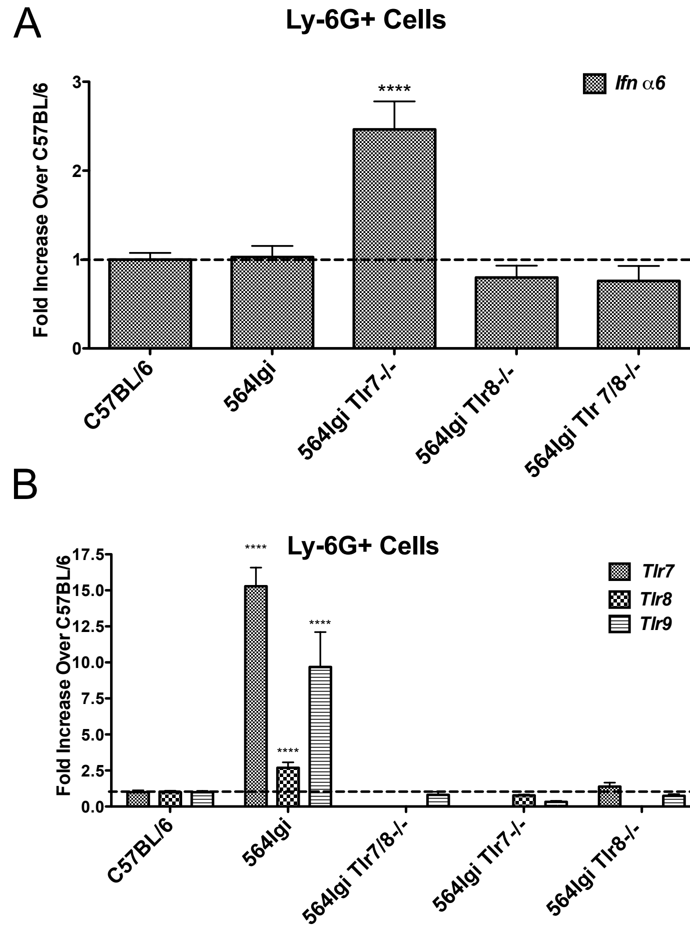

Figure 49. Increase in *Ifn-I* transcript levels in neutrophils from 564Igi *Tlr7*$^{-/-}$, but not from 564Igi *Tlr8*$^{-/-}$ or *Tlr7/8*$^{-/-}$. mRNA transcript levels of *Ifn-a6* **(A)** or *Tlr7*, *Tlr8* and *Tlr9* **(B)** relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G^{+} cells (neutrophils). Fold increase over C57BL/6. Arithmetic means +/- SEM. Significance was determined by two tailed Student's t-test. * p< .05, ** p< .01, *** p< .001 and **** p< .0001.

Results 3.4. *Tlr8* expression is critical for heightened *Ifn-I* transcript levels in neutrophils stimulated by ICs.

Human neutrophils express high levels of TLR8 and low levels of TLR7 (Berger et al., 2012). Other groups have shown that human neutrophils can be stimulated to produce IL-8 through TLR8, but not TLR7 (Janke et al., 2009). In chapter 2 it was shown that TLR8 plays a major role in accumulation of neutrophils in the BM of 564Igi (Fig. 31). This made us question whether TLR8 mediated IFN-I production on neutrophils in 564Igi.

Neutrophils express TLR8 in C57BL/6 mice. TLR8 is also expressed on B cells of C57BL/6 mice, but the expression is lower compared to neutrophils. *Tlr8* transcript levels are higher in C57BL/6 neutrophils compared with B cells (Fig. 50A). Similarly, TLR8 protein levels by western blot are higher in C57BL/6 neutrophils compared with C57BL/6 B cells (Fig. 50B). Therefore, murine TLR8 is highly expressed on neutrophils in mice.

Next it was investigated whether TLR8 in neutrophils could mediate IFN-I production. CLO-97 is a small molecule that stimulates TLR7 at low concentrations and both TLR7 and TLR8 at high concentrations. CLO-75 stimulates TLR8 at low concentrations and both TLR7 and TLR8 at high concentrations. CD11b$^+$ Ly6G$^+$ neutrophils were sorted using flow cytometry. *In vitro* neutrophils were treated with either CLO-75 or CLO-97 at 1μg/ml. C57BL/6 neutrophils treated with either CLO-75 or CLO-97 have higher levels of *Ifn-α6* mRNA transcripts compared with untreated C57BL/6 neutrophils (Fig. 51A). C57BL/6 *Tlr8*$^{-/-}$ neutrophils treated with either CLO-75 or CLO-97 at 1μg/ml had lower levels of *Ifn-α6* mRNA transcripts compared with untreated C57BL/6 *Tlr8*$^{-/-}$

neutrophils (Fig. 51B). Together this implies that TLR8 was critical for an increase in neutrophil *Ifn-I* production by either CLO-75 or CLO-97 at 1μg/ml. This is true despite the fact that CLO-97 induces activation through TLR7 at lower concentration than TLR8 in HEK293 cells (Gorden et al., 2005). These data further imply that TLR8 was critical for mediating increased IFN-I responses from neutrophils.

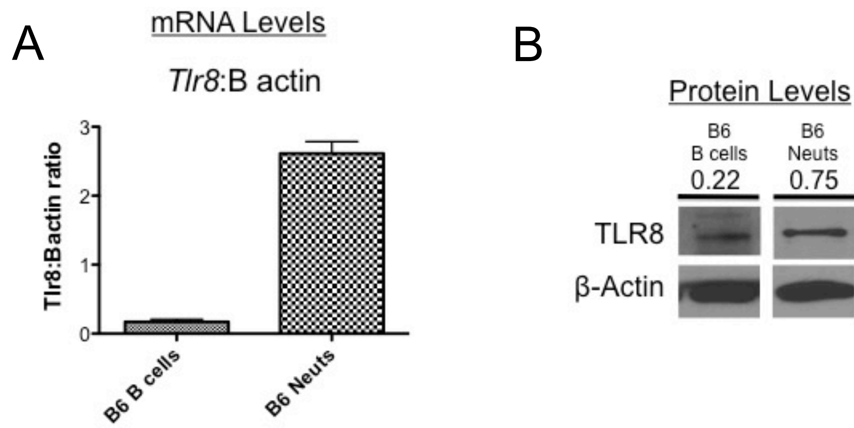

Figure 50. TLR8 is highly expressed in neutrophils, but not in B cells. A. mRNA transcript levels of *Tlr8* relative to *Actb* was determined by qPCR with cDNA from BM Ly-6G$^+$ cells (neutrophils) compared to BM B220$^+$ (B cells). Fold increase over C57BL/6. Arithmetic means +/- SEM. **B.** Western blots for TLR8 and BETA ACTIN. TLR8/BETA-ACTIN ratios are above blots. Representative of at least three experiments.

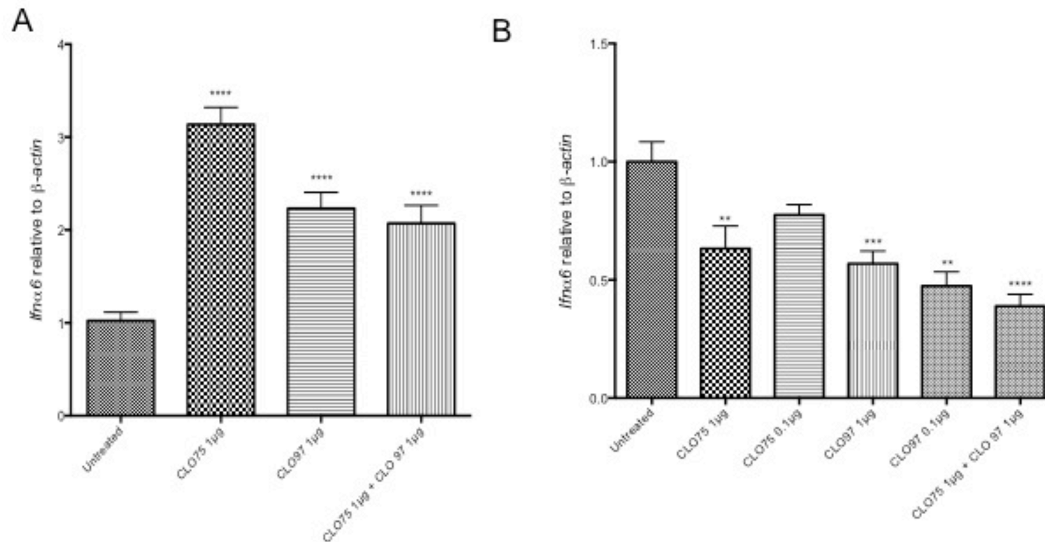

Figure 51. TLR8 is critical for neutrophils IFN-I induction by CLO-75 and CLO-97. Neutrophils sorted from C57BL/6 **(A)** or B6-*Tlr8*$^{-/-}$ **(B)** were treated with CLO-75 and CLO-97 at 1µg/ml. mRNA transcript levels of *Ifn-a6* relative to *Actb* were determined by qPCR with cDNA from BM Ly-6G^{+} cells (neutrophils). Fold increase over untreated samples. Significance was determined by two tailed Student's t-test. ** p< .01, *** p< .001 and **** p< .0001. Data was collected by Nick Cabal.

Results 3.5. IFN-I directly stimulates B cells and neutrophils in 564Igi mice.

IFN-induced signature genes are genes that they are up-regulated when cells are acted upon by IFN-I. PBMCs and BM cells from SLE patients have increased expression of the IFN-signature genes (Bennett et al., 2003) (Baechler et al., 2003) (Nakou et al., 2008). Therefore we were able to ask whether IFN-I acted on neutrophils and B cells in the BM of 564Igi mice by measuring the expression of three IFN-signature genes: Myxovirus resistance 1 (*Mx1*), interferon-induced protein with tetratricopeptide repeats 1 (*Ifit1*), and 2'-5' oligoadenylate synthetase 1A (*Oas1a*) (Sadler and Williams, 2008).

Immature B cells (B220$^+$AA4.1$^+$$\kappa^+$$\lambda^+$) were sorted from the BM of C57BL/6 mice and 564Igi Rag1$^{-/-}$. Using 564Igi *Rag1*$^{-/-}$ ensured that the immature B cells had not undergone secondary recombination, and therefore, a higher percentage of the immature B cells tested expressed an RNA-reactive Id$^+$ BCR than the immature B cells of 564Igi. *Mx1*, *Ifit1* and *Oas1a* are highly expressed in immature B cells from 564Igi *Rag2*$^{-/-}$ mice compared with immature B cells from C57BL/6 (Fig. 52A). Therefore, autoreactive immature B cells in 564Igi *Rag2*$^{-/-}$ were activated by IFN-I in the BM.

In 564Igi neutrophils the transcripts level of *Mx1* and *Ifit1* are significantly increased compared with C57BL/6 neutrophils (Fig. 52B). It is suggested that IFN-I acts directly on neutrophils in 564Igi, inducing activation.

