The Role of TLR7/8 in Autoantibody Production and Granulopoiesis in Murine SLE

A Dissertation

submitted by

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that is characterized by the presence of IgG autoantibodies to nuclear antigens in the sera of patients. Utilizing a novel SLE mouse model "564Igi" with knocked-in genes for the heavy and light chain of an anti-RNA autoantibody, our laboratory has previously reported that autoantibody production in 564Igi is T cell independent and partially dependent on the RNA receptor TLR7. In this thesis I provide evidence that female 564Igi mice with intact (X-linked) *Tlr8* but deficient in *Tlr7* and *Tlr9*, have more IgG bearing lymphocytes and produce more autoantibody than males. This phenotype is reminiscent of human lupus where the disease occurs in females nine times more frequently than in males. Additionally I also show that the concurrent deficiency of *Tlr7* and *Tlr8* in 564Igi mice leads to a drastic reduction of serum IgG2a and IgG2b autoantibodies, similar to what is observed in *Myd88* deficient mice. Lastly, I have evidence that granulopoiesis, which is observed in both human SLE patients and in 564Igi mice, is *Tlr7/8* and *Myd88* dependent and IL1R type 1 independent. Collectively the data indicate that TLR8 in mice is important for the pathogenesis of SLE. The phenotypic similarities described in this thesis between human and mouse SLE suggest that the potential involvement of TLR8 in human SLE should be seriously considered.

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Abbreviations

AID	Activation-induced cytidine deaminase
ANA	Anti-nuclear antibody
AP	Alkaline phosphatase
APC	Antigen presenting cell
BAFF	B-cell activating factor
BAFFr	B-cell activating factor receptor
BCR	B-cell receptor
BM	Bone marrow
C	Complement
DC	Dendritic cell
DKO	Double Knock-out
ds DNA	Double stranded DNA
dsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activating cell sorting
HSC	Hematopoietic stem cell
IC	Immune complex
Id	Idiotype
Ifi202	Interferon-inducible gene, 202 kDa
IFN-I	Type I interferon [IFN-α, interferon-α; IFN-β, interferon-β]

Ig	Immunoglobulin
IL-1RA	Interleukin type-1 receptor antagonist [IL-1R1, IL-1α, IL-1β]
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITAM	Immunoreceptor tyrosins-based motif
κ	kappa
KO	Knock-out
λ	lambda
MyD88	Myeloid differentiation primary response gene 88
MZ B cells	Marginal zone B cells
NK	Natural killer cells
PAMPs	Pathogen associated molecular patterns
PAS	Periodic acid schiff
pDC	plasmacytoid dendritic cells
PnP	*p*-Nitrophenyl Phosphate
qPCR	Quantitative polymerase chain reaction
RAG	Recombination activating Genes
RSS	Recombination signal sequences
SEM	Standard error of the mean
SHM	Somatic Hypermutation
SLE	Systemic Lupus Erythematosus
ssRNA	single stranded RNA
SPL	Spleen
SHp-1	Src homology domain containing phosphatase 1

Tg	Transgene
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VDJ	Variable, Diversity and Joining gene sections
WT	Wild Type
Yaa	Y-linked autoimmune accelerator

Chapter I: Regulation of autoantibody production in 564Igi mouse model of SLE

Overview of Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, which can affect any organ. In humans, some of the symptoms of SLE include a malar rash, also known as a butterfly rash, fever, fatigue, photosensitivity, generalized malaise and arthritis. These symptoms are nonspecific, unpredictable and can flare at different points in time making diagnosis difficult. SLE most commonly affects the heart and lungs, blood vessels, joints, skin, liver, kidneys and the nervous system (Pisetsky, D.S., 2007).

Due to the fact that many symptoms of this disease are common features of a number of other diseases, not all of them are used as diagnostic criteria. In order to diagnose SLE, there are eleven criteria of which four must be fulfilled. These are: Malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, hematologic disorder, anti-double stranded (dsDNA), anti-Smith (Sm), and/or anti-phospholipid and antinuclear antibodies (Sabatine M.S. 2007). There is no cure for SLE, and the disease can be fatal. The development of immunosuppressive and anti-inflammatory drugs has made survival rates quite high and fatalities rare in the developed world (Rahman A. and Isenberg D.A., 2008, Pisetsky D.S., and Rönnblom L. 2009).

Patients with SLE have auto-reactive B cells that predominantly produce high levels of immunoglobulin (Ig) G autoantibodies. Defective clearance of these autoantibodies allows for circulating antibodies to bind self-antigens forming immune complexes. These immune complexes eventually deposit into end organs, such as the glomeruli of kidneys, where they can activate effector cells to produce cytokines and chemokines. These will attract other immune cells such as neutrophils that will result in inflammation, leading to tissue damage as evidenced in the kidney with lupus nephritis (Pisetsky, D.S., 2007).

The population most affected by SLE is women of childbearing age. While all women are at risk, Asian and African American women are five times more likely to develop this disease than their Caucasian counterparts. SLE affects females nine times more frequently than males, implicating sex hormones in the pathogenesis of SLE (Rahman A. and Isenberg D.A., 2008). There has been a great deal of research done on the role and effect of sex hormones on the development and severity of disease. One such study indicated that estrogen-induced autoimmunity might be due to the DNA instability caused by dsDNA breaks made by activation induced cytidine deaminase (AID) (Paulkin *et al.* 2009). Another important factor in the development of SLE is genetics. Human studies on monozygotic twins have shown the concordance of SLE to be 25%. This indicates that genetics, while important, is not the only determinant for the development of SLE.

B-cell Development

One initiating event of SLE is the production and retention of auto-reactive B cells. In order to explore the origins of these auto-reactive B cells, it is crucial to understand the development of the B cell lineage.

V (D) J recombination

B cells originate as hematopoietic progenitor cells and differentiate with the help of stromal cells that provide the appropriate microenvironment for B cell maturation. One important aspect of B-cell development and adaptive immunity is to establish antibody diversity, which allows for the recognition of a high variety of pathogens. A high degree of B cell receptor (BCR) diversity is achieved through V (D)J recombination. The immunoglobulin heavy chain locus is composed of variable (V), diversity (D), and joining (J) gene segments, which recombine to form a single variable region of the heavy chain. Similarly two light chain loci λ and κ have several V and J gene segments that assemble to form the light chain of the BCR. In a process known as allelic exclusion most often functional rearrangement occurs at only one allele at a time, preventing further rearrangement of the second allele (Inlay and Xu 2003). V(D)J recombination is mediated by Recombination Activating Genes, RAG-1 and RAG-2, which carry out recombinase function upon binding to recombination signal sequences (RSS) located on either side of each V (D)J gene segment (Bassing *et al.* 2002; Inlay and Xu 2003).

B-cell development is a highly regulated process. Heavy chain rearrangement occurs at the pro-B cell stage, and the cell progresses to the next stage only if the heavy chain is functional and associates with a surrogate light chain. Only then does the light chain locus begin to rearrange. The process continues until a functional BCR is formed (Figure 1).

The high degree of diversity achieved as a result of V(D)J recombination allows for the immune system to respond to a vast array of pathogens: however this unfortunately also results in the production of a significant number of self-reactive B-cells. It has been shown that 55% to 75% of emerging early immature B cells are auto-reactive, the majority of which are removed from the B cell repertoire in healthy individuals (Wardemann *et al.* 2003).

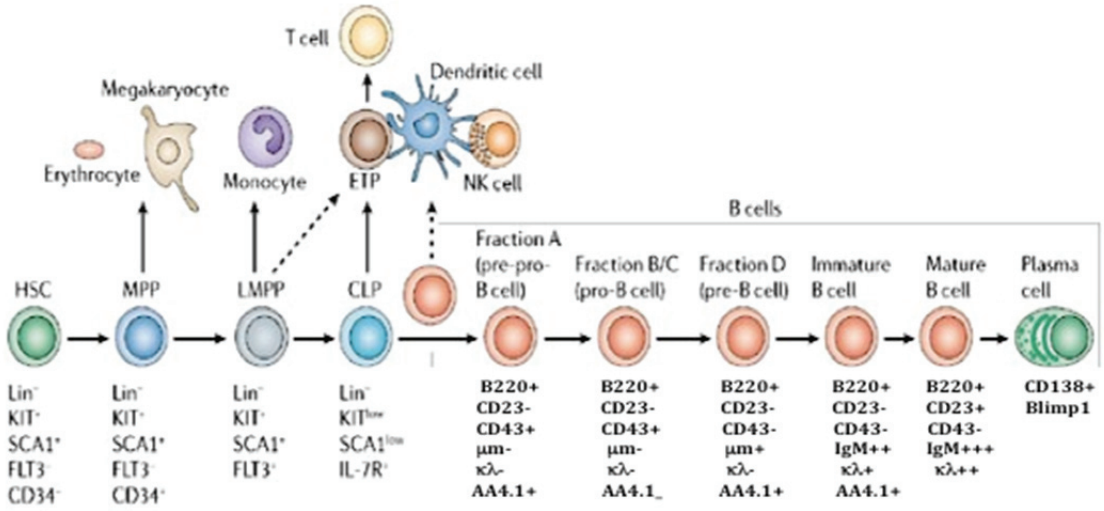

Figure 1: B-cell development in the bone marrow

Hematopoietic stem cells (HSC), identified by the listed stem cell markers, differentiate into multipotent progenitor (MPP) cells. MPP cells can either progress to terminally differentiated erythrocytes and megakaryocytes or

lymphoid-primed multipotent progenitor (LMPP) cells. LMPP cells go on to differentiate into common lymphoid progenitor (CLP) cells or monocytes. The CLP gives rise to T-lymphocytes, B-lymphocytes, natural killer cells and dendritic cells. Each of these stages of development can be identified by differences in surface markers.