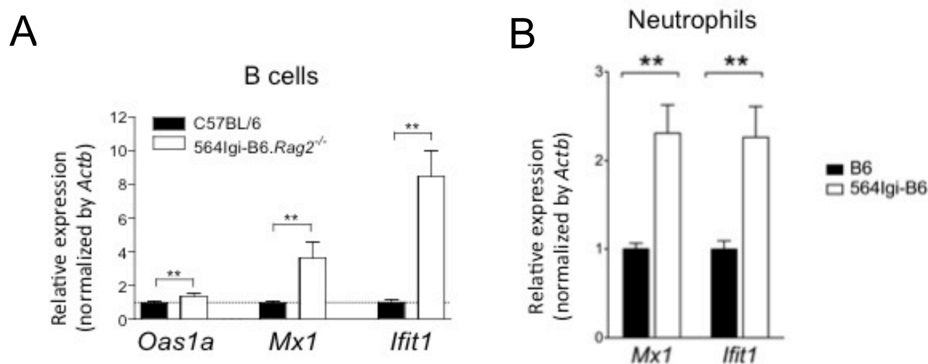

Figure 52. IFN-I signature genes were up regulated in 564Igi B cells and neutrophils compared to C57BL/6 neutrophils. mRNA transcript levels of *Oas1a, Mx1 and Ifit1* relative to *Actb* were determined by qPCR with cDNA from **A** immature B cells and **B** neutrophils. Fold increase over C57BL/6. Significance was determined by two tailed Student's t-test. ** p< .01. Data in A was collected by Jin Hwan Han.

Results 3.5. Glomerulonephritis is TLR7 and TLR8 dependent in 564Igi

There are many signs of SLE–like pathology in 564Igi, including kidney disease in the form of glomerulonephritis. The role of specific TLRs is different in many phenotypic aspects of disease in 564Igi. In order to investigate which TLR dependent traits of 564Igi affected kidney pathology, kidneys from 564Igi *Tlr* knockouts were analyzed. After staining kidneys by PAS, kidneys were examined for signs of disease. Kidney nephritis was determined using the International Society of Nephrology/Renal pathology Society (ISN/RPS) classification of Lupus nephritis (Weening et al., 2004). Kidneys were also stained for the presence Id+ antibodies.

The classification of glomerulonephritis in SLE is as follows: Class I (or negative) minimal to no mesangial lupus nephritis. Class II is proliferative mesangial lupus without any subendothelial deposits or scarred glomeruli. Class III has focal lupus nephritis in less than 50% of the glomeruli, usually with subendothelial deposits or scarred glomeruli. Class IV has focal lupus nephritis in more than 50% of the glomeruli. Class IV can be defined as segmental (IVS) if there are a majority of glomeruli with segmental lesions. Also, Class IV can be diffuse and global (IVG) if the majority of glomeruli have global lesions (Weening et al., 2004). More severe lupus nephritis (Classes V and VI) is not observed in 564Igi mice.

564Igi *Myd88*$^{-/-}$ mice have no IgG autoantibody circulating in their sera. Likewise, there are no 564 Id+ antibody deposits in the kidneys of 564Igi *Myd88*$^{-/}$. The glomeruli of 564Igi *Myd88*$^{-/-}$ had no signs of nephritis (Fig 53 and Table 2).

There are high levels of glomerulonephritis observed in the kidneys from *Tlr7* and *Tlr7/9* deficient 564Igi female mice (Table 2). This is despite the fact that both of these mouse strains have relatively low circulating anti-RNA IgG antibody in their sera.

Young 564Igi *Tlr7/8*$^{-/-}$ mice had no signs of glomerulonephritis and no Id^{+} ICs were detected in their kidneys. However, as 564Igi *Tlr7/8*$^{-/-}$ mice aged, several had signs of inflammation, including enlarged spleens. Also several 564Igi *Tlr7/8*$^{-/-}$ mice developed lymphoma. Some of these mice that have clear signs of inflammation when aged had class II glomerulonephritis. However, 564Igi *Tlr7/8*$^{-/-}$ mice that have no signs of infections or cancer have no Id^{+} antibody or kidney damage. Therefore, we propose that having no *Tlr7* and *Tlr8* increases the susceptibility of mice to infections and cancer. However, in the absence of infection and cancer, 564Igi *Tlr7/8*$^{-/-}$ had no signs of lupus nephritis.

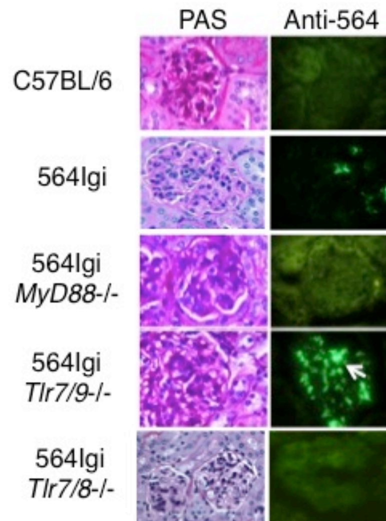

Figure 53. Glomerulonephritis is found in the kidneys of 564Igi and 564Igi *Tlr* knockouts. Kidneys harvested from mouse strains indicated were sectioned and then stained with Periodic Acid Schiff (PAS) (left panels) and Alexa 488 conjugated anti 564-idiotype antibodies (right panels) Collected by with the help of Monika Pilichowska, Shauna Andersson.

Table 2.

Degree of Lupus Nephritis In 564Igi Mouse Lineages	
Genotype	**Class**
C57BL/6	Negative
564 Igi	Class II – IVS
564 *Myd88* KO	Negative
564 *Tlr7* KO	Class III – IVG
564 *Tlr9* KO	Class IV – S
564 *Tlr7/Tlr9* DKO	Class III* - IVG
564 *Tlr7/Tlr8* DKO	Negative-Class II

TABLE 3 Phenotypes of 564Igi *Tlr* knockout mice

Neutrophils	C57BL/6	564Igi	564Igi *Tlr7*$^{-/-}$	564Igi *Tlr8*$^{-/-}$	564Igi *Tlr9*$^{-/-}$	564Igi *Tlr7/9*$^{-/-}$		564Igi *Tlr7/8*$^{-/-}$
	Female	Female	Female	Female	Female	Female	Male	Female
Number of BM neutrophils	+	+++	+++	+++	++++	+++	+	+
Ifn-I expression in neutrophils	+	+	+++	+	+++	++++	+	+
Tlr7 expression in neutrophils	+	++++	-	+	-	-	-	-
Tlr8 expression in neutrophils	+	++	+	-	+	+	+	-
Tlr9 expression in neutrophils	+	++++	-	+	-	-	-	+
Pathology								
Glomerulonephritis	-	++	++++	nd**	++++	++++	nd**	-
Antibody Production								
Anti-RNA IgG antibody	-	+++	+	+++	+++	+	-	-

- **No antibody or detectable mRNA transcript**

\+ **Low levels of antibody or mRNA transcript**

++ Moderate level of antibody or mRNA transcript

+++ High level of antibody or mRNA transcript

++++ Very high level of antibody or mRNA transcript

nd: no data

DISCUSSION

This section will discuss the results published in three papers, Han et al., 2013, Umiker et al., 2014a, and Umiker et al., 2014b, along with further findings presented in the results section. The data will be discussed in the context of previous work from the Imanishi-Kari laboratory and other groups that study SLE. This dissertation addresses questions pertaining to SLE pathology observed in the 564Igi mouse model. Disease in 564Igi mice results from the expression of an autoreactive antibody, encoded by two knock-in genes.

The results of Han et al., 2013 demonstrated that, in 564Igi mice, the expression of an anti-RNA antibody leads to many aspects of pathology that are also observed in human SLE. In particular, there is an increase in the number of BM and peripheral neutrophils and monocytes in 564Igi mice. These cell types are the primary producers of IFN-I in 564Igi mice, which challenges the current assumption that pDCs are the main cell type responsible for heightened IFN-I levels in the sera of SLE patients.

In Umiker et al., 2014a, a dual role for AID expression in early developing B cells is described. First, AID in early developing B cells is critical for the expression of autoreactive IgG antibody in 564Igi mice. Secondly, AID expression in early developing B cells prevents the accumulation of anti-RNA IgM antibody in the sera. We demonstrated that the low levels of autoreactive IgM in the sera of 564Igi mice is at least partially due to SHM of autoreactive genes in early developing B cells.

Umiker et al., 2014b investigated the role of nucleic acid-sensing TLRs in the etiology of 564Igi mice. TLR8 is not critical for autoantibody production in 564Igi mice; yet, TLR8 has the ability to mediate autoantibody production in the absence of TLR7 and TLR9. Additionally, TLR8 is critical for an increase in BM neutrophils as well as increased IFN-I production by neutrophils. The copy number of the *Tlr8* gene in male and female 564Igi *Tlr7/9*$^{-/-}$ mice determines the phenotypic difference between the sexes. Together, these findings contribute a clearer understanding of the specific roles of endosomal TLRs in the development of SLE.

D.1. Anti-RNA antibody in SLE and 564Igi

SLE patients produce autoantibodies reactive to a variety of self-antigens (Isenberg et al., 2007). In SLE patients, autoantibodies are most often reactive to nucleic acid or nucleic acid-associated molecules. The two most common autoantibody specificities used in the diagnosis of SLE are double stranded DNA (dsDNA) and Smith (Sm) Antigen. Other common autoantigens in SLE include, histones, cardiolipin, and RNA associated proteins such as RNP, Ro, La, and ribosomal P (Riemekasten and Hahn, 2005).

A portion of autoantibodies produced in SLE is directly pathogenic. Their production leads to an inflammatory immune response that causes a diverse set of symptoms. In 564Igi mice, SLE-like disease - including lupus nephritis - is the result of anti-RNA antibody expression. There are many indicators that anti-RNA antibodies are potentially more pathogenic than anti-DNA antibodies in SLE.

Plasma from SLE patients stimulates healthy peripheral blood mononuclear cells (PBMCs) to transcribe IFN-alpha signature genes. Plasma from either healthy controls or rheumatoid arthritis patients does not increase PBMC IFN-I-regulated gene expression. There is an association between upregulated IFN-I signature genes in PBMCs and autoantibodies that target the RNA-associated proteins in the sera SLE patients (Crow, 2014; Kirou et al., 2005). Plasma from patients with anti RNA-binding protein (anti-RBP) antibody in their sera induced the highest levels of IFN-I signature gene expression in PBMCs (Hua et al., 2006). This finding demonstrates a correlation between anti-RNA and -RNA-associated molecules antibody production as well as the induction of IFN-I.

RNA has been shown to be a potent inducer of IFN-I via TLR7 recognition on pDCs (Savarese et al., 2006). In addition, TLR7 knockout SLE mouse models have significantly less severe disease. This indicates that RNA-mediated TLR7 signaling is important for SLE disease progression. In contrast, TLR9 is critical for high levels of anti-DNA antibodies in MRL/*lpr* mice, but is not critical for severe disease. In fact, TLR9 deficient MRL/*lpr* mice have exacerbated disease, which is most likely due to an increase in anti-RNA antibody (Nickerson et al., 2010).

In sum, autoantibodies produced in 564Igi mice are reactive to RNA and 564Igi anti-RNA antibody production drives pathogenesis. The potential mechanisms by which tolerance is broken in 564Igi mice, allowing for the

production of anti-RNA antibody and SLE-like pathology is discussed in the next section.

D.2. T cell-independent and -dependent autoantibody production

Antigen-driven and AID-mediated SHM occurs during GC reactions. These processes lead to affinity maturation and high affinity antibody production. SHM is also an important process in the immune response that leads to the clearance of pathogens. However, there are possible negative consequences for AID expression. For instance, B-cell lymphomas are often derived from GC reactions (Kuppers et al., 1999; Stevenson et al., 1998). Such lymphomas are often associated with AID-mediated c-myc/IgH translocations (Ramiro et al., 2006).

Another negative consequence of AID expression in GCs is the potential for the genesis of auto-reactive antibody genes derived from non-autoreactive genes. Studies have shown that in both humans and mice anti-dsDNA autoantibodies are created by the introduction of point mutations in non-autoreactive germline antibody genes (Lambrianides et al., 2007) (Rahman et al., 2001) (Wellmann et al., 2005). Pathogenic IgG anti-nuclear antibodies can be produced by T cell-dependent activation of B cells within GCs in MRL/*lpr* mice (Connolly et al., 1992) (Wen et al., 1996).

The predominant hypothesis is that the GC reaction is the main source of autoantibody generation in SLE. However, there is evidence that autoreactive antibody can be produced outside of GC reactions. SHM occurs in dividing extra-

follicular B cells. These B cells are outside GC reactions and have been shown to be a source of autoantibody in a mouse model of SLE (William et al., 2002).

The production self-reactive IgG antibody has been shown to be T cell-independent in 564Igi mice (Berland et al., 2006) and MRL/*lpr* mice (Herlands et al., 2008). In 564Igi mice deficient in RAG1, there are no T cells. There are, however, B cells because the 564 transgene is prearranged allowing for B-cell development. Interestingly, in 564Igi *Rag1*$^{-/-}$ mice there are detectable levels of IgG anti-RNA autoantibody. This demonstrates that class-switched autoantibody production in 564Igi mice is not dependent on T cells. Nonetheless, this does not rule out a role for T cells in other disease factors in 564Igi mice.

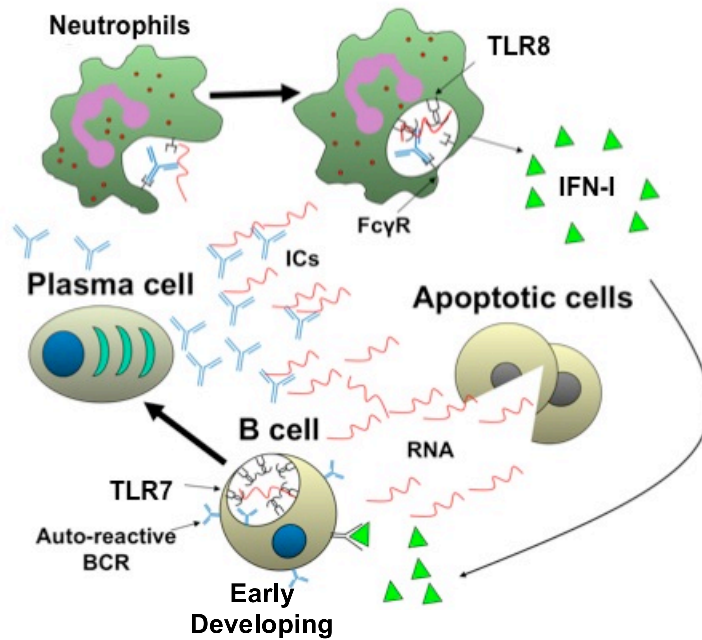

Figure D.1. Schematic of proposed mechanism for T cell-independent autoantibody production leading to IFN-I production by neutrophils. Developing B cells in the bone marrow recognize host RNA shed from apoptotic cells through an auto-reactive BCR and primarily TLR7. BCR/TLR crosslinking and IFN-I stimulation allows for the activation of immature B cells and differentiation into autoantibody producing plasma cells. Secreted autoantibody forms ICs with RNA antigen. These ICs can be recognized by neutrophils through activating FcgRs (I and IV) and primarily TLR8. This stimulates the neutrophils to produce IFN-I.

T-independent B-cell activation requires BCR/TLR coengagement and stimulation by cytokines. An autoreactive BCR can recognize nucleic acids that are shed from cells undergoing apoptosis. Upon recognition by the BCR, the autoantigen is endocytosed, thereby delivering the autoantigen to the endosome where recognition by nucleic acid-sensing TLRs can occur (Rahman and Eisenberg, 2006).

In 564igi BM, there are a large number of neutrophils producing IFN-I

(Han et al., 2013). The mechanism by which 564-autoantibody leads to the generation of large populations of neutrophils producing IFN-I in 564Igi is unknown. It is possible that ICs are recognized through a combination of FcγR-I, -IV and TLR8 on neutrophils. TLR8 is highly expressed on neutrophils and TLR signaling is crucial for the production of IFN-I in SLE and has the potential to induce an increase in granulopoiesis (Lee et al., 2008a; Marshak-Rothstein, 2006). IFN-I can stimulate auto-reactive developing B cells leading to the differentiation of autoantibody-producing cells (Braun et al., 2002).

D.3. Dual role for AID in early developing B cells

AID is expressed in pre-B cells and immature B cells

Immature B cells express AID. Their antibody genes can undergo CSR when activated. CSR in immature B cells is T-independent, but requires both BCR and MyD88 signaling (Han et al., 2007). Self-antigens, such as self-DNA and self-RNA, signal through both self-reactive BCRs and Myd88 dependent TLRs. The coengagement of BCRs and TLRs leads to activation of B cells and subsequent expression of *Aicda* in immature B cells (Han et al., 2007).

Pre-B cells can be activated to produce AID. The pre-BCR can drive the proliferation of pre-B cells when stimulated by self-antigen (Eschbach et al., 2011; Ubelhart et al., 2010) (Herzog and Jumaa, 2012). Selection of autoreactive pre-B cells could lead to escape to the periphery before further B-cell differentiation. There is a subset of circulating B cells that expresses a surrogate light chain and these cells are often autoreactive even in healthy patients (Meffre

et al., 2004). It is tempting to speculate that these cells came from pre-B cells stimulated in the BM.

Interestingly, in pre-B cells the requirements for AID expression are different than they are in immature B cells. Pre-B cells do require MyD88-mediated signaling, but Bruton tyrosine kinase (Btk) is not required for CSR (Han et al., 2007). Btk is critical for conventional BCR-mediated signaling (Craxton et al., 1999; Takata and Kurosaki, 1996). Therefore, in pre-B cells BCR-mediated signaling is not required for *Aicda* expression and CSR. There must be an alternative mechanism for the activation of pre-B cells leading to the expression AID other than conventional BCR/TLR coengagement.

There are a few potential mechanisms that could account for the production of AID in pre-B cells that are independent of pre-BCR signaling. Transcribed endogenous retroviral elements may play a role in activating B cells in SLE. RNA transcribed from retroviral elements is able to activate B cells through TLR7 (Eilat and Wabl, 2012) (Stetson, 2009) and possibly also through TLR8. TLR7 was shown to control ERV activation in Tlr7-/- animals(Yu et al., 2012) . How endosomal TLRs would have access to retroviral RNA is unknown, but there is work supporting the hypothesis that retroviral element transcription mediates autoimmunity (Crow, 2014).

Aicardi-Goutieres sydrome (AGS) is an autoimmune disorder with many characteristics similar to those in SLE. AGS is caused by a mutation in the TREX1 gene (Crow et al., 2006). TREX1 is a DNA exonuclease that has been shown to degrade retroviral elements (Stetson et al., 2008; Yan et al., 2010).

Reverse transcriptase inhibitors ameliorate autoimmune symptoms in TREX1 deficient mice (Beck-Engeser et al., 2011). This suggests that reverse transcription of retroviral elements is a key mediator of autoimmunity in AGS. Since TREX1 mutations have also been associated with SLE (Lee-Kirsch et al., 2007) (Namjou et al., 2011), it is tempting to speculate that similar retroviral elements play a role in SLE.

Endogenous murine leukemia virus (MLV) can initiate a TLR7-dependent immune response in healthy mice (Kane et al., 2011). In normal mice, MLV is not particularly infectious between cells. However, NZB x NZW autoimmune mice have a mutated endogenous MLV that can readily infect B cells. MLV activation of B cells may contribute to autoimmunity in NZB x NZW mice (Levy, 1973) (Eilat and Wabl, 2012).

Another potential source of RNA autoantigen are retrotransposons. Retrotransposons are ubiquitous components of eukaryotic genomes. Active retrotransposons replicate via a RNA intermediate. Replication can rapidly increase the copy numbers of retrotransposons in a genome. One class of retrotransposons is called long interspersed nuclear elements (L1). Dysregulation of L1 retrotransposons is common in both AGS patients and TREX1-deficient mice. This is due to a lack of RNA degradation leading to the replication of L1 elements (Stetson et al., 2008; Zhao et al., 2013).

Could RNA transcription of L1 elements contribute to the activation of cells in SLE patients? In patients with either SLE or Sjögren’s syndrome, increased expression of L1 RNA transcripts is highly correlated with IFN-I production (Crow,

2010, 2014) This implicates L1 transcription as an activator of IFN-I-producing cells. It is possible that L1 transcription could lead to B-cell activation.

It has been established that AID is expressed in early developing B cells (Mao et al., 2004, Han et al., 2007). The mechanisms that potentially could lead to AID expression in immature and pre-B cells was discussed in this section. In the next section, the roles for AID in early developing B cells in 564Igi will be presented.

AID expression in early developing B cells is required for CSR of autoreactive genes

AID is functional in early developing B cells. Circle transcripts, the result of recent CSR, are expressed in developing BM B cells (Han et al., 2007). µMT mice provide further suggestive evidence for CSR in early developing B cells. Without maturation beyond very earliest B-cell stages, µMT mice produce IgG antibody (Macpherson et al., 2001) (Hasan et al., 2002). Thus, CSR and antibody production must occur in very early B-cell stages in µMT mice.

The effect of B-cell stage-specific *Aicda* expression on the production of autoantibodies produced in 564Igi mice was investigated. Anti-RNA autoantibodies are produced in mice that express AID throughout B-cell development including 564Igi, 564Igi mb1-cre, and 564Igi CD19-cre mice. On the other hand, the vast majority of 564Igi CD21-cre mice do not express IgG anti-RNA autoantibody. In 564Igi CD21-cre mice, there is no expression of AID in early developing B cells. In 564Igi CD21-cre mice, there is however AID expression in non-B cells such as FDCs. Together, these data suggest that AID

expression during early B-cell development is critical for the production of IgG class-switched autoantibody.

564Igi CD19-cre mice have high titers of anti-RNA IgG in their sera. The CD19-cre promoter is inefficient for Cre recombination. Few B cells express AID at any stage of B-cell development in 564Igi CD19-cre mice. These results suggest that a small number of AID-expressing B cells is responsible for a high titer of anti-RNA IgG antibody in the sera.

Autoantibody production by B cells is crucial for SLE disease. It was therefore logical to try to deplete B cells in SLE patients as a potential treatment. Rituximab is an anti-CD20 monoclonal antibody used to successfully treat B-cell lymphomas. Many clinical trials using rituximab on SLE patients have been conducted; however, the clinical outcomes have been mixed (Sfikakis et al., 2005b).

Plasma cells do not express CD20 and therefore are not targeted by rituximab. While plasmablasts are short lived, antibody-producing plasma cells stay alive without cell division. Plasma cells continue to produce antibody throughout their long lives. Anti-DNA antibody producing plasma cells have been shown to be resistant to anti-proliferative immunosuppressive drugs (Hoyer et al., 2004).