B220, a pan-B cell marker, is expressed from pre-pro B cell stage until the mature cell stage of differentiation. CD23 is expressed during transitional and mature cell stages, while CD43 is expressed only at the pre-pro B cell and pro-B cell stage. Mυ heavy chain is expressed at the pre-B cell stage, while surface IgM is expressed from immature to mature stage. AA4.1 is a maker that identifies immature B cells from pre-pro-B to surface IgM positive immature B cells. Blimp-1 is a transcription factor expressed by pre-/plasma cells. (Figure adapted from Nature Reviews Immunology 2006)

Antibody production

Unlike the innate immune response that relies on a general pattern of pathogen recognition, the adoptive immune response leads to the production of antibodies that are highly specific for pathogens. B cells produce antibodies upon proper activation. There are two main pathways for B-cell activation: T-cell dependent and T-cell independent.

During the T-cell dependent response, antigen presenting cells (APC), such as dendritic cells, travel from the site of infection to the secondary lymphoid organs where they present antigen to naive T cells via the major

histocompatibility complex (MHC) class II. Antigen-specific T cells become activated and differentiate into armed effector T cells, either T-helper 1(T_H1) or T-helper 2(T_H2), depending on the nature of the APC. Effector T cells can in turn activate B cells by providing co-stimulatory signal through CD40-CD40 ligand interaction. The activated B cell proliferates, forms germinal centers where it undergoes affinity maturation and differentiates into either clonal pre-plasma cell, capable of making antigen-specific antibodies, or a memory B cell, available for a rapid secondary response.

The T-cell independent B-cell activation is divided into two types: Type 1 and Type 2. Type 1 leads to the activation and proliferation of certain B cells (marginal zone (MZ)) to produce polyclonal low affinity IgM in response to poorly degradable large molecular weight antigens such as 2,4,6-trinitrophenyl-aminoethylcarboxymethyl lipopolysaccharide (TNP-LPS) (Ishida et al. 2007). Type 2 relies on macrophages' or natural killer cells' help to present multivalent antigens in a manner that leads to antigen receptor cross-linking.

The mechanism of T-cell independent type 2 B-cell activation relies on the formation of a few antigen receptor clusters on the surface of the B cell which then interact with Brutons tyrosine kinase (BTK), leading to prolonged calcium flux (Vos et al. 2000, Mond et al. 1995). This persistent calcium flux is believed to be the first signal that allows B-cell activation and proliferation. Engagement of toll-like receptor (TLR) family members has been shown to be important for the second (costimulatory) signal that leads to B-cell secretion of antibodies. T-

independent activation combines the speed of the innate immune response with the specificity and strength of the adaptive immune response (Vos et al. 2000).

Cellular markers of B cell development

As B cells develop and undergo differentiation they express cell surface markers that indicate where they are in the developmental timeline. These surface markers have been utilized by a generation of scientists to differentiate cells, determine cellular percentages in various organs and also to identify and track diseases that are either caused by or affect the hematopoietic compartment. Many of these markers, such as CD45R also known as B220, are found on B-cells from the earliest B-cell progenitor until the mature B-cell stage as well as on activated T cells and lymphokine activated killer cells (LAK) (Coffman 1982) (Figure 1).

However, there are many markers that are unique to certain cell types and to cells that are at certain stages of development. For example, AA4.1 recognizes mouse C1qRp a c-type lectin-like transmembrane protein, which is an early B-cell marker as well as a marker of megakaryoblasts and platelets (Mckearn *et al.* 1984). Another marker of early B-cell development is CD43. CD43 also known as sialophorin, is an anti-adhesive molecule and is thought to be involved in leukocyte repulsion. CD43 is found on pro B cells (fraction A-C), mature T cells, NK cells, platelets and myeloid lineage cells (Figure 1 and Pallant *et al*. 1990). Not all markers begin at an early stage and then recede. CD23 is a low affinity receptor for IgE and its activity is modulated by B-cell activation. This

marker is only found on mature B cells and transitional B cells (Figure 1). B cells can also be identified by their BCR using anti-IgM and anti-IgG or anti-IgA for cells that have undergone class switch recombination (CSR).

B-cell tolerance mechanism

Two main checkpoints lead to B-cell tolerance: Central tolerance is when self-reactive B cells are removed before the cell exits the bone marrow, and peripheral tolerance, which occurs in secondary lymphoid tissues and blood vessel. Loss of tolerance leads to the development of self-reactive B cells and autoimmune disease. Treatment regimens involving the depletion of B-cells in patients with lupus and rheumatoid arthritis have recently proven effective, highlighting the importance of understanding how B cells contribute to the break in tolerance to self-antigens (Sanz *et al.* 2007, Crampton *et al.* 2010).

Approximately 55%-75% of early immature B cells in the bone marrow (BM) are self-reactive (Wardemann *et al.* 2003), and are eliminated through the process of negative selection. During B-cell development the BCR of developing B cells are tested to determine if they are responsive to self-antigens. Lymphocytes with BCRs that have a strong interaction with self-antigen undergo deletion (Nemazee and Bürki 1989). BCRs that have a weak interaction with self-antigen, either undergo receptor editing or anergy (Gay et al. 1993; Tiegs et al. 1993; Goodnow et al. 1988).

Receptor editing is a process where the self-reactive BCR undergoes secondary V(D)J rearrangement or somatic hypermutation in the attempt to

create a new non self-reactive BCR (Han *et al.* 2007; Mao *et al.* 2004). One important consideration of continuous receptor rearrangement during B-cell development is the increased risk of allelic inclusion, which is defined as a B cell expressing more than one BCR (Sonoda *et al.* 1997). An interesting fact is that self-reactive B cells originally associated with a kappa (κ) light chain have been found to express another light chain namely, lambda (λ) on the same cell (Hasan et al., 2002; Oberdoerffer et al., 2003).

Utilizing the second BCR on the cell surface, self-reactive B cells can escape the selection process and possibly become an antibody-secreting cell. Such a cell could contribute to the development of autoimmune disease (Liu et al., 2005). If receptor editing proves successful and the BCR ceases to be self-reactive, the B cell is then allowed to continue through the checkpoint. If, however the B cell still has an auto-reactive BCR the cell is then deleted or becomes anergic (Nemazee and Bürki 1989; Goodnow et al. 1988; Goodnow et al. 2005). By this method, in normal mice a significant number of functional self-reactive cells are eliminated. In a recent report it was determined that immature B cells are preferentially tolerized to membrane antigens by receptor editing rather than undergoing deletion (Halverson et al. 2004). Without effective secondary rearrangement the percentage of self-reactive B-lymphocytes would increase drastically.

Anergy is defined as functional unresponsiveness to activating stimuli preventing B-cell differentiation. An anergic B cell has several identifying characteristics such as: B-cell receptor downregulation on the cell surface,

exclusion of the anergic B cells from follicles, decreased cell survival due to weak activation of NF-κB1, expression of the cell surface marker CD5 to recruit SHP1, leading to inhibition of BCR activation and lack of calcium flux in response to BCR cross-linking (Goodnow *et al*., 1988; Goodnow *et al*., 2005, Crampton *et al.* 2010). If any cell escapes central tolerance they are still subject to the second checkpoint of peripheral tolerance (Basten *et al*., 1991; Hartley *et al*., 1991: Crampton *et al*., 2010).

During peripheral tolerance, B cells must undergo both positive and negative selection in order to survive and mature. (Stadanlick and Cancro 2008). Unlike central tolerance where the avidity of BCR binding to self-antigen determines whether a B cell is deleted, receptor edited or becomes anergic, in the periphery a partnership between BCR interaction and B-cell activating factor (BAFF) signaling determines B-cell maturation and survival. In the periphery, B cells compete for resources in order to survive and undergo further differentiation.

Using mixed bone marrow chimeras it was shown that cells with transgenic (Tg) self-reactive BCRs could survive in the absence of competition. When these cells were mixed with non-transgenic B cells (normal in regards to receptor diversity), self-reactive cells died rapidly, unable to effectively out-compete the non Tg B cells for vital survival resources (Cyster *et al.* 1994). One such resource is BAFF/BLyS (Harless *et al.* 2001). BAFF is important for maintenance of normal peripheral B cell populations, as BAFF/BAFF receptor (BAFFr) engagement dictates mature B cell lifespan (Schiemann *et al.* 2001; Yan

et al. 2001;Harless *et al.* 2001). BCR signaling is still important for peripheral B cell differentiation and survival, however the signal strength required for successful differentiation varies and is dictated by the amount of available BAFF (Miller et al. 2006; Stadanlick and Cancro 2008). These various tolerance mechanisms have evolved to reduce the overall percentage of self-reactive lymphocytes in the circulation. However in about 5% of the population these mechanisms are not functional, and autoimmune diseases result.

Class switch recombination

The production of IgG autoantibodies in SLE is critical to the pathology of the disease. In order to activate B cells to switch from producing IgM antibodies to other classes of antibodies (IgG or IgA etc.), B cells must undergo the process of CSR. This recombination alters the antibody effector function without changing the antigen specificity. Antibody effector functions are encoded within the constant region of immunoglobulin heavy chain (IgH) locus. CSR involves DNA breaks which are generated in switch regions located upstream of constant gene segments and then joined through recombination events. This allows downstream constant gene segment to come into close proximity with the antigen specific variable gene segments (Stavnezer 2008 and Figure 2).

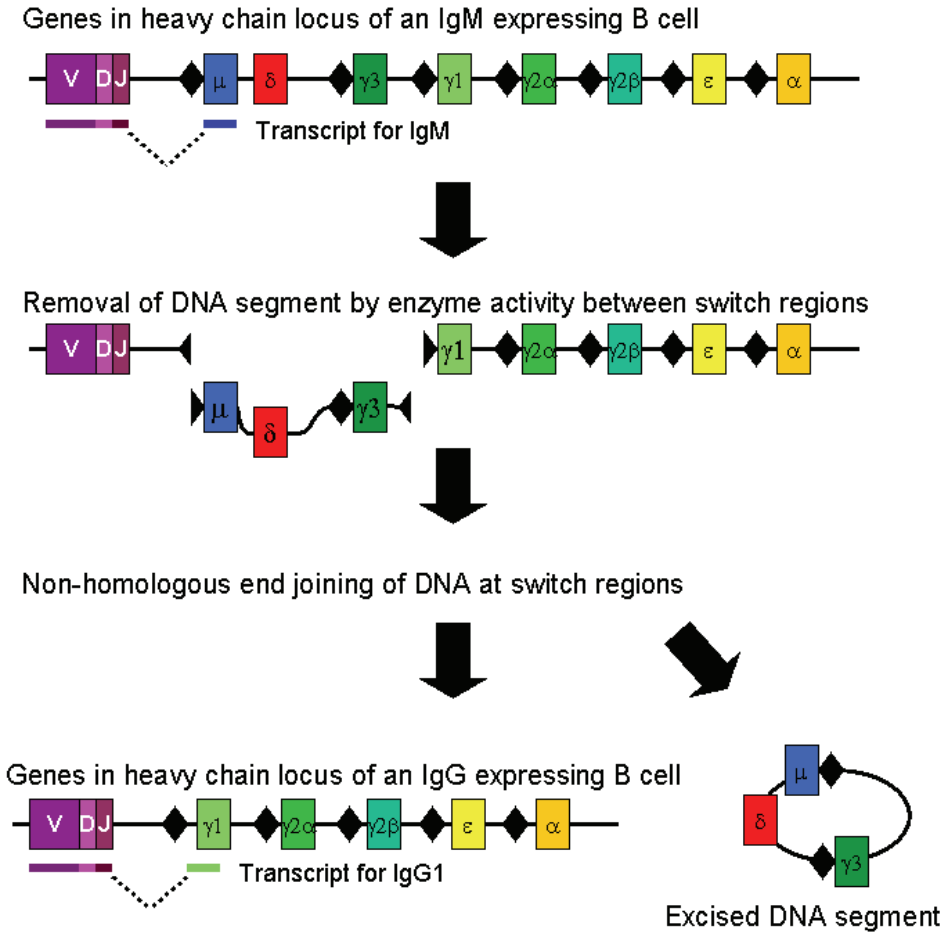

Figure 2: Rearranged heavy chain locus undergoes class switch recombination from IgM to IgG1

Activation induced cytidine deaminase (AID) activity between switch regions causes excision of the upstream DNA segment containing mυ, delta and gamma that then forms a short-lived circle transcript. DNA repair mechanism such as non-homologous end joining repairs the double strand break and the VDJ region is now within close proximity to the gamma 1 gene segment.

Activation induced cytidine deaminase (AID) is responsible for both SHM and CSR (Muramatsu et al., 2000). AID has been shown to convert cytosine to uracil in single-stranded DNA (Chaudhuri et al., 2003; Dickerson et al., 2003; Muramatsu et al., 2000; Petersen-Mahrt et al., 2002). AID introduces U-G nucleotide mispairing in switch region which are thought to recruit DNA repair factors such as mismatch repair and base excision repair proteins and act as substrates for these DNA damage repair proteins. Current models suggest that activity of base excision repair and mismatch repair proteins at AID-induced U-G mispairings leads to the generation of DNA breaks needed for the recombination events involved in CSR and SHM (Imai et al., 2003).

Briefly SHM is initiated, when upon B-cell activation, the action of AID leads to U-G mispairs. Aberrant repair of these mispairs lead to a mutation rate that is one hundred thousand to million times higher than spontaneous mutation rates of other genes (Wang *et al.* 2004). These mutations can lead to further diversification of immunoglobulin genes and the production of high affinity antibodies that can be used to protect the host against pathogen attack (Gearhart *et al.* 1981).

During CSR, AID is required for the generation of DNA breaks in switch regions (Muramatsu et al., 2000). There are five classes of immunoglobulins in humans and mice, in order they are IgM, IgD, IgG, IgE and IgA. The different immunoglobulin isotypes have different properties, enabling B cells to produce antibodies with the same antigen specificity but different effector functions. Antibody effector functions include the ability to neutralize pathogens and toxins

by blocking their entry into host cells as well as enhancing phagocytosis through opsonization or via complement activation. Another function is to facilitate the destruction of pathogens by inducing natural killer (NK) cell mediated cytotoxic killing. The antibody classes differ in half-life, ability to activate complement, and their ability to bind various Fc receptors and trigger different innate immune responses (Snapper *et al.* 1993).

When a B cell is first activated by antigen, IgM is secreted. IgM has a short half-life and tends to be a low affinity antibody whose effects depend on activating complement factors (Heyman et al. 1988; Hjelm et al. 2006). One interesting aspect of IgM is its ability to form pentamers, which generates ten antigen binding sites per molecule. IgM is also effective at complement fixation (Stavnezer 1996a). IgA can form dimers and are found at high levels in respiratory, digestive and urogenital secretions, making it a primary defense against ingested or inhaled pathogens (Monteiro and Van De Winkel 2003). IgE is detected at low levels in serum and is found mainly associated with FcεRs on mast cells. This isotype is involved in mediating hypersensitivity reactions and host defenses against parasite infections by inducing degranulation of mast cells when bound to antigen (Stavnezer 1996b).

Lastly, IgG is the most abundant isotype found in the blood and lymph and it can be sub-divided into four sub-classes known as IgG3, IgG1, IgG2b and IgG2a in mice. In murine SLE, IgG2a and IgG2b (IgG1 and IgG3 in humans) are the most abundant autoantibody subclass detected in the sera and they have been implicated in tissue damage. IgG provides long term immunity and

protection, and it also can cross the placenta barrier providing immunity for the fetus.

The IgG subclasses bind to Fc receptors with varying affinities. Such receptors include, the activating FcγRI, FcγRIII, FcγRIV as well as the inhibitory FcγRIIB, which are differentially expressed on innate immune cells. Interaction of IgG antibodies with FcγRs can trigger phagocytosis in macrophages, neutrophil activation, inhibition of B-cell activation and NK cell cytotoxic killing (Ravetch and Bolland 2001). In addition, interaction of IgG immune complexes with FcγRs on dendritic cells (DC) can trigger internalization of the immune complexes and subsequent MHC-class-II-restricted antigen presentation and cross presentation on MHC class I molecules, thus priming both CD4 and CD8 T cell responses (Regnault et al. 1999). It is also important to note that in mice, activation of neutrophils require FcγRIII / FcγRIV and these cells are highly specific for immune complexes (IC) formed with antigen and IgG2a and IgG2b (Nimmerjahn *et al*., 2005; Janus *et al.,* 2008). The multiple functions of IgG allow for modulation of both the adaptive and innate immune system effector responses.

General features of the innate immune system

The innate immune system is found in all classes of animal life. It has evolved to immediately respond to infection, however, it does not confer long lasting or protective immunity in the host organism. The receptors of the innate immune system recognize conserved molecules that are shared by a large number of pathogens and are essential for pathogen survival (Medzhitov and Janeway 1997). The cells that compose the innate immune system are granulocytes (composed of neutrophils, eosinophils, and basophils), mast cells, macrophages, dendritic cells (DC), monocytes, NK cells and gamma delta T cells.

The Innate immune system is unrivaled in the speed at which it responds to infections. Upon infection, the innate immune response leads to inflammation, mediated by the chemicals released by injured or infected cells. These chemicals (histamine, bradikinin, serotonin, leukotrienes and prostaglandins) lead to vasodilation, pain and recruitment of phagocytes to the site of active infection. These phagocytes consume and destroy pathogens and present antigen to the cells of the adaptive immune system. These adaptive immune cells secrete cytokines and chemokines to enhance the immune response (Janeway *et al.* 2001).