Rituximab is effective at depleting most B-cell stages as demonstrated by a lack of $CD19^+$ cells (Kneitz et al., 2002; Looney et al., 2004; Sfikakis et al., 2005a). It is important to note that neither plasma cells, pro- or pre- B cells express CD19; therefore, all three of these cell types could still be present in

rituximab treated SLE patients. After rituximab treatment SLE patients often have small decreases in circulating anti-dsDNA antibody (Sfikakis et al., 2005b). We have shown that a small number B cells can mediate significant levels of autoantibody production. It is possible that a small remaining population of B cells and plasma cells are responsible for autoantibody production and the mixed results of rituximab treatment in SLE.

In 564Igi CD21-cre mice, AID is fully functional in mature B cells. First, AID expression in mature B cells is sufficient for CSR of antibody genes to IgG_1. In 564Igi mice, IgG_1 antibody is not autoreactive. There is no Id^+ anti-RNA IgG_1 in 564Igi sera (Berland et al., 2006). Similarly, IgG_{2a} and IgG_{2b} antibody is present in 564Igi CD21-cre mice; however, the IgG_2 antibodies are not RNA reactive. Therefore, in 564Igi CD21-cre antibody genes in mature B cells are fully capable of CSR to IgG. However, autoreactive IgG antibody is not expressed in 564Igi CD21-cre mice.

Antibody genes in CD21-cre mice also undergo AID-mediated SHM. Ig genes from $CD138^+$ plasma cells isolated from 564Igi CD21-cre mice have a high frequency of mutations in V_H and V_L genes. IgG antibody genes in 564Igi CD21-cre mice have replacement mutations and these mutations could be responsible for a loss of autoreactivity. No hybridomas from the BM or from the spleen of 564Igi CD21-cre mice produce IgG anti-RNA antibodies.

It has been demonstrated by others that a loss of central B-cell tolerance contributes to the etiopathology of both SLE and rheumatoid arthritis (RA). There is a risk-allele of protein tyrosine phosphatase non-receptor 22 (PTPN22) for

several autoimmune diseases including SLE and RA (Begovich et al., 2004) (Kyogoku et al., 2004). The risk-allele of PTPN22 has been shown to lead to defects in central B-cell tolerance. In patients with the risk-allele there are more autoreactive immature B cells than normally are found (Menard et al., 2011). It would be interesting to investigate AID expression levels in immature B cells from patients with the PTPN22 risk-allele. One may expect an increase in AID expression in immature B cells from PTPN22 risk-allele patients.

Signaling through surface IgG_1 can mediate the differentiation of all subsets of B cells. IgG in developing B cells can promote activation, longer life spans, and increased antibody production by B cells. This was demonstrated with a transgenic mouse, which expressed surface IgG_1 throughout B-cell development. In this mouse model, transgenic IgG_1^+ B cells differentiate into B cells. Transgenic circulating plasma B cells produce more antibody than WT B cells (Storb et al., 1986) (Waisman et al., 2007). IgG^+ B cells enhance and suppress various markers compared with IgM^+ B cells. The balance of activation and suppression markers in IgG^+ B cells leads to an overall enhancement of plasma cell differentiation (Horikawa et al., 2007). There is more robust signaling through IgG compared with IgM demonstrated by an increase in ERK1/2 phosphorylation (Irish et al., 2006). I am proposing that signaling through IgG in early developing autoreactive B cells in 564Igi could lead to increased plasma cell differentiation.

564Igi CD21-cre mice do not produce self-reactive IgG, but do produce non-autoreactive IgG. Fully mature B cells that are spared during central B-cell

tolerance may have undergone RAG-mediated receptor editing before they reach the periphery in 564Igi CD21-cre mice. The secondary rearranged antibody sequences could gain the ability to recognize conventional antigen. Mature B cells that recognize convention antigen in the periphery could undergo affinity maturation through SHM and differentiate into plasma cells. These plasma cells would produce class switched antibody that is no longer self-reactive.

What is the role of AID in early developing B cells? The data presented here implies that CSR of autoreactive genes occurs in 564Igi, 564Igi CD19-cre, and 564Igi mb1-cre mice in early developing B cells. It is tempting to speculate that the activation of early developing B cells induces CSR to IgG and then differentiation to plasma B cells, thus allowing for a break in central B-cell tolerance. The differentiation of immature B cells to plasma cells is an intriguing, but as of yet unproven mechanism.

Crouch et al., 2007 defined a new system to track the expression patterns of AID (Crouch et al., 2007). This report states that there is very little AID expression in BM immature B cells in naïve mice and that there are no mutated clones in emigrant B cells. Minimal levels of AID expression in developing B cells makes it difficult to imagine how CSR could occur in order to produce class switched autoantibodies. It is possible that in 564Igi more early developing B cells express AID than in wild-type mice. This could be caused by the stimulation of cells through the highly autoreactive BCRs expressed in 564Igi B cells. Heightened AID expression observed in BM B cells in 564Igi supports this claim.

AID is a multi-functional protein. In early developing B cells, AID-mediated SHM occurs along with CSR of antibody genes. The role of SHM in early B-cell development will be the subject of the next sections.

AID is required for central B-cell tolerance in 564Igi

Hyper IgM type 2 (HIGM2) is a rare immune deficiency disorder. There are three common features for patients with HIGM2: a lack of class switched IgG, a lack of SHM and lymph node hyperplasia with enlarged GCs. HIGM2 is caused by mutations in the *Aicda* gene (Revy et al., 2000). Patients with HIGM2 often have recurrent bacterial infections and have a dysregulated gut flora (Fagarasan et al., 2002).

Interestingly, in one study a percentage of HIGM2 patients also developed a variety of autoimmune disorders (Quartier et al., 2004). This included diabetes mellitus, polyarthritis, autoimmune hepatitis, hemolytic anemia, immune thrombocytopenia, Crohn's disease and chronic uveitis (Quartier et al., 2004). The mechanism by which AID deficiency causes a predisposition to autoimmunity in humans is under investigation.

In mice, AID deficiency can cause autoimmunity. IgM in the sera of *Aicda*$^{-/-}$ wild-type mice are specific for autoantigen in the gastric system (Hase et al., 2008). MRL/*lpr* mice deficient in AID have significantly higher levels of IgM autoreactive antibodies than wild-type MRL/*lpr*. In the absence of IgG, MRL/*lpr* *Aicda*$^{-/-}$ mice have symptoms of SLE, such as glomerulonephritis, demonstrating

that IgG autoantibody is not critical for disease in MRL/*lpr* mice (Chen et al., 2010).

AID inhibits the development of B cells. This was shown by a competitive reconstitution of equal parts *Aicda*$^{-/-}$ and *Aicda*$^{+/+}$ stem cells into an irradiated mouse. The majority of B cells in mice reconstituted this way are AID deficient. This demonstrated a selective advantage for *Aicda*$^{-/-}$ B cells (Kuraoka et al., 2011). The expansion of *Aicda*$^{-/-}$ B cells was observed early in B-cell development. The majority of immature B cells were also AID-deficient (Kuraoka et al., 2011).

When an autoreactive antibody transgene referred to as 3H9 is inserted into C57BL/6 mice the normal mechanisms of central B-cell tolerance result in a low percentage of immature B cells carrying the transgene. However, when 3H9 is crossed to *Aicda*$^{-/-}$ background an increase in 3H9^{+} immature B cells and increased serum autoreactive IgM are present (Kuraoka et al., 2011). These results suggest that AID expression in developing B cells plays a role in B-cell tolerance.

Other studies in humans support the idea that AID plays a role in B-cell tolerance by preventing autoreactive IgM. Meyers and colleagues isolated single B cells from patients with HIGM2 with no functional AID. Mature B cells express autoreactive antibody at an abnormally high rate in these patients. This indicated that peripheral tolerance was dependent on AID. Antibody genes in transitional B cells have high rates of auto-reactivity, indicating that central tolerance is dependent on functioning AID in humans (Meyers et al., 2011).

In the sera of 564Igi mice there are low titers of anti-RNA IgM antibody. IgM$^+$ B cells in the spleen of 564Igi mice have many signs of anergy and do not effectively flux calcium (Berland et al., 2006). In 564Igi, *Aicda*$^{-/-}$ anti-RNA IgM autoantibody serum titers are high. AID is therefore critical to prevent anti-RNA IgM accumulation in the sera of 564Igi.

564Igi CD21-cre mice have high titers of serum IgM autoantibodies. 564Igi mb1-cre mice have low titers of anti-RNA IgM autoantibody similar to wild-type 564Igi mice. AID in mature B cells does not prevent high titers of IgM autoantibody in the sera. Together, this implies that AID early in B-cell development is required for central B-cell tolerance.

One possible mechanism by which AID is important for central B-cell tolerance is through SHM. Hypothetically, autoreactive antibody genes could be mutated away from self-reactivity. On the other hand, many in the field believe that SHM is in fact needed for autoantibody production. The prevailing theory is that non-autoreactive B cells are stimulated to produce AID by foreign antigen (Lambrianides et al., 2007; Rahman et al., 2001; Wellmann et al., 2005). Clonal expansion of activated cells and SHM of antibody genes drive the genesis of self-reactive anti-dsDNA antibody (Neeli et al., 2007; Shlomchik et al., 1990).

SHMs are found in many autoantibody genes from human SLE patients. As SHMs accumulate, the affinity for dsDNA increases. This indicates that dsDNA reactivity is acquired through positive selection by self-antigen (Wellmann et al., 2005). Anti-dsDNA antibodies with SHMs were mutated back to their germline sequence. The germline sequence produced antibody that was not

reactive to dsDNA (Wellmann et al., 2005). It was therefore presumed that these auto-reactive B cells are generated from nonautoreactive antibody responding first to foreign antigen. Further it was presumed that SHM leading to the generation of autoreactive antibody genes occurs in T-cell dependent GC reactions.

Whether GC reactions are necessary for autoantibody production has come into question. One study found that extra-follicular autoantibody production takes place at the splenic T zone-red pulp border. Extra-follicular B cells are actively dividing and their antibody genes are undergoing SHM independent of GC reactions (William et al., 2002). In addition, more recent work demonstrating that SHM can occur in early B-cell development (Mao et al., 2004) (Han et al., 2007) puts into question the assumption that SHM of autoantibody genes must occur during GC reactions.

There is evidence that SHM is not critical for autoantibody production. Patients without functioning AID (HIGM2) have autoreactive antibodies. Interestingly, patients with intact SHM capabilities but no CSR capabilities do not develop autoimmunity, and these patients are immune-compromised (Durandy et al., 2013). Together this points to the potential for SHM to be a driver of central B-cell tolerance.

Furthermore, we have exciting data that supports the idea that SHM can drive autoreactive antibody genes away from self-reactivity, and that this plays a contributing role in the loss of tolerance in 564Igi *Aicda*$^{-/-}$ and 564Igi CD21-cre mice. Contrary to our findings there is evidence there is very little AID expression

in early developing B cells and no mutations in emigrant B cells (Crouch et al., 2007). It is possible that mutations occur in a very small population of early developing B cells that never leave the BM.

In 564Igi mb1-cre mice, Igμ and Igκ genes from the 564 transgene have many replacement mutations. Pre-B cells and immature B cells from 564Igi mb1-cre mice have mutated clones of the 564 gene that when expressed had lost reactivity to RNA. Therefore, SHM driven by the *Aicda*^{tg} can cause a loss of self-reactivity in early developing B cells. AID at physiological levels in 564Igi *Rag1*$^{-/-}$ can also be responsible for the loss of self-reactivity of 564 antibody genes in early developing B cells (Umiker et al., 2014b).

564 Igμ and Igκ variable region gene sequences cloned from single pre-B and immature B cells in 564Igi mb1-cre mice had significant numbers of point mutations. Selected variable region sequences were expressed in HEK293T cells with human $IgG_{1.}$ Multiple mutated 564 antibody genes from early developing B cells had lost reactivity to RNA.

In 564Igi CD21-cre mice, early developing B cells do not express AID. The vast majority of IgM^+ hybridomas obtained from 564Igi CD21-cre BM B cells were reactive to RNA. Fewer IgM^+ hybridomas obtained from 564igi mb1-cre BM B cells were reactive to RNA. AID expression in early developing B cells therefore decreases the number of B cells with autoreactive IgM genes. SHM in early developing B cells contributes to this difference.

There is also suggestive evidence for another putative role for AID in the prevention of autoreactive IgM production. Namely, that the absence of AID in

early developing B cells affects the efficiency of RAG-mediated secondary recombinations.

Igκ genes can be amplified using 564-Vκ primers from most 564Igi mb1-cre BM B cells. However, from a large percentage of the same cells neither Igμ nor Igγ genes amplified with 564-V_H primers. Heavy chain genes that do not amplify have potentially been altered so that 564-V_H primers no longer can recognize them. During B-cell development, RAG-mediated secondary recombination have been shown to edit anti-dsDNA antibody genes so that they lose self-reactivity (Chen et al., 1995b). In 564Igi mb1-cre mice, H chain genes in early developing B cells are altered so that they could no longer be amplified with 564H chain specific primers, our hypothesis, yet unproved, is that this is due RAG-mediated secondary recombinations.

On the other hand, in 564Igi CD21-cre mice Igμ genes in immature and pre-B cells can be amplified at a high rate using 564-V_H primers. It follows that AID in early developing B cells could potentially be necessary for RAG-mediated secondary recombinations. RAG dependency on AID is a novel explanation for AID's role in central B-cell tolerance.

The potential mechanistic relationship between AID activity and RAG activity is not understood. However, a co-dependency of RAG and AID has been reported by Michael Lieber's group, but in a different context (Tsai et al., 2008). Their work demonstrates that RAG-mediated DSBs in pro- and pre-B cells occur frequently in hotspots. The DSBs can lead to translocations of genetic elements, a cause of human leukemia and lymphoma. Interestingly, the majority of

hotspots are located at CpG dinucleotides that have previously been deaminated by AID. The RAG complex preferentially acts on these sites and causes DSBs that lead to chromosomal translocations (Tsai et al., 2008). It is possible that AID activity on specific sites in the antibody loci could increase the potential for RAG-mediated secondary recombinations. This would account for the increase in anti-RNA IgM production in 564Igi *Aicda*$^{-/-}$. Without AID the frequency of RAG-mediated secondary recombination would potentially decrease. Fewer incidences of secondary recombinations would increase the B-cell pool that expresses the autoreactive 564Ig antibody, eventually leading to more IgM autoantibody production in 564Igi *Aicda*$^{-/-}$ mice.

Our work sought to establish the role of AID expression in early B-cell development. Two conflicting roles for early B-cell AID expression are established. First, AID expression in early developing B cells is required for CSR to IgG for autoreactive antibody genes. CSR of autoreactive antibody genes would in theory be detrimental to an organism and therefore unlikely to be selected for evolutionarily. Second, in early developing B cells AID plays a role as a mechanism of B-cell tolerance. AID expression in early in B-cell development helps in avoiding IgM-mediated autoimmunity. AID as a mechanism of central B-cell tolerance may provide a rationale for the evolutionary selection of AID expression in early developing B cells.

D.4. Innate Immunity in 564Igi

It has become clear that innate immune sensing of nucleic acids plays a central role in the initiation of SLE pathology (Crow et al., 2014). Nucleic acid-

sensing TLRs and resulting inflammation are critical for the induction of the adaptive immune response. The production of IFN-I is the focus of much attention as a main contributor to SLE (Crow et al., 2014). Here I will discuss the findings acquired from the study of 564Igi regarding TLRs and IFN-I in the context of what has previously been discovered from other mouse models and human SLE patients.

Neutrophils and monocytes in SLE

In 564Igi mice, there is an increased number of neutrophils and monocytes in the BM compared with C57BL/6. This is potentially caused by many factors. Lymphopenia in the blood is often associated with SLE with less than 1500 lymphocytes per microliter of blood (Rivero et al., 1978). On one hand, there is a decrease in total lymphocytes in the blood of SLE patients. At the same time, there is often an increase in the population of circulating pre-GC B cells (Arce et al., 2001). Lymphopenia in the BM of 564Igi mice could allow for an increase in the number of neutrophils and monocytes by merely taking up the space left by the decreased population of B cells.

It is also possible that there is an increase in granulopoiesis that leads to the differentiation of neutrophils in the BM. High levels of circulating IFN-I leads to an increase in the serum levels of granulocyte colony-stimulating factor (G-CSF) (Fukuda et al., 2000). G-CSF stimulates the growth of neutrophil progenitors (Burgess and Metcalf, 1980). Furthermore, IFN-I expression can induce an increase in the number of low-density neutrophils in circulation

(Corssmit et al., 1997). Therefore, IFN-I-dependent mechanisms may increase granulopoiesis and lead to high numbers of neutrophils.

There are multiple pieces of evidence that suggest granulopoiesis is increased in 564Igi mice. First, as shown in this report GM-CSF production by B cells is higher in 564Igi compared with C57BL/6 B cells. GM-CSF recognition and signaling by HSCs can lead to the differentiation of neutrophils and other granulocytes (Kimura et al., 2009). Granulopoiesis signature genes are upregulated in patients with SLE (Bennett et al., 2003). This strongly implies that heightened granulopoiesis is occurring in SLE patients and it is possibly also occurring in 564Igi mice.

An increase in BM monocytes is also observed in 564Igi. These monocytes are significant producers of IFN-I (Han et al., 2013). Immature monocytes have been shown to produce significant amounts of IFN-I in SLE and mouse models (Feng et al., 2010; Lee et al., 2008b).

TLR7, TLR8 and TLR9 in murine SLE

Nucleic acid antigen engagement of the BCR and TLRs leads to the activation and proliferation of self-reactive B cells *in vitro* (Leadbetter et al., 2002) (Lau et al., 2005). TLR9 was the first TLR identified that effectively activates autoreactive B cells *in vitro* with synergistic engagement of the BCR (Leadbetter et al., 2002). In these studies, B cells from a transgenic mouse line called AM14 RF^+ were tested *in vitro*. AM14 RF^+ B cells were treated with an IgG_{2a} anti-dsDNA antibody. Presumably, the DNA in the culture supernatant associates

with the IgG_{2a} anti-dsDNA leading to the formation of ICs. ICs coengage BCRs/TLRs, activating B cells to proliferate (Leadbetter et al., 2002). DNase treatment abrogated the proliferation response, indicating that DNA was required for B-cell activation. They established that the antibody treatment was making IgG ICs with DNA chromatin in culture. Using *Tlr9* deficient AM14 cells they demonstrated that these ICs could access TLR9 in the endosome after binding with the BCR resulting in proliferation (Leadbetter et al., 2002).

TLR9 was thought to be a primary mediator of anti-dsDNA autoantibody production and, therefore, SLE pathology. On an MRL/*lpr* background *Tlr9*-deficient mice do have fewer anti-dsDNA reactive autoantibodies (Christensen et al., 2006). Unexpectedly, however, many signs of SLE pathology actually increased in MRL/*lpr* *Tlr9*-deficient mice, including increased inflammatory infiltrates in the salivary gland and kidney, proteinuria, and mortality. There is also an increase in antibodies reactive to RNA and RNA associated proteins in *Tlr9* deficient MRL/*lpr* mice (Wu and Peng, 2006) (Christensen et al., 2006; Lartigue et al., 2006). A later section will discuss TLR9 as a negative regulator of pathology in 564Igi.

Tlr7$^{-/-}$ MRL/*lpr* mice develop a distinct disease phenotype compared with *Tlr9*$^{-/-}$ MRL/*lpr*. *Tlr7*$^{-/-}$ MRL/*lpr* mice do not produce anti-RNA autoantibody though they do have high levels of antibodies reactive to dsDNA in their sera. *Tlr7*$^{-/-}$ MLR/*lpr* mice have a decrease in kidney disease compared with wild-type MRL/*lpr* mice (Christensen et al., 2006). Similarly, in 564Igi mice *Tlr7* deficiency leads to a decrease in anti-RNA autoantibody production (Berland et al., 2006). A

recent study demonstrated that divergent signaling after TLR7 or TLR9 engagement in B cells caused the difference in autoantibody production in *Tlr7* and *Tlr9* deficient SLE mouse models (Jackson et al., 2014).

MRL/*lpr MyD88*$^{-/-}$ mice have no circulating autoantibody (Nickerson et al., 2010) indicating that TLR signaling is crucial for SLE-like disease in MRL/*lpr*. Combined deficiency of *Tlr7* and *Tlr9* in MRL/*lpr* male mice completely abrogates autoantibody production (Nickerson et al., 2010). In *Nba2* autoimmune mice the combined deficiency of *Tlr7* and *Tlr9* decreases anti-DNA antibody production, but has little effect on anti-RNA associated protein antibodies (Santiago-Raber et al., 2010). This leaves the possibility that another TLR is responsible for the mediation of anti-RNA antibody production. Since TLR8 recognizes ssRNA, it is tempting to propose TLR8 as another mediator of anti-RNA antibody production. Autoantibody production in female 564Igi *Tlr7/9*$^{-/-}$ mice, but not in female 564Igi *Tlr7/8*$^{-/-}$ demonstrates that TLR8 can mediate autoantibody production in the 564Igi model.

IgG autoantibody production in 564Igi mice is partially dependent on TLR7 (Berland et al., 2006). However, autoantibody production is completely dependent on MyD88 in 564Igi mice. These results indicate that another MyD88 dependent factor besides TLR7 mediates autoreactive IgG production in the absence of TLR7. MyD88 is also an adaptor for TLR signaling. In addition, MyD88 mediates IL-1 signaling. IL-1-R deficient 564Igi mice did not have any difference in autoantibody production compared with 564Igi. Therefore, IL-1 signaling is not critical for autoantibody production. Thus, another MyD88-

dependent TLR, in addition to TLR7, must mediate autoantibody production in 564Igi.

TLR8 was once thought to be non-functional in mice (Jurk et al., 2002). Although this has been shown not to be the case (Gorden et al., 2006), it is still thought to be true amongst many in the field. Recent work has begun to define the role of TLR8 in both human and murine SLE. Our data demonstrates that TLR8 can mediate IgG anti-RNA antibody production in 564Igi. Furthermore, TLR8 plays a major role in IFN-I production by neutrophils and their prevalence in the BM of 564Igi mice.

TLR8 in 564Igi mice

TLR8 can mediate the production of autoreactive IgG in 564Igi *Tlr7/9*$^{-/-}$ female mice; nonetheless, TLR8 is dispensable for autoantibody production as there are high levels of IgG autoantibody in 564Igi *Tlr8*$^{-/-}$ mice. TLR8 is responsible for increases in BM neutrophils and IFN-I neutrophil expression in the absence of TLR7 and TLR9. These heightened disease factors occur with low titers of circulating anti-RNA autoantibody. Female 564Igi *Tlr7/9*$^{-/-}$ mice have severe kidney disease, indicating that low levels of autoantibody are sufficient to cause pathology.