Toll like receptors and their ligands

Toll like receptors (TLR) are part of the innate immune system and are activated by pathogen associated molecular patterns (PAMPs). TLRs are located either on the outer cell membrane or, in the case of nucleic acid sensing TLRs, in endolysosomal compartments located within the cell. This endosomal localization is important for the function of these receptors as well as helping to ensuring self/non-self discrimination (Gay et al. 2006; Barton et al. 2006). As there are always cells undergoing apoptosis in the body, the possibility of mistaken activation of nucleic acid sensing TLRs would be far greater if these TLRs were localized on the cell surface.

Nucleic acid sensing TLRs are closely related in sequence in that they all have conserved regions, such as an insert in LLR8 that is cysteine-rich (Gay *et al.* 2006). These TLRs' also share their mechanism of transport from the endoplasmic reticulum to the endolysosomes. This transport mechanism is regulated by a molecule encoded by the Unc93B1 gene that binds to and directs the transport of TLR3, 7, 8 and 9 in both dendritic cells and B cells (Fukui *et al.* 2009; Brinkmann *et al.* 2007). Toll receptors were initially discovered in Drosophila and were later found to be conserved in humans (Anderson et al., 1985a; Anderson et al., 1985b). Human TLRs are type I transmembrane proteins with a leucine-rich repeat (LRR) domain in the extracellular portion and an IL-1 receptor (IL-1R) homologous domain in the cytoplasmic region.

Utilizing a mouse CD4/hToll chimera fusion with a constitutively active mutant of hToll transfected into a human monocyte cell line, it was shown that

hToll activated NF-κB. NF-κB activation in this system also led, to increased expression of inflammatory cytokines, such as IL-1, IL-6 an IL-8 (Anderson et al., 1985a; Anderson et al., 1985b). This finding indicated that TLRs trigger downstream signaling cascades that ultimately result in the initiation and persistence of the host inflammatory response. It is largely accepted that most mammalian species have between10 to 15 TLRs. TLR ligands were identified by experiments done on mutant and transgenic mice: TLR4 detects LPS (Poltorak et al., 1998), TLR2 detects bacterial lipoproteins (Takeuchi et al., 2000), TLR5 detects flagellant (Hayashi et al., 2001), TLR9 detects the unmethylated CpG DNA of bacteria and viruses (Hemmi et al., 2000), TLR3 detects double-stranded RNA (Alexopoulou et al., 2001) and TLR7 and TLR8 detect single-stranded viral RNA (Lund et al., 2004).

Both the TLR and the IL-1R signaling pathways utilize the same downstream components, with the exception of TLR3. One important protein that serves as a master adapter/regulator of TLRs and IL-1R is myeloid differentiation primary response gene 88 (MyD88) (Medzhitov *et al.,* 1998; Browne and Littman 2009 and Figure 3). MyD88 is known to be involved in T cell independent autoimmunity (Groom *et al.,* 2007). It has also been shown that MyD88 deficiency reduces autoantibody production in autoimmune MRL/*lpr* mice (Nickelson *et al.,* 2010).

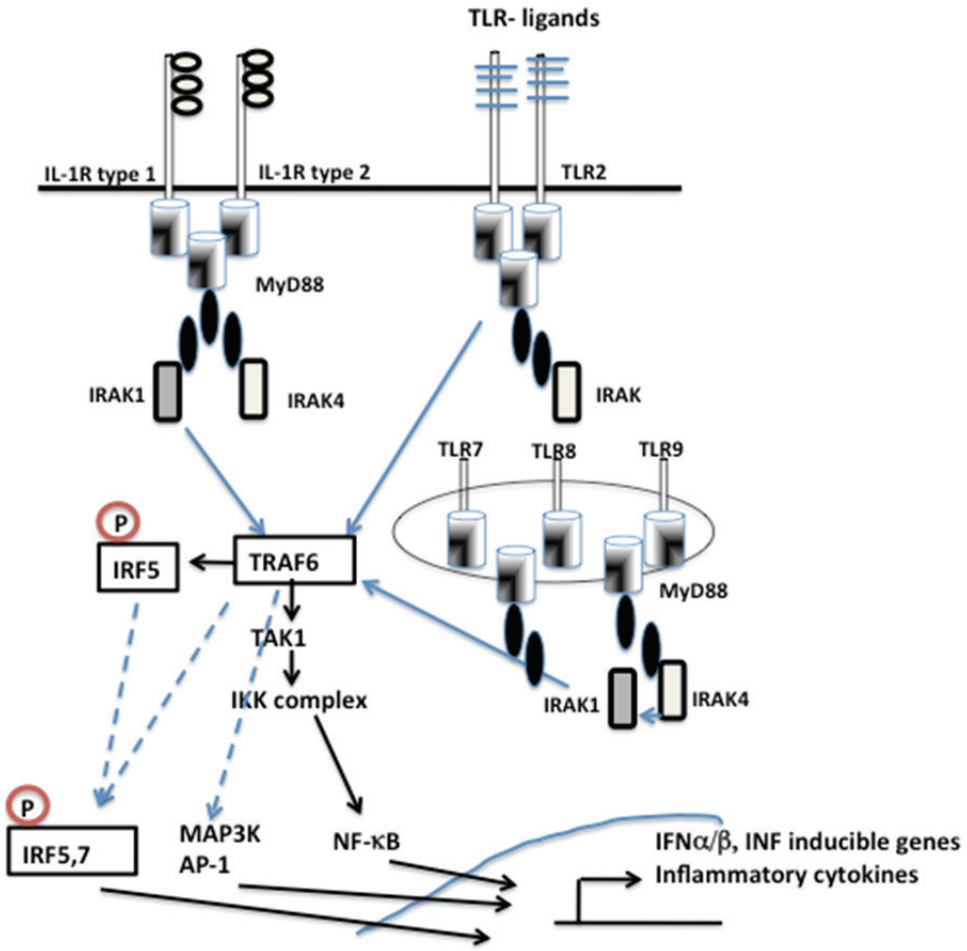

Figure 3: TLR/ IL-1 receptor signaling cascade

Depicted is a cartoon of the MyD88 downstream signaling cascade and the interconnection of the TLR and IL-1R signaling pathway. Upon activation of either TLR or IL-1R the adapter protein MyD88 interacts with IRAK-1 and IRAK-4 through its death domain resulting in phosphorylation of those molecules. IRAK-1/IRAK-4 then activate TRAF-6 which is ubiquitinated ultimately leading to either the phosphorylation of IRF5 or IRF7, or activation of TAK1 through TAB-2 resulting in either the activation of MAP kinases and AP-1 or the IKK complex

which activates NF-κB. Activation of IRF5, IRF7, AP-1 or NF-κB results in their translocation into the nucleus where they can trigger the transcription of genes encoding INFα/β and inflammatory cytokines.

Aberrant TLR recognition of self-nucleic acids is an important aspect of the pathogenesis of SLE. To that end, understanding the mechanism of action, the function and the roles that nucleic sensing TLRs play in autoimmunity is critical. The connection between the function of TLRs and self-reactive B cells was initially discovered by Leadbetter *et al.* using the AM14 system that was derived from the MRL/*lpr* mouse model of SLE. The AM14 system was created using a rheumatoid factor (RF) isolated from a hybridoma made from an ill autoimmune MRL/lpr mouse (Shlomchik et al., 1987). B cells from the AM14 transgenic mice express a BCR that recognizes IgG2a of the a allotype ($IgG2a^a$) but not IgG2a of the b allotype ($IgG2a^b$ = IgG2c) (Shlomchik *et al*., 1993).

It was discovered that the immune complexes (IC) associated with the $IgG2a^a$ antinucleosome antibodies that were being used to stimulate AM14 splenic cells *in vitro* were DNase sensitive. Immune complexes made with other antigens did not stimulate the AM14 cells, indicating that specific antigens are required for autoreactive B cell activation (Leadbetter et al. 2002). Due to the observed DNase sensitivity, a factor that sensed DNA was implicated in the activation of self-reactive AM14 B cells.

In MyD88 deficient AM14 Tg mice, ICs composed of nucleosome and antinucleosome antibodies failed to activate B cells, indicating a role for TLR in autoreactive B-cell activation (Leadbetter et al. 2002). The DNase sensitivity of

the IC in AM14 mice implicated TLR9. To determine if TLR9 was responsible for this autoreactive B-cell activation, endosomal maturation was chemically blocked by chloroquine and concanamycin B. This resulted in a subsequent decrease in the activation of autoreactive B cells in the AM14 Tg mouse model (Leadbetter et al. 2002). Similar to T-cell independent B-cell activation, self-reactive B cells require two signals to become activated, in this case dual BCR/TLR engagement (Vos *et al.* 2000; Leadbetter *et al.* 2002).

Another TLR was implicated in the pathogenesis of SLE utilizing the FcγRIIb deficient C57BL/6 (B6.RIIb$^{-/-}$) mice. B6.RIIb$^{-/-}$ mice produced anti-DNA and anti-nucleosome autoantibodies and developed glomerulonephrites. When the B6.RIIb$^{-/-}$ mice were bred to mice bearing the Y chromosome linked autoimmune accelerator (*Yaa*), male mice developed autoantibodies specific for the nucleoli and developed accelerated disease. *Yaa* is only present in male mice. When it is present in mice also expressing genes that promote autoimmunity such as MRL/*lpr* or B6.RIIb$^{-/-}$ there is acceleration of the autoimmune disease (Deane et al., 2007).