Further supporting a key role for TLR8 in SLE is that the identification of a polymorphism in the *Tlr8* promoter has been identified that is associated with

SLE (Armstrong et al., 2009). Also, human TLR8 overexpressed in mice causes autoimmune symptoms similar to arthritis (Guiducci et al., 2013). Together, this suggests that TLR8 may be important for the development of autoimmune inflammation in humans that leads to autoantibody driven diseases such as SLE.

564Igi *Tlr8*$^{-/-}$ mice have high levels of autoantibody in their sera. However, 564Igi *Tlr8*$^{-/-}$ mice have lower numbers of BM neutrophils. Together, the phenotype of *Tlr8*$^{-/-}$ mice demonstrates that, in the absence of TLR8, the production of autoantibodies is not sufficient for a heightened number of BM neutrophils or for increased IFN-I expression from neutrophils on a per cell basis. TLR8 mediates the activation of granulocytes to increase their population and produce IFN-I.

In contrast to our results, another group observed that C57BL/6 *Tlr8*$^{-/-}$ mice had SLE-like symptoms. In the absence of TLR8, TLR7 is upregulated on pDCs (Demaria et al., 2010). An increase in TLR7 could mediate an increase in IFN-I production and drive SLE pathology. We do not observe an increase in TLR7 expression in B cells and neutrophils, or an increase in IFN-I production in neutrophils in the absence of TLR8 in 564Igi mice. Also, in our mouse facility C57BL/6 *Tlr8*$^{-/-}$ do not have autoantibody in their sera at 3 months, contrasting with earlier findings (Demaria et al., 2010). Exposure to infection may be a possible cause of inflammation and autoantibody production in C57BL/6 *Tlr8*$^{-/-}$ mice. A small percentage of 564Igi *Tlr7/8*$^{-/-}$ mice were susceptible to infection after 12 months. Some aged 564Igi *Tlr7/8*$^{-/-}$ mice with clear infections also produce autoreactive IgG. It is possible that in aged C57BL/6 *Tlr8*$^{-/-}$ mice, the

absence of TLR8 increases the likelihood of viral infections. Upon infection B cells could be activated to produce autoantibody. In 564Igi, TLR7 is critical for high levels of autoantibody produced by autoreactive B cells, while TLR8 is critical for high levels of BM neutrophils and IFN-I production by neutrophils.

Cell specific expression patterns of TLR7 and TLR8

The expression of TLR8 is higher on neutrophils than on B cells in C57BL/6 mice, measured by both qPCR and western blot. TLR7 expression is higher on B cells than neutrophils. Together our data suggest that TLR8 plays more of a role on neutrophils, and TLR7 on B cells, in the pathogenesis of 564Igi mice.

Cell type specific expression of TLR7 and TLR8 has been a subject of investigation. TLR8 can be directly responsible for the activation of human granulocytes including both eosinophils and neutrophils (Janke et al., 2009). TLR7 is expressed at very low levels on granulocytes and does not activate these cell types (Janke et al., 2009). Neutrophils express high levels of TLR8 along with cytoplasmic RNA sensors RIG-I and MDA-5. However, they do not express TLR7 or TLR3 (Berger et al., 2012).

In humans, TLR8 is expressed in monocytes/macrophages and myeloid DCs. In fact, between TLR7, TLR8, and TLR9, TLR8 is expressed highest in monocytes (Hornung et al., 2002). TLR8 is, however, not thought to be expressed on pDCs (Bauer et al., 2010; Luber et al., 2010); though, there is a conflicting report claiming that pDCs can be stimulated through TLR8 (Martinez

et al., 2010). TLR7 is highly expressed on human B cells and pDCs (Hornung et al., 2002). TLR9 is expressed highly on pDCs, but at low levels on monocytes/macrophages (Hornung et al., 2002).

It has also been shown that TLR7 and TLR8 have functionally different signaling events when triggered by different ligands (Colak et al., 2014). Both cell type and ligand specificity determines the response after TLR signaling (Colak et al., 2014). This will have important implications in the role of nucleic acid-sensing TLRs in SLE.

Neutrophils and IFN-I production

In mice induced to develop SLE-like symptoms by the chemical pristane, there is a significant increase in monocytes and neutrophils. The increase in cell populations is dependent on MyD88, but not TLR9 (Lee et al., 2008a). The possibility that TLR7 and TLR8 are important for increases in neutrophils and monocytes is a possibility in the pristane-induced SLE model.

There is an increase in neutrophils in the BM, blood and spleen of 564Igi mice compared with C57BL/6 mice. In 564Igi mice, the neutrophils express inflammatory activating FcγRIV *in vivo* (Han et al., 2013). This indicates that neutrophils could be recognizing IgG ICs. In 564Igi *Ifnar1*$^{-/-}$ neutrophils, FcγR expression is diminished compared with 564Igi neutrophils (Han et al., 2013). IFN-I signaling is therefore important in neutrophils for an increased expression

of activating FcγRs (Han et al., 2013). In the sera of 564Igi mice there are high levels of circulating ICs with RNA specific IgG antibody. ICs may lead to an increase in IFN-I production through coengagement of TLRs and FcγRs. 564Igi B cells and neutrophils have upregulated IFN-I signature genes meaning that IFN-I acts directly on neutrophils. This indicates that high levels of IFN-I are acting directly on B cells and neutrophils in 564Igi mice.

Neutrophils have upregulated FcγR-IV, TLR7 and TLR8 in 564Igi compared with C57BL/6 neutrophils. Together FcγR-IV, TLR7, and TLR8 have the potential to recognize RNA ICs and activate neutrophils in 564Igi mice. Activating FcγR receptors are upregulated after inflammatory cytokine stimulation or TLR stimulation *in vitro* (Nimmerjahn and Ravetch, 2005) (Weinshank et al., 1988). Therefore, signaling from TLR7/8 may contribute to the increase in FcγR-IV expression in 564Igi neutrophils.

IFN-I can be produced after stimulation of nucleic acid specific TLRs in the endosome. Alternatively, nucleic acid recognition in the cytosol can also lead to the production of IFN-I. Cytosolic recognition of self-nucleic acids has been implicated in SLE pathogenesis. Cytosolic receptors including RIG-I and MDA5 can recognize RNA. Both of these receptors signal through MAVs leading to IRF and Nf-kB induced IFN-I production. RIG-I binds the 5’ triphosphate on a RNA molecule while, MDA5 binds long dsRNA molecules (Loo and Gale, 2011) (Kono et al., 2013). cGAS recognizes cytosolic self-DNA, and induces IFN-I production through STING signaling (Kono et al., 2013; Sun et al., 2013). Together, cytosolic

recognition of self-nucleic acids is an alternative root for the synthesis of IFN-I with possible implications for SLE.

In 564Igi mice, the most abundant IFN-I-producing cells *in vivo* are neutrophils and monocytes (Han et al., 2013). Previous members of the lab developed a novel ELISPOT assay that detected the abundance of IFN-I-producing cells. This assay demonstrated that in 564Igi myeloid phagocytic cells, such as monocytes and neutrophils, are the major IFN-I-producing cells. On a per cell basis the mRNA transcript level of *Ifn-I* is similar in neutrophils from C57BL/6 and 564Igi mice. In the BM, IFN-I produced by neutrophils is increased in 564Igi compared with C57BL/6 mice, which is due to an increase in the number of IFN-I producing neutrophils. On the other hand, pDCs represented a small percentage of the IFN-I-producing cells in both C57BL/6 and 564Igi mice (Han et al., 2013).

One possible reason that there is no up-regulation of IFN-I on a per cell basis in neutrophils is negative regulation by FcγR2B. FcγR2B is expressed highly in 564Igi neutrophils compared with C57BL/6 neutrophils. Stimulation of FcγR2B by ICs leads to the suppression of cytokine responses, including IFN-I production (Ravetch and Bolland, 2001; Ravetch and Lanier, 2000). *In vitro* stimulation of C57BL/6 neutrophils with ICs leads to very modest to no increase in *Ifn-I* expression. However, when C57BL/6 *Fcγr2b*-deficient neutrophils are stimulated by RNA ICs there are substantial increases in *Ifn-I* mRNA levels. This supports the idea that FcγR2B suppresses IFN-I production in neutrophils. TLR9

will be discussed in a later section as another potential suppressor of IFN-I production in 564Igi mice.

The role of Tlr8 in the sex-disparity of SLE

Females are diagnosed with SLE approximately nine times more frequently than males (Stewart, 1998). Female 564Igi *Tlr7/9*$^{-/-}$ mice have low autoantibody sera titers, a significant increase in the number of BM neutrophils, and severe kidney disease. However, male 564Igi *Tlr7/9*$^{-/-}$ do develop not any of these disease factors. Therefore, 564Igi *Tlr7/9*$^{-/-}$ mice recapitulate the preponderance of disease in females observed in human SLE patients.

Male *Tlr7*/*Tlr9*-deficient MRL/*lpr* mice also do not produce anti-nuclear autoantibodies. Christensen et al 2006, concluded that TLR8 could not mediate autoantibody production in MRL/*lpr* mice. There was no analysis of autoantibody production in female *Tlr7*/*Tlr9*-deficient MRL/*lpr* mice. However, they do mention that female *Tlr7*/*Tlr9*-deficient MRL/lpr mice develop skin rashes, indicating that there may be a similar sex disparity in SLE symptoms (Christensen et al., 2006).

There are many potential factors that contribute to the predilection of SLE in females. Hormonal differences between men and women may lead to increased disease incidence in females. Both men and woman with SLE have similar levels of estrogen as their healthy counterparts (Lahita, 1992a, b). There have been associations, however, with SLE and estrogen metabolism leading to the formation of 16 alpha-hydroxyesterone (Lahita, 1992a, b). Female SLE patients have lower levels of plasma androgens and SLE flares have been

associated with high levels of prolactin (Blanco-Favela et al., 1999). These associations indicate that hormones may play a role in female SLE development, but do not provide a mechanism.

Another possibility is that genes on the X-chromosome contribute to disease in females, but not males. For this to be the case, genes on the X-chromosome would have to be transcribed from both X-chromosomes in females. Both *Tlr7* and *Tlr8* are on the X-chromosome. When human peripheral blood lymphocytes (PBLs) are stimulated with the human TLR7/8 agonist R848 there is an increase in IFN-I production. Interestingly, the production of IFN-I is higher in female PBLs compared with male PBLs treated with R848. (Berghofer et al., 2006).

X-inactivation of *Tlr7* is normal in a human B cell transformed cell line (Berghofer et al., 2006). This does not rule out the possibility that escape of X-activation is cell type or microenvironment dependent. This type of variability and cell-type specificity has been demonstrated for other genes that escape X-inactivation (Carrel and Willard, 2005). It would be interesting to investigate the X-inactivation pattern for *TLR7* and *TLR8* in human PBLs to explain the findings of Berghofer et al., 2006.

In female 564Igi *Tlr7/9*$^{-/-}$ mice there are two potentially active copies of *Tlr8.* One copy of the *Tlr8* gene is not sufficient in either male 564igi *Tlr7/9*$^{-/-}$ or female 564Igi *Tlr8*$^{+/-}$ *Tlr7/9*$^{-/-}$ mice for autoantibody production. This shows that the disparity of disease between male and female mice is due to the difference in

the copy number of *Tlr8*. This implies that in certain situations the *Tlr8* gene may not undergo X-inactivation on one chromosome in females.