The Yaa mutation is a translocation of the telomeric end of the X-chromosome onto the Y-chromosome and includes 17 genes, two of which are *Tlr7* and *Tlr8* (Subramanian et al. 2006), suggesting that they might contribute to the phenotype. Further evidence that *Tlr7* is partially responsible for the autoimmune phenotype came with the observation that mice transgenic for multiple copies of *Tlr7* develop severe autoimmunity (Pisitkun *et al.* 2006, Deane *et al.* 2007).

Initially it was thought that the Yaa phenotype could be solely attributed to *Tlr7* duplication (Deane *et al.,* 2007, Subramanian *et al.,* 2006, Pisithun *et al.,* 2006, Fairhurst *et al.* 2008). This hypothesis was called into question by a study stating that some aspects of the Yaa mutation were due to an additional unknown factor (Santiago-Raber *et al.* 2008). As both *Tlr7* and *Tlr9* have been shown to be involved in murine SLE autoantibody production (Pisitkun *et al.* 2006, Berland *et al.* 2006, Christensen *et al.* 2006, Deane *et al.* 2007, Santiago-Raber *et al.* 2009, Nickelson *et al.* 2010), we surmised that *Tlr8* was an excellent candidate to investigate since it is part of the Yaa translocation, senses single stranded RNA (ssRNA) and is MyD88 dependent.

Recently, a follow-up study has shown that the loss of marginal zone (MZ) B cells, a known aspect of the Yaa phenotype, is due to DC-mediated enhanced B-cell activation (Santiago-Raber et al. 2010). These authors noted that a role for *Tlr8* in this effect was an attractive possibility, but claimed that anlyses using C57BL/6.Yaa mice (B6.Yaa) that were null for the *Tlr8* gene on the endogenous X-chromosome (only 1 copy of the *Tlr8* gene present) still showed loss of the MZ B-cells (data was not shown). However, the authors could not rule out that MZ B cell loss in this model was due to the combined effect of both *Tlr7* and *Tlr8* gene duplication.

It is known that *Tlr7* and *Tlr8* are important for human DC sensing of ssRNA and subsequently their maturation. Larangé *et al.* showed that while both human TLR7 and 8 are important for DC maturation, they trigger different signaling pathways to achieve their goal (Larangé *et al.* 2009). Therefore it is

possible that gene duplication of both *Tlr7 and Tlr8* are likely involved in the observed DC-mediated enhanced B-cell activation (Santiago-Raber et al. 2010), ultimately leading to MZ B-cell loss.

Due to the fact that one of the hallmarks of human lupus is high levels of anti-DNA autoantibodies and TLR9 was found to be important for anti-DNA autoantibody production in mice (Christensen *et al.* 2005), it was believed that deleting *Tlr9* would lead to a significant reduction of serum autoantibodies and ameliorated disease in the MRL/*lpr* SLE mouse model. Unexpectedly, the *Tlr9* deficient MRL/*lpr* mice developed severe disease with increased mortality (Christensen *et al.* 2006). In addition, in male MRL/*lpr* mice double deficient in *Tlr7*/*Tlr9* there was a drastic reduction of autoantibodies in the sera, similar to MRL/*lpr Myd88* deficient animals. These findings led the authors to conclude that *both Tlr7* and *Tlr9* are necessary and sufficient for the development of disease in MRL/*lpr* mice (Nickelson et al. 2010). These findings, however, were made using male mice, and since SLE predominantly affects women, it is conceivable that there are other factors that are important for the development of SLE in females.

TLR8 in humans is expressed predominantly in lung tissue and in peripheral blood leukocytes (Chaung and Ulevitch 2000). As previously mentioned, TLR8 senses ssRNA, it is MyD88 dependent and it is part of the Yaa translocation. Recently, several reports have emerged that show h*TLR8* plays a distinct role in monocyte activation and subsequent inhibition of TL1A. TL1A is a member of the TNF superfamily and it is a ligand for the death domain-containing

receptor DR3 (Migone et al. 2002). TL1A is expressed mainly on endothelial cells and DR3 is expressed on lymphocytes. It has been shown that TL1A can be a costimulatory signal for T-cell activation (Migone et al. 2002; Bamias et al. 2003). Therefore hTLR8 may inhibit T-cell activation. Human *TLR8* has also been shown to be involved in h*TLR7* independent dendritic cell maturation (Saruta *et al.* 2009; Larangé *et al.* 2009; Ganguly *et al.* 2009). In the 293T cell line, co-transfected with human TLR7 and TLR8 or mouse TLR7 and TLR8, it has been found that in both humans and mice TLR8 inhibits TLR7 but not vice versa. In this same study it was shown that TLR9 inhibits both TLR7 and TLR8 (Wang *et al.* 2006; Krieg and Vollmer, 2007). Elucidation of the role of TLR8 in autoimmune disease has been limited by the fact that TLR8 in mice was thought to be non-functional (Heil et al. 2004).

Previous studies utilized Resiquimod (R848), an agonist for human TLR7 and TLR8; however, cells from TLR7 deficient mice were not responsive to R848, which lead to the conclusion that TLR8 was non-functional (Jurk et al. 2002). However, a pivotal study by Gorden *et al.,* found that murine TLR8 was in fact functional when stimulated with a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides (Gorden *et al.* 2006). These results indicated that the original claim was premature due to the inadequacy of the ligand for mouse TLR8.

In human SLE patients, one common feature is the elevated levels of IFN-α in the blood. Stimulation of human TLR7 by R848 has been shown to lead to increased IFN-α secretion in females more so than males (Berghofer et al. 2006).

Stimulation of hTLR8 using R848 was observed to increase NF-κB activation and IL-8 secretion from peripheral blood neutrophils. This response was enhanced when stimulated neutrophils were exposed to oxidative stress (Yanagisawa *et al.* 2009). Directly stimulating TLR8 in human peripheral blood mononuclear cells (PBMC) using imiquimod or resiquimod, human TLR7 and TLR8 agonists, leads to increased production and secretion of proinflammatory cytokines such as IL-12, MIP-1α and TNF-α when compared to TLR7 (Gorden *et al.* 2005). TNF-α has been linked to proinflammatory effects in autoimmune diseases and renal tissue damage (Vielhauer and Mayadas 2007; De Bandt *et al.* 2006).

Lastly, TLR8 mRNA was found to be up-regulated in patients with Sjögren's syndrome implicating a role for TLR8 in this and possibly other autoimmune diseases (Gottenberg et al. 2006; Krieg and Vollmer, 2007). In mice, *Tlr8* has been found to function as a negative regulator of neurite outgrowth and as an inducer of neuronal apoptosis (Ma *et al.* 2006). *Tlr8* could simply play a compensatory role for *Tlr7;* however, it is more likely that it has separate and distinct functions of its own. The results presented in this thesis clearly indicate that TLR8 plays a distinct and important role in mouse SLE.

Non-TLR pattern recognition receptors

There are many different non-TLR pattern recognition receptors. These receptors recognize microbial proteins, bacterial endotoxins and viruses independent of the known toll like receptors. The scavenger receptor family members are not structurally related however they share a ligand binding profile.

There are scavenger receptor class A and class B. Class A receptors do not bind to specific individual moieties but to a group of different ligands. These receptors include, lipoproteins, chemically modified lipids, apoptotic cells and the proteins that they release and a variety of microorganisms (*N.meningitidis and E.coli*). CD36 is a class B receptor that has a role in host defense as well as lipid metabolism. CD36 is best known for recognizing a surface molecule of P. falciparum, which is important in the sequestration of red blood cells. Mannose binding lectin (MBL) is a member of a family of secreted proteins named collectin. MBL binds to complicated ligands found predominately in microorganisms. This is important so that this ligand can recognize the difference between self and non-self-ligands. MBL can bind to viral, bacterial, fungal and protozoan ligands. It is even posited that it can recognize and bind to bacterial lipopolysaccharides (Jack et al., 2001).

NOD-like receptors have 20 related family members. Unlike many TLR receptors NDL's are located in the cytosol and recognize intracellular ligands such as invasive bacteria. There are two groups of NLR's containing either a CARD or a Pyrin motif and they regulate NF-κB activation and IL-1β and IL-18 production (Rietdijk et al., 2008).

There are also many non-TLR pattern recognition receptors that can recognize nucleic acids. While these receptors have not been linked to the pathogenesis of lupus it is still an important aspect of innate immune system.

These receptors appear to be triggered when nucleic acid is introduced into the cytoplasm as opposed to being triggered by extracellular DNA like TLR9.

"Cytoplasmic DNA activates three main pathways: first, DNA can trigger several, partially redundant, DNA receptors with transcriptional activity; second, DNA can be transcribed by RNA polymerase III into immune-stimulatory RNA that is sensed by the RIG-I pathway; and third, formation of the AIM2 (absent in melanoma 2)-containing inflammasome triggers a proteolytic pathway culminating in the activation of members of the pro-interleukin-1β (IL-1β) cytokine family.(Hornung and Latz, 2010)"

Within cells, DNA can exist in different states/conformations based on the cells hydration status. B-DNA (right handed spiral DNA), specifically the homo-copolymer poly (dA:dT) has been shown by Akira and colleagues to trigger the IFN regulatory factor transcription factor IRF3 and well as the INFβ promoter in a signaling pathway involving TANK binding kinase 1 (TBK1) (Akira et al., 2006).

Similar conclusions were found by Stetson and Medzhitov, when they found that a synthetic 45mer dsDNA without CPG motifs and having a random sequence could trigger potent TLR-independent type I INF responses in a IRF3 dependent manner in macrophages and dendritic cells. There were discrepancies in the study in that they were not able to replicate this response in HEK293 cells nor were they able to activate NF-κB or mitogen activated protein kinase pathways. Despite these discrepancies intracellular DNA appears to have the ability to activate different non-TLR pattern recognition receptors.(Stetson et al., 2008)

Retinoic acid inducible gene-1 like receptors (Rig-1) are in the helicase family and they have been reported to be involved in viral recognition. These receptors are also located within the cytoplasm. Glomerular endothelial cells have been studied to determine the onset on glomerulonephritis in response to viral infections. It was shown that dsDNA enters these cells and the RIG-1 located in the cytoplasm activates the cells and initiates the immune cascade to fight the infection. This immune activation contributes to the resulting glomerulonephritis (Hagele et al., 2009). There are many pathways that the immune system uses to fight off infections from viruses, bacteria and other pathogens and while TLR's are an important aspect of the innate immune systems defense, other receptors have an important role to play in our bodies' defense.

Interleukin -1

Interleukin-1 (IL-1) was initially identified as a leukocytic pyrogen whose actions lead to fever and degranulization of the peripheral blood neutrophils (Dinarello *et al.* 1977a;b; Klempner *et al.* 1978). In the intervening years many other molecules with structural homology to IL-1 have been identified forming the IL-1 superfamily. The original members of the super family are IL-1α, IL-1β and the IL-1 receptor antagonist IL-1RA. IL-1α and IL-1β are acute phase pro-inflammatory cytokines that are released from macrophages, dendritic cells, monocytes and fibroblasts in response to infection. These cytokines bind to IL-1 receptor type 1 in order to facilitate the immune response. IL-1RA or IL-1 receptor type II competes for receptor binding to IL-1α and IL-1β, effectively blocking their role in the immune response. The end result of IL-1 signaling is sterile inflammation (no cytotoxicity), fever, hyperalgesia, and lastly, increased expression of endothelial cell adhesion factor, which allows for transmigration of leukocytes (O'Neill *et al.* 2000a,b; Cohen *et al.* 2010).

IL-1 has also been implicated in human disease such as autoimmune rheumatoid arthritis (O'Neill *et al.* 2000a,b). Due to the intricate relationship between the IL-1R and TLR signaling pathways, I questioned if there may be a role for IL-1 in SLE. Evidence that IL-1 might be involved in the pathogenesis of SLE can from a report by Ueda *et al.* where C57BL/6 mice and IL-1R type1 deficient mice were immunized with alum precipitated antigens and the levels of different blood leukocytes were followed for eight days. After this eight-day period the mice were sacrificed and granulocytes and hematopoietic stem cells from the

BM were analyzed and compared. This comparison showed that; IL-IR type 1 dependent proliferation of hematopoietic stem cells was necessary for inflammatory granulopoiesis and reactive neutrophilia (Ueda *et al.* 2009).

Studies from our laboratory, using the 564Igi SLE mouse model, have also shown that these mice, similar to humans, have BM and peripheral granulopoiesis (Han Unpublished data, figure 18). The absence of IL-1R type 1 in Ueda *et al.* had a significant impact on the neutrophil and granulocyte population in the alum induced inflammatory mouse model. These results suggest that the IL-1R signaling pathway might also play a role in the autoimmune granulopoiesis observed in 564Igi mice.

Complement system

Another important component of innate immunity is the complement system. Complement factors assists antibodies in bacterial opsonization and in some cases it allows antibodies to kill the bacteria. Complement consists of a large number of heat labile plasma proteins that help fight infection by reacting with each other to bind and attack bacteria and recruit inflammatory cells.

Complement factors are initially zymogens that are activated by proteolytic cleavage. Interestingly, these factors are proteases as well. Complement factors are activated locally via an enzyme cascade. Each factor has a specific task that varies from binding antibody-antigen immune complexes and pathogen surfaces (C1q) to activating enzymes (C1r, C1s, C2b, Bb, D, MASP-1, and MASP-2) to

membrane attack proteins (C5b, C6, C7, C8 and C9) (Janeway et al. 2001,Prodeus et al 1998).

There are three separate complement pathways, the classical pathway, the alternative pathway and the MB-Lectin pathway. These three pathways converge into a common pathway resulting in complement 3b (C3b) deposition. In brief the classical pathway is triggered by IC binding or by coming into contact with pathogens, the alternative pathway is triggered by coming into contact with pathogens and the MB-Lectin pathway is activated by binding to mannose found on the surface of pathogens. All three pathways lead to the cleavage and activation of C3 before going on to recruit phagocytes, binding to phagocytes, removing ICs, opsonization of pathogens, and lastly, membrane attack and pathogen lysis (Janeway *et al.,* 2001).

Deficiency of complement factors can lead to massive infection, sepsis and in the case of C1q deficiency a 93% increased risk of developing SLE (Manderson et al. 2004). Specific deficiencies in complement regulatory proteins lead to over-activation of the complement system. Two clinical outcomes of complement over-activation are paroxysmal nocturnal hemoglobinuria and hereditary angioedema. One interesting mouse model of autoimmune disease is based on C4 deficiency.

In humans, C4 deficiency is associated with a 75% increased risk of developing SLE (Prodeus et al 1998; Manderson et al. 2004). C4 is involved in the early steps of activation of the classical complement pathway and is critical for the maintenance of self-tolerance. Deficiencies of complement C4 has also

been shown to lead to a decrease in negative selection of auto reactive lymphocytes (Prodeus et al., 1998).

Thus, on an autoimmune background (*lpr/lpr*), C4 deficiency causes a dramatic increase in anti-nuclear and dsDNA antibodies, lymphadenopathy and glomerulonephritis (Carrol M.C., 1998). 564Igi mice, with deficiencies in complement C4, result in an exacerbation of the SLE phenotypes and serve as a useful model of autoimmunity (Han unpublished data). The complement system is not only a vital component of the innate immune system, but it also provides an important link to / and regulation of the adaptive immune system.

Fragment crystallizable gamma receptors (FcγR)

Fcγ receptors are found on hematopoietic cells and are important for the recognition of the Fc portion of IgG molecules. They are important for recruitment and activation of effector cells. Fcγ receptor cross linking on effector cells such as NK cells, neutrophils, basophils, eosinophils, monocytes and monocyte derived dendritic cells results in the activation of the effector function of these cells. These effector functions include phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and the release of cytokines and chemokines whose ultimate result is amplification of the inflammatory response and cellular destruction.

The complement system described above was previously thought to be solely responsible for the pathogenesis of IC mediated disease (Cochrane *et al.* 1970). Utilizing complement C3 and C4 deficient mice in comparison to the common γ chain Fcγ deficient mice, Sylvestre *et al.* determined that Fcγ was

critical of IgG mediated inflammatory responses. C3 and C4 deficient mice treated with anti-red blood cell and anti-platelet antibodies or with soluble immune complexes had a normal inflammatory response. If complement factors were indeed solely responsible for IC recognition, this would not be the case. In contrast, common γ chain deficient animals had markedly deficient responses to either stimulus. This established Fcγ receptors as crucial for the development of many IC-mediated inflammatory diseases (Sylvestre *et al.* 1996).

In mice there are three known activating Fcγ receptors (FcγRI, FcγRIII, and FcγRIV) and one known inhibitory Fcγ receptor (FcγRIIB). Humans, in contrast have five activating Fcγ receptors (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB) and one inhibitory Fcγ receptor (FcγRIIB) that have been identified thus far (Nimmerjahn and Ravetch, 2008).

In mice the activating Fcγ receptors consist of two polypeptide chains: one unique α-chain and a common γ-chain that have an immunoreceptor tyrosine-based activating motif (ITAM). The inhibitory Fcγ receptor, FcγRIIB, is expressed as a single chain and has an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Nimmerjahn and Ravetch, 2008).

As the Fcγ receptors are crucial for enhancing the cellular inflammatory response, the activation of these receptors must be tightly controlled. The inhibitory receptor FcγRIIB has been shown to be a critical checkpoint for humoral tolerance. FcγRIIB was shown to be important for excluding low-affinity autoreactive B cells from entering the follicle, preventing B cells with higher affinity autoreactive BCR's from class switching and becoming IgG secreting

plasma cells and for initiating apoptosis of plasma cells, thus regulating plasma cell homeostasis (Nimmerjahn and Ravetch, 2008).