If X-inactivation is found to be fully functional for the *Tlr8* gene there is an alternative explanation for the lack of autoantibody production in female 564Igi *Tlr8*$^{+/-}$ *Tlr7/9*$^{-/-}$ mice. It is possible that half of the B cells inactivate the X-chromosome that contains the *Tlr8* gene. These B cells would not express TLR7 and TLR8 and, therefore, they would not produce autoantibody. The remaining B cells would inactivate the X-chromosome that does not contain the *Tlr8* gene. These B cells would be able to produce autoreactive antibody through TLR8. It is possible that only having half the B cells producing TLR8 is insufficient for autoantibody serum titers in 564Igi *Tlr8*$^{+/-}$ *Tlr7/9*$^{-/-}$ mice. Refuting this possibility is the observation that few B cells can be responsible for IgG autoantibody production in 564Igi mice. Future experiments to determine whether the *Tlr8* gene escapes X-inactivation are now underway.

TLR9 negatively regulates IFN-I production by neutrophils

MRL/*lpr* autoimmune *Tlr9*-deficient mice have exasperated SLE-like pathology (Nickerson et al., 2010). Similarly, 564Igi *Tlr9*$^{-/-}$ mice have severe disease. These results were confounding as it was previously shown that TLR9 could mediate the activation of autoreactive B cells *in vitro.* The most common explanation for the increased severity in disease in *Tlr9*-deficient animals is a resulting increase in TLR7-mediated cell activation (Nickerson et al., 2010).

The protein Unc93b1 provides a plausible explanation for the increase in TLR7-mediated cell activation in the absence of TLR9. Unc93B1 is a chaperone molecule that is needed for TLR3, TLR7, and TLR9 signaling (Tabeta et al., 2006). More recently, Unc93B1 was found to bind TLR8 (Itoh et al., 2011). Unc93B1 is involved in the trafficking of nucleotide-sensing TLRs from the endoplasmic reticulum to the endolysosome (Kim et al., 2008).

Unc93B1 preferentially binds to TLR9 over TLR7 (Fukui et al., 2009b). There is a mutated version of Unc93B1 (D34) that preferentially binds TLR7 rather than binding to TLR9. This mutated Unc93B1 increases the expression of TLR7 in the endolysosome. (Fukui et al., 2011). Heightened TLR7 expression leads to anti-RNA-mediated systemic inflammation in mice expressing the D34 mutated Unc93b1 (Sasai and Iwasaki, 2011). D34 mutated Unc93B1 also binds TLR8 preferentially (Fukui et al., 2009b). However, the role for TLR8 in D34 mutated Unc93B1 mice has not been investigated.

Wild-type Unc93B1 preferentially binds to TLR9, leading to heightened cellular sensitivity to DNA antigen (Fukui et al., 2009b). The question is why does changing this balance towards TLR7 (and possibly TLR8) expression lead to autoimmunity? There are many possible explanations. TLR7/8 may lead to a more robust pro-inflammatory response than TLR9. Also there may be more abundant or available TLR7/8 antigens compared with TLR9 antigens. Therefore, TLR7/8 overexpression would lead to greater cell activation compared with TLR9 overexpression due to antigen availability. Lastly, there may be cell type specificity in TLR7/8 and TLR9 responses, meaning that skewing the expression

of one TLR in a particular cell subset may affect the immune response (Sasai and Iwasaki, 2011). There may also be different signaling mechanisms responsible for different responses by each individual TLR.

In 564Igi mice, the absence of TLR9 has an effect on neutrophils. *In vivo*, the absence of TLR9 in 564Igi mice leads to a large increase in the number of BM neutrophils as well as increased expression of IFN-I by neutrophils. *In vitro,* when C57BL/6 *Tlr9*-deficient neutrophils are stimulated by RNA ICs there are substantial increases in *Ifn-I* expression. This supports the idea that TLR9 suppresses IFN-I production in neutrophils. The increase in TLR9 expression in 564Igi neutrophils may be a means to keep IFN-I from being expressed. Furthermore, severe kidney disease was observed in *Tlr9*-deficient 564Igi mice.

It is unclear if intrinsic TLR7/8 signaling in neutrophils is critical for the induction of IFN-I expression and increased cell numbers in the BM. In order to address whether neutrophil and monocyte TLR signaling leads to SLE pathology conditional knockouts of MyD88$^{fl/fl}$ with Ly6Zcre on a 564Igi background have been bred. In these mice Ly6G+ and Ly6C+ cells will have no MyD88 driven TLR signaling. MyD88$^{fl/fl}$/Ly6Zcre will be tested for autoantibody production, granulocyte cell populations, and IFN-I expression.

Concluding remarks

I have defined multiple roles for AID expressed in early developing B cells in an SLE mouse model. The work presented here challenges the prevailing hypothesis that the genesis of pathogenic autoantibody is only dependent on mature B-cell activation by conventional antigens and subsequent SHMs of antibody genes (Shlomchik et al., 1987). My results strongly suggest a novel mechanism for autoantibody production in SLE. Namely, that AID not only has the ability to mediate CSR of pathogenic autoantibody genes in early stages of B-cell development, but that AID expressed in early B-cell development is also critical for expression of IgG autoantibody. Furthermore, the current studies define critical roles for AID in early developing B cells to preserve B-cell tolerance through SHM. In addition, my studies have added to the present understanding of the roles of specific TLRs in SLE.

In this thesis, I present data indicating that TLR8 expression is critical for an increase in granulopoiesis and IFN-I production by neutrophils. TLR9, on the other hand, is a negative regulator for both granulopoiesis and neutrophil IFN-I production. These results indicate that more effective treatment for SLE using TLR antagonists for TLR7 and TLR8. In conclusion, my thesis presents multiple molecular and cellular mechanisms that are critical for SLE pathogenesis in 564Igi and suggest novel therapeutic approaches for SLE. Thus, I believe that this work has made a significant and important contribution to the understanding of the etiology and pathogenesis of SLE.

Materials and Methods

Mice

All experiments with mice were performed in accordance with the regulations and with the approval of Tufts/NEMC IACUC. The creation of 564Igi mice was previously described (Berland et al., 2006, Li, et al., 2001). *Aicda*$^{-/-}$ and *Aicda*^{tg} mice were obtained from Dr. T. Honjo (Kyoto University). C57BL/6, BALB/c, CD21-cre, CD19-cre and *Rag2*$^{-/-}$ mice were purchased from Jackson laboratories. Mb1-cre mice were obtained from Dr. M. Reth. 564Igi was bred to *Aicda*$^{-/-}$ and to *Rag2*$^{-/-}$ mice. 564Igi *Aicda*$^{-/-}$ mice were bred to CD21-cre, CD19-cre and mb1-cre mice on *Aicda*$^{-/-}$ background. The males of these strains were bred to females 564Igi *Aicda*^{tg} on *Aicda*$^{-/-}$ background. This tactic was used to prevent Cre-mediated recombination early during embryogenesis (Schmidt-Supprian, 2007). The offspring were selected by genotyping for Cre, *Aicda*^{tg}, 564 IgH and IgL genes; the mice are designated 564Igi CD21-cre, 564Igi CD19-cre and 564Igi mb1-cre. Experiments were performed with female and male offspring.

The creation of 564Igi mice was previously described (Berland et al., 2006). 564Igi WT mice were bred in house to create homozygous, *Tlr7* deficient (Hemmi et al., 2002), *Tlr9* deficient (Hemmi et al., 2000) *Tlr7*/*Tlr9* double deficient, *Tlr7/Tlr8* double deficient (Valenzuela et al., 2003) and *MyD88* deficient (Adachi et al., 1998) *Aicda*$^{-/-}$ mice were bred in house. C57BL/6 and BALB/c mice were purchased from Jackson laboratories. Experiments were performed with female and male offspring.

ELISA

ELISAs were run to determine the levels of total antibodies, 564 Idiotype$^+$ (Id$^+$) antibodies and anti-RNA antibodies in the sera of various mouse strains. Id$^+$ assays were performed using monoclonal anti-564 idiotypic antibodies (B6-256). B6-256 anti-id antibodies recognizes the combination of 564Ig H and L chains only [54]. B6-256 anti-id were coated on ELISA plates (Immulon 1B) at 5 µg/ml in borate buffer as previously described (Berland et al., 2006). Plates were blocked with 1% BSA in borate buffer and serum samples were added in serial dilutions in 1% BSA blocking buffer. Bound serum antibodies were detected using alkaline phosphatase (AP)-coupled goat-anti-mouse-IgG2a, -IgG2b and -IgM antibodies (Southern Biotech) diluted to 1μg/mL in 1% BSA blocking buffer. The plates were developed with the AP substrate 4-nitrophenyl phosphate disodium salt hexadrydrate (pNPP) at 1mg/mL in 0.1M glycine/1mM $ZnCl_2$/1mM $MgCl_2$ ELISA buffer. For measurement of total serum antibodies of different isotypes, a similar protocol was followed. Wells were coated with isotype-specific goat-anti-mouse-IgG2a, -IgG2b and -IgM antibodies at 1 µg/ml in borate buffer and bound serum antibodies were detected with AP-conjugated isotype-specific goat-anti-mouse antibodies (Southern Biotech) in 1% BSA blocking buffer. Anti-RNA ELISAs were performed according to Dr. Keith Elkon (University of Washington, Seattle, Washington). Nunc MaxiSorb flat bottom (eBiosciences) ELISA plates were coated with 100ml of poly-L-Lysine (Sigma Aldrich #P8920), incubated 4hrs at room temperature, and then coated with 50µl of 10µg/ml of yeast RNA (Ambion/Invitrogen) in borate buffer and incubated overnight at 4°C. ELISA

plates were blocked in 1x borate buffer/ 0.05% tween 20/ 5% goat serum/0.1% NaN_3 and washed 3 times with 1x borate buffer/ 0.05% tween 20. Serum samples were added in serial dilutions in goat serum blocking buffer and developed as for 564 idiotype ELISA. The data was acquired using a Spectra Max 340 ELISA plate reader (Molecular Devices) at the optical density of 405 nm (OD_{405}).

Immunofluorescence Staining of HEp-2 Cells

Fixed human HEp-2 cells (Antibodies Inc.) were coated with mouse serum according to the manufacturer's instructions, and anti-nuclear antibodies were detected using an Alexa 488® goat-anti-mouse IgG secondary antibody (Invitrogen). Slides were mounted with ProLong Gold anti-fade reagent (Invitrogen) and digitally photographed with a Nikon E400 fluorescence microscope. Pixel Fluorescent intensity was measured by MetaXpress® software.

Flow Cytometry

Cells were stained for flow cytometry according to standard procedures. Tissue samples were homogenized to generate single cell suspensions and diluted to $1x10^6$ cells/mL in 1% heat-inactivated rabbit serum / 0.1% NaN_3 / 1X DPBS with Ca^{2+} and Mg^{2+} FACS staining buffer. Cells were centrifuged and re-suspended in 50μL of fluorescent anti-IgM, anti-B220, anti-CD138, anti-CD21, or anti-AA4.1 antibodies (BD Biosciences/Southern Biotech) at 1μg/mL in FACS

staining buffer. Each sample was washed in 2mL FACS staining buffer and re-suspended in 500μL FACS staining buffer for analysis. Propidium iodide (PI) was added to a final concentration of 10ng/mL just prior to analysis on a FACScalibur flow cytometer (BD Biosciences) to assess cell viability.

Isolation of B lymphocytes

Fluorescently labeled B220$^+$ idiotype$^-$ B cells were sorted from the spleen of a *Rag*$^{-/-}$564Igi mouse by flow cytometry using the MoFlo sorter. Cells were stained for flow cytometry according to standard procedures. B cells were stained with B6-256 anti-Id, as described (Berland et al., 2006), which was coupled to the Alexa-647® fluorophore according to the manufacturer's instructions (Invitrogen Molecular Probes). B cells were also stained with an anti-CD45 (B220) antibody labeled with R-Phycoerythrin (PE) from Southern Biotech as a marker for B cells. Fluorescent antibodies were generally used at 1 μg/ml. B220$^+$ GFP$^-$ from 564Igi, 564Igi mb1-cre, 564Igi CD19cre and 564Igi CD21-cre BM and spleen cells were sorted and RNA prepared as described below. B cells from the spleen of an *Aicda*$^{-/-}$564Igi mouse were purified using the EasySep Mouse B Cell Enrichment kit (StemCell 19754), which isolates B cells by magnetic negative selection.

Cloning and Sequencing

Total RNA was isolated from the cells using TRIZOL reagent (Invitrogen 15596-018) and converted to cDNA using the iScript cDNA synthesis kit (BioRad 170-8890). The μ heavy chain and the κ light chain genes were PCR amplified using primers specific for the 564 μ heavy chain and κ light chain genes with the

following sequences: forward 564 VH 5’-CTG-CAA-CCG-GTG-TCC-ACT-CCC-AGG-TC-3’ reverse Cμ 5’-AGG-GGG-CTC-TCG-CAG-GAG-ACG-3’, forward 564 Vκ 5’-CTG-CAA-CCG-GTG-TAC-ATT-CCC-AAA-TT-3’ and reverse Cκ 5’-GCC-ACC-GTA-CGT-TTC-AGC-TCC-3’. The amplified genes were then transfected into competent E. coli cells using the TA cloning kit (Invitrogen K2020-20). Positive transformants were selected based on ampicillin resistance and blue-white colony screening with the **β-**galactosidase gene, *lacZ*. The plasmids were isolated from the bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen 27106) and the presence of the insert was verified by restriction mapping. The inserts were sequenced using the standard M13 forward (5'-TGT-AAA-ACG-ACG-GCC-AGT-3') and M13 reverse (5'-CAG-GAA-ACA-GCT-ATG-AC-3') primers on the ABI 3130XL Automated DNA sequencer.

Antibody production

Cloning strategy, expression vectors, antibody expression in HEK293 cells, and antibody purification were as described (Wardemann et al., 2003)

Real-time PCR

All qPCR experiments were as follows: first-strand cDNA synthesis was performed on four-fold serial dilutions of purified RNA. Triplicates were amplified with commercially available mouse *Aicda* or *Actb* (endogenous control)-specific TaqMan primer/probe sets (Applied Biosystems) in an iQ5

real-time PCR system (Bio-Rad). Quantification was determined using standard curves for genes of interest and the *Actb* control.

Mice

All experiments with mice were performed in accordance with the regulations and with the approval of Tufts/NEMC IACUC. The creation of 564Igi mice was previously described (Berland et al., 2006). 564Igi WT mice were bred in house to create homozygous, *Tlr7* deficient (Hemmi et al., 2002), *Tlr9* deficient (Hemmi et al., 2000) *Tlr7*/*Tlr9* double deficient, *Tlr7/Tlr8* double deficient (Valenzuela et al., 2003)and *MyD88* deficient (Adachi et al., 1998) *Aicda*$^{-/-}$ mice were bred in house. C57BL/6 and BALB/c mice were purchased from Jackson laboratories. Experiments were performed with female and male offspring.

Flow Cytometry

Cells were stained for flow cytometry according to standard procedures. Propidium iodide (PI) was added just prior to analysis on a FACS calibur flow cytometer (BD Biosciences). B6-256 anti-Id was generated as described (Berland et al., 2006) and coupled to Alexa 488 and Alexa 647 according to the manufacturer's instructions (Invitrogen Molecular Probes). Granulocytes in the BM or spleen were stained with Alexa 488 labeled anti-CD11b, and PE labeled anti-Ly6-C or with anti-Ly6-G (Biolegend). CD11b_Ly-6Cin or CD11b _Ly-6G+ represents mainly neutrophils (Zhu et al., 2007). B cells were stained with Fluorescent Anti-IgM, anti- IgG2a, anti-B220 antibodies (Southern Biotech) and

anti-Idiotype Alexa 647 B6-256. Other fluorochrome-conjugated antibodies were from BD Biosciences or Southern Biotech and were generally used at 1 μg/ml.

Cell purification

B cells from bone marrow cells were treated with biotinylated anti-B220 (Biolegend #127604) then separated by magnet after conjugation to Dynabeads® Biotin Binder (Invitrogen #110.47). The remaining cells were treated with biotinylated anti-B220 (Biolegend #103204) and B cells were extracted. Tryzol (Ambion/invitrogen) was added to purified cells and RNA purification performed subsequently.

Quantitative Real Time Taqman PCR

All qPCR experiments were carried out and analyzed using first-strand cDNA synthesis for 3 dilutions of purified RNA. Triplicates for each cDNA reaction were used for amplification with a predesigned mouse *Tlr7*, *Tlr8, Tlr9*- or *β-actin* FAM primer probe (Life Technologies) in a Biorad IQ5 quantitative PCR system. Relative quantification was determined after establishing standard curves for mRNA expression using serially diluted Raw cell cDNA. qPCR data represent 3-4 mice ± SD. Significance was determined by the Student's t-test.

Western Blot

Purified B220+ B cells were re-suspended in 5 times more lysis buffer. Cell lysates were made by slowly rocking the cells for 45 min at 4 degrees Celsius.

After centrifugation at 4 degrees Celsius for 15 min at 5000 rpm the supernatants were collected and boiled at 100 degrees Celsius prior to determination of protein concentration utilizing the 1ml of lysate in a Bradford assay. Lysates were combined with 1x Laemmli loading buffer, and run overnight at 46 volts on a 10% acrylamide gel. The protein was transferred to a nitrocellulose membrane (Thermo Scientific #Z613657- A83) using a semidry transfer apparatuses for 3hrs at 150 volts. After transfer the membrane was blocked with 5% milk in TBS (20mM Tris, 0.14M NaCl, 0.1% Tween 20 pH 7.6-8.0) for 1 hr and then washed with TBST 3 times for 5 min each. The membrane was then incubated for 1-2 hrs at room temperature with rabbit polyclonal anti-TLR7 (Abcam #ab45371) or anti-TLR8 antibody (Abcam #ab24185) and with mouse anti-β-Actin monoclonal antibody (Sigma #A3441). The membrane was washed 3 times for 5 min each wash in TBST. The membrane was incubated for 1hr with goat anti-mouse IgG-HRP (Thermo Scientific #31432) antibody diluted 1:5000 in TBS and anti goat secondary IgG-HRP (Santa Cruz Biotechnology) antibody diluted 1:5000 in TBS and then washed for 15 min 3 times in TBST. West Pico Chemiluminescent substrate (Thermo Scientific #34079) was placed on the membrane for 5 sec then the membrane was wrapped in plastic. Autoradiography film (Denville Scientific #E3018) was placed in a cassette with the membrane for less then 1min and developed on a Kodak M35A X-OMAT processor.

C1q immune complex (IC) binding ELISAs

Levels of serum immune complexes were determined using a protocol provided by Dr. Ricardo Gazzinelli (UMass Medical School, Worcester, MA). ELISA plates were coated with 5 μg/ml of C1q (Complement Tech) overnight at 4°C, blocked with 1% BSA in PBS, and washed 3 times with 1xPBS/ 0.05% Tween 20. Serum samples, or immune complexes, were added and incubated for 1hr at 37°C. Detection of IC with AP-labeled anti-IgG2a and anti-IgG2b (Southern Biotech) was as previously described. Immune complexes were made by incubating C57BL/6 bone marrow cells RNA with 564 hybridoma (IgG2b) and S7.1 hybridoma (564 IgG2a) monoclonal antibodies (RNA-specific) at a 1:10 ratio for 3 hrs at room temperature.

Immunofluorescence and Light Microscopy Analysis of Kidney Sections

Fresh kidney samples for immunofluorescence studies were frozen in OCT and 4μm sections cut on a cryostat and mounted on glass slides. The sections were air-dried for 1hr, dehydrated in PBS, and incubated with Alexa 488 anti-idiotype antibody solution for 1hr at room temperature. The sections were rinsed in PBS, mounted in Aquamount, and examined and photographed with a Zeiss fluorescence microscope.

For light microscopy, fresh kidneys were fixed in 10% buffered formalin, and embedded in paraffin. 5 μm paraffin sections were stained with periodic acid-Schiff (PAS) and evaluated blindly by light microscopy.

To score the PAS sections for morphologic evidence of lupus, the clinical scale of the International Society of Nephrology/Renal pathology Society (ISN/RPS-2003) was used. Classification of Lupus Nephritis was used (1 through

6). Class 1 indicated minimal mesangial lupus, with normal glomeruli as seen with light microscopy, but with mesangial immune deposits revealed by Immunofluorescence. Class 2 is indicated by mesangial proliferative lupus nephritis; class 3 is focal lupus nephritis, class 4 is diffuse lupus nephritis, class 5 is defined as membranous lupus nephritis and class 6 indicated advanced sclerotic nephritis where greater than 90% of the glomeruli are globally sclerosed (Gupta et al., 2005). IC deposits were evaluated by immunofluorescence and scaled from 1 to 3, 3 being the highest degree of deposits. At least 10 glomeruli were examined for negative samples (Gupta et al., 2005).

ACKNOWLEDGEMENTS

First and foremost I would like to thank my thesis advisor, Dr. Thereza Imanishi-Kari. She is a wonderful mentor, advisor and friend and her support and care have been unwavering since I joined the lab.

I would like to thank the members of my committee, Dr. Erik Selsing, Dr. Henry H. Wortis, and Dr. Noami Rosenberg. The committee provided me with helpful advice and criticisms. I would also like to thank my outside examiner, Dr. Eric Meffre, for accepting my invitation to evaluate my dissertation.

Many wonderful people in our laboratory made this possible. Gabrielle McDondald has been particularly helpful as my fellow student. Amma Labri, Luis Fernadez and Parimal Korgaokar were very helpful as I first entered the lab. Nick Cabal provided a large amount of work that was presented in this thesis and helped with critical reading. Carlos Medina, Marisa Zuluaga, Julian Quinones, and Steven Kusi were all great help during their stays in lab. Angel Leung was a great friend and colleuge in the neighboring lab. Rotating students also contributed to my work including Maria Repass, Caitlin Lui, Megan McPhillips and Nicole Castagnozzi. Former members of the lab, Jin-Hwan Han and Shauna Andersson were critcial for my projects and publications. I would also like to thank Steven Kwok, Allen Parmlee, Di Pierce and Albert Tai for their kind support and hard work.

I would like to thank my parents, brother and sister for all they have done. Lastly, I would like to thank Holly Ponichtera for her encouragement, care, affection, advice and critical reading of this dissertation.

The following initials were used to give credit for contrbutions in particular figures in the results section:

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