Considering the importance of the inhibitory FcγRIIB receptor in both regulating the activation of the innate and adaptive immune systems, late peripheral checkpoint loss or deletion of this Fcγ receptor can have severe consequences especially in the arena of autoimmunity.

Type 1 interferon

Type 1 interferon (INF-I) is a family of pro-inflammatory cytokines that are produced and secreted by a number of cell types in response to activation by endogenous ligands. These cell types include granulocytes (Han et al. unpublished data Figure 17) and plasmacytoid dendritic cells (pDC) (Vallin et al. 1999). INF-1 was initially described as a cytokine that enhanced antiviral resistance in mammals (Kyogoku and Tsuchiya 2007). The mammalian type 1 INF family consists of INF-α (13 subtypes), INF-β, INF-κ, IFN-δ, IFN-ε, INF-τ, IFN-ο and INF-ζ (Hardy *et al.,* 2004). INF-I promotes cell proliferation, B-cell differentiation into plasma cells, survival and cytotoxic activity of CD8+ T cells and NK cells, and the activation of dendritic cells.

Treatment of patients with viral Hepatitis or carcinoid tumors with INF-α was found to cause autoimmunity in 19% of patients, highlighting the role of INF-α in SLE disease development (Kalkner et al. 1998; Ronnblom et al. 1991).

Elevated levels of INF-α were found in the sera of SLE patients (Shi et al. 1987), and increased percentages of INF-α producing cells were found in the BM of 564Igi mice (Han et al. unpublished figure16A and 16B).

Recent work has uncovered an "IFN-I signature" of gene expression that correlated with the presence of SLE autoimmune disease. Peripheral blood leukocytes from SLE patients were found to express both IFN-I and IFN-I signature genes (Krug 2008; Baechler et al. 2003; Bennett et al. 2003; Crow et al. 2003). Treatment of patients with viral Hepatitis or carcinoid tumors with INF-α was found to cause autoimmunity in 19% of patients highlighting the role of INF-α in disease development (Kalkner et al. 1998; Ronnblom et al. 1991)

Several established mouse models were utilized to elucidate the role of IFN-I in autoimmune disease. New Zealand Black (NZB) mice are known to have lupus like disease and deletion of the INFα receptor resulted in an abrogation of autoantibody production (Santiago-Raber *et al.* 2003; Kono *et al*. 2003). The NZB x NZW F1 (B/W) mouse model is known to spontaneously develop autoimmune disease characterized by the production of anti-nucleic acid antibodies and fatal immune complex glomerulonephritis that is accelerated in female mice (Howie and Helyer 1968). A recent study using pre autoimmune B/W mice treated with IFN-α from a recombinant adenovirus, found rapid induction of severe dose dependent SLE-like disease that was not evident in WT Balb/c mice (Mathian *et al.* 2005). Anti-DNA antibodies appeared within 10 days of INFα treatment, and nine to 18 weeks post treatment all mice in the treatment group died due to glomerulonephritis. This study provided direct evidence that

prolonged *in vivo* expression of INFα leads to the development of lethal lupus in susceptible mice (Mathian *et al.* 2005).

Linkage analyses done in mice have found 31 SLE susceptibility loci distributed over 21 non overlapping-genetic intervals (Wakeland *et al.* 1999). Through all of this analysis, one region, the telomeric end of chromosome 1 or 1q21-44 has shown strong linkage in both humans and NZM2410 (derived from B/W) mice respectively. In mice, this region called Sle1 has three loci; NZW-derived Sle1 (Morel *et al.* 1994), NZB-derived Lbw7 (Kono *et al.* 1994), and Nba2 (Drake *et al.* 1995). Several genes have been identified in this region that are known to play a role in the susceptibility of SLE such as FcγRIIb and FcγRIIIA (Salmon *et al.* 1996; Wu *et al.* 1997). Two other susceptibility loci were also detected and named Sle2 (located on chromosome 4) and Sle3 (located on chromosome 7) (Morel *et al.* 1994; Morel *et al.* 2001).

Each of these three loci has unique functions in mice, but Sle1 has been shown to be required in bicongenic mice for the development of clinical glomerulonephrits. B6 mice congenic for each of these three loci were created and utilized to dissect the functions of these susceptibility loci. Sle1 can cause loss of tolerance specific for nuclear antigens due to B6.Sle1 mice developing high titers of spontaneous IgG (Morel *et al.* 1997). Sle2, while not sufficient for generation of antinuclear antibodies or nephritis, does lead to elevated levels of polyreactive / polyclonal IgM, elevated B1 cell formation and B-cell hyperactivity (Mohan *et al.* 1997). Lastly Sle3 had elevated CD4:CD8 ratios, stronger proliferation and expansion of CD4+ T cells, reduced apoptosis after CD3

stimulation and accumulation of activated CD4+ T cells. In general Sle3 leads to T-cell activation but no overt disease on its own (Mohan et al. 1999).

The triple congenic mouse model of C57BL/6.Sle1Sle2Sle3 (B6.Sle 123) combines three SLE susceptibility loci that contain the minimal set of genes necessary to reconstitute murine SLE (Morel *et al.* 2000). Utilizing a similar experimental design to what was described above, with B/W mice, WT B6 and B6.Sle 123 mice were treated with an adenovirus construct containing a recombinant IFN-α gene cassette and were found to have similar IFN-α induced phenotypes. The notable exceptions were B6.Sle123 had increased B1 and plasma cells and severe glomerulonephritis. The main effect of INF-α treatment in these mice is a worsening of the mechanism that led to end organ damage (Fairhurst *et al.* 2008).

The MRL/*lpr* mouse model of lupus has yielded significant information about SLE as the disease these mice have is quite similar to human lupus. The *lpr* mutation was found spontaneously and is a point mutation at nucleotide position 786 from T to A in the *Fas* gene. It changes the amino acid asparagine to isoleucine in the cytoplasmic domain of the Fas protein, disrupting the protein conformation (Watanabe-Fukunaga *et al*., 1992). Mice with this mutation have increased development of systemic autoimmune disease and short lifespan. C57BL/6 mice bred to *lpr* (B6.*lpr)* treated with a potent inducer of type 1 interferon, polyinosinic:polycytidylic acid (Poly IC), developed severe renal disease, higher titers of autoantibodies, activated lymphocytes and ten-fold increased serum Ig accumulation. Conversely, B6.lpr mice with null mutation for

the IFN-I receptor gene had reduced lymphadenopathy and marked decrease in IC deposits in the kidney (Braun *et al.* 2003).

The information gathered from these models of lupus underscore the importance of IFN-I and it shows that the disease is quite polygenic. One important gene locus that has been identified as being important in lupus development is the Nba2 loci. The interferon signature gene Ifi202 is localized to the Nba1 locus and it was found to be highly expressed in NZB mice. Ifi202 is a transcription factor important for B-cell proliferation, differentiation and survival and is induced by type 1 interferon (Rozzo *et al*. 2001; Min *et al.* 1996). It is quite possible that in an autoimmune background, induction of IFN-I and activation of genes such as ifi202 can lead to severe autoimmune disease.

564Igi mouse model

In order to elucidate the mechanisms involved in the development of SLE, we have utilized the 564Igi mouse model, which was created in our laboratory (Berland *et al.* 2006). 564Igi is a knock-in mouse model in which rearranged heavy chain and light chain genes from the 564 hybridoma derived from an autoimmune (SWR X NZB)F_1 mouse were introduced into the IgH and IgL loci of a C57BL/6 mouse. The antibodies produced by the 564 hybridoma are polyreactive, as they bind nucleoli and cytoplasmic antigens (ssDNA, ssRNA, and nucleosomes) and are pathogenic, as the injection of these antibodies was found to accelerate the appearance of glomerulonephrites in young (pre-autoimmune) female F1 (SWRxNZB) mice (Gavalchin *et al.* 1987). In general,

injection of self-reactive antibodies was found to cause glomerular damage (Vlahakos *et al.* 1992; Mohan *et al.* 1993).

564Igi mice produce self-reactive 564-idiotype (564Id) B cells, which carry the 564Igi BCR. In 564Igi mice on the non-autoimmune C57BL/6 background (wild type (WT) mice) 564Id B cells cells are anergic. Nevertheless large amounts of 564Id-positive T_H1-skewed IgG2a and IgG2b autoantibodies were found in the sera of these mice. Production of autoantibodies in the 564Igi mouse model has been shown to be partially *Tlr7* dependent, and in Hep2 cells nucleoli and cytoplasmic antigens are stained with 564Igi antibodies, suggesting that these antibodies are RNA specific or specific for RNA associated antigens. While deletion of*Tlr7* significantly reduces autoantibody levels, it does not completely eliminate it (Berland *et al.* 2006). These results suggest that another nucleic acid sensing TLR such as *Tlr8* or/and another factor might be involved in the activation of B cells in these autoimmune mice.

Chapter II: MyD88, TLR7/TLR8 Dependent Autoantibody Production in 564Igi mice Introduction

Introduction

It has been determined that TLR7 plays a major role in the production of RNA specific auto-antibodies (Berland et al., 2006; Pisitkun et al., 2006; Subramanian et al., 2006). However, deficiency in *Tlr7* does not completely inhibit autoantibody production in 564Igi mice. Berland *et al.* showed that TLR7 deficient 564Igi mice have a significant decrease in 564Id+ antibodies. This decrease was seen in 564 Id+ IgG2a, IgG2b, IgM, IgG3 and IgA; however, it was not due to a decrease in total serum IgG2a, IgG2b, IgM, IgG3 or IgA (Berland et al. 2006).

In order to determine whether additional TLRs or other factors are involved in antibody production in 564Igi mice, we created mice that are deficient for *Tlr4*, *Tlr9*, IL1Rtype 1 and *MyD88,* as well as mice double deficient for *Tlr7/Tlr9* and *Tlr7/Tlr8.* We hypothesize that TLR8 plays a role in the residual autoantibody production that is observed in 564Igi Tlr7 KO mice.

Results

The production of RNA specific 564Id+ antibodies is MyD88 dependent, partially Tlr7 dependent and suppressed by Tlr9

As previously described B-lymphocytes from 564Igi mice produce pathogenic RNA specific autoantibodies. The production of these autoantibodies was found to be T cell independent and TLR7 dependent (Berland et al 2006). TLR7 deficiency in MRL/lpr mice has also been shown to ameliorate autoimmune disease (Christensen et al. 2006). In the 564Igi mouse model, MyD88, the adaptor molecule for TLRs 2, 4, 5, 7, 8 and 9 was found to be required for autoantibody production (Figure 4A and B).

TLR9, a MyD88 dependent TLR, has been found to control anti-DNA and anti-chromatin autoantibody production, but not anti-smith or other autoantibodies in the MRL/*lpr* mouse model (Christensen et al. 2005). MRL/*lpr* TLR9 deficient mice also had exacerbated disease, increased activation of lymphocytes and pDCs and increased IgG and IFN-α production (Christensen et al. 2006). In 564Igi mice, TLR9 deficiency resulted in increased autoantibody production in both female (Figure 4B) and male mice (data not shown) when compared to 564Igi WT mice. These results suggest that TLR9 can contribute to the prevention of autoimmunity by possibly suppressing RNA-specific autoantibody production in 564Igi mice.

Deletion of TLR7 significantly reduces but does not eliminate the production of autoantibodies in 564Igi mice (Figure 4B and Berland *et al.* 2006). These data are applicable to both male and female 564Igi *Myd88* KO, 564Igi *Tlr7*

KO and 564Igi *Tlr9* KO mice as there was no significant difference in antibody titers between them (Figure 4C). In 564Igi *Tlr7* KO male there is a slight decrease in the titer of IgG2b antibodies; however, it is not statistically significant.

Anti-RNA autoantibody production is completely MyD88 dependent, and the only MyD88 dependent TLRs known to be activated by nucleic acids are *TLR7*, 8*,* and 9 (Hemmi *et al.* 2000; Diebold *et al.,* 2004; Heil *et al.* 2004). Autoantibody production in 564 is only partially decreased by a loss of *Tlr7,* while loss of *Tlr9* augments it. This implied that *Tlr8* might contribute to autoantibody production in 564Igi.

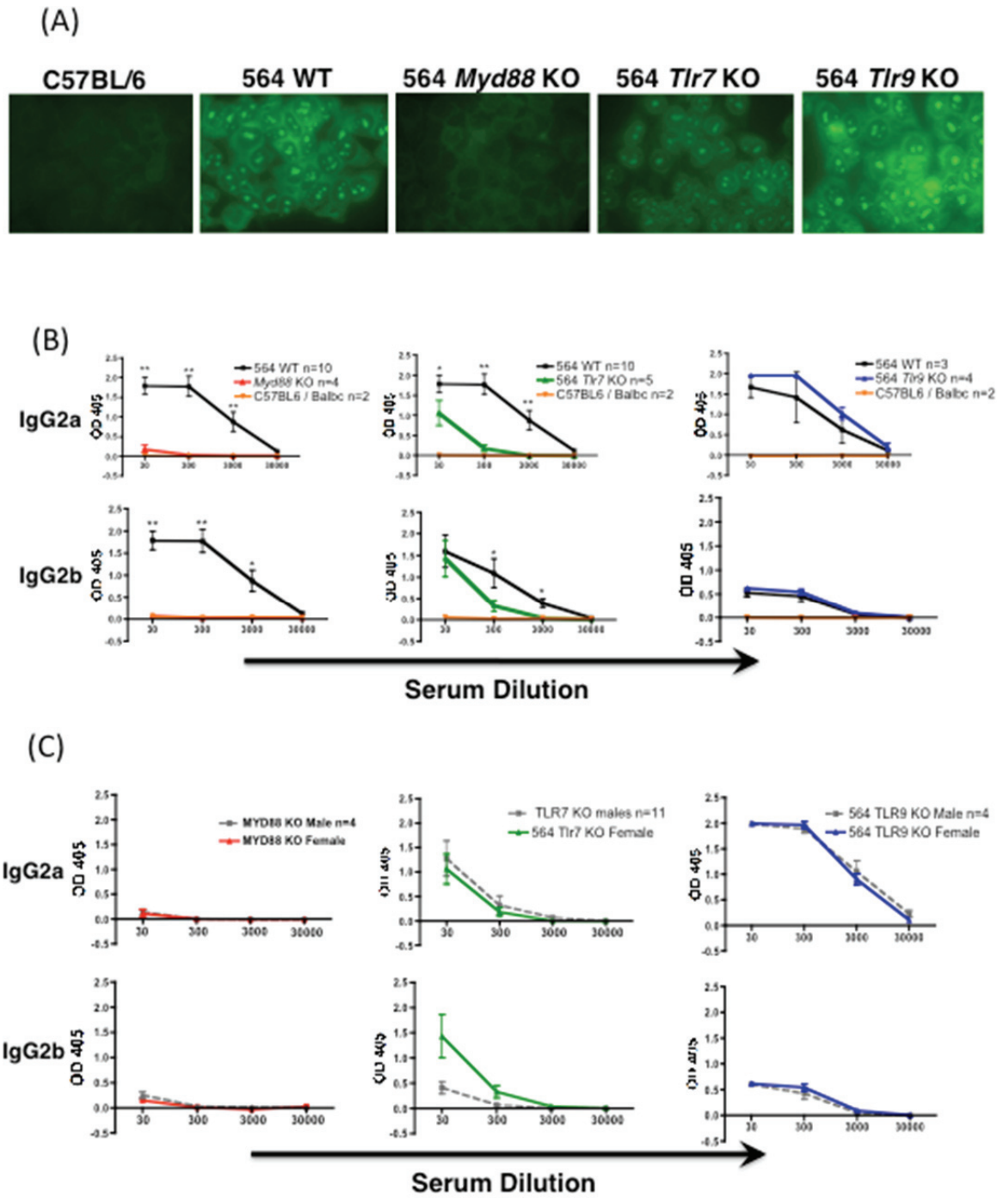
(A)
C57BL/6
564 WT
564 Myd88 KO
564 Tlr7 KO
564 Tlr9 KO
(B)
IgG2a
IgG2b
564 WT n=10
Myd88 KO n=4
C57BL6 / Balbc n=2
564 WT n=10
564 Tlr7 KO n=5
C57BL6 / Balbc n=2
564 WT n=3
564 Tlr9 KO n=4
C57BL6 / Balbc n=2
OD 405
Serum Dilution
(C)
IgG2a
IgG2b
MYD88 KO Male n=4
MYD88 KO Female
TLR7 KO males n=11
564 Tlr7 KO Female
564 TLR9 KO Male n=4
564 TLR9 KO Female
OD 405
Serum Dilution

Figure 4: The production of 564Id+ antibodies in 564Igi mice is *Myd88* dependent and partially *Tlr7* dependent

Autoantibody RNA specificity is retained despite *Tlr7* and *Tlr9* deficiency (A) Antinuclear antibody (ANA) testing was performed using sera from 3 month old C57BL/6, 564Igi WT, *Myd88* KO, *Tlr7* KO, *Tlr7/Tlr9* KO. Shown are 1:25 dilutions. (B) Sera from 3 month-old female 564Igi *Myd88* KO, *Tlr7* KO and *Tlr9* KO mice were tested by ELISA. (C) Sera from 3 month-old female and male 564Igi *Myd88* KO, *Tlr7* KO and *Tlr9* KO mice were tested by ELISA. Plates were coated with borate buffer and serial dilutions were done as indicated in borate buffer with 1% BSA, and the plates were developed using alkaline phosphatase (AP) labeled anti-IgG2a and IgG2b and treated for 2hrs with PnP substrate. Upper panel represents IgG2a and the lower panel is IgG2b. Significance was determined by the student t-test. **, < .01, *, < .05. 564Igi *Myd88* KO female mice (n=4), 564Igi *Myd88* KO male mice (n=4), 564Igi WT mice (n=10,) (564Igi WT mice in TLR9 ELISA n=3), 564Igi *Tlr7* KO female mice (n=5), 564Igi *Tlr7* KO male mice (n=11), 564Igi *Tlr9* KO female mice (n=4), 564Igi *Tlr9* KO male mice (n=4) and C57BL6 / Balbc mice (n=2). Error bars represent the SEM.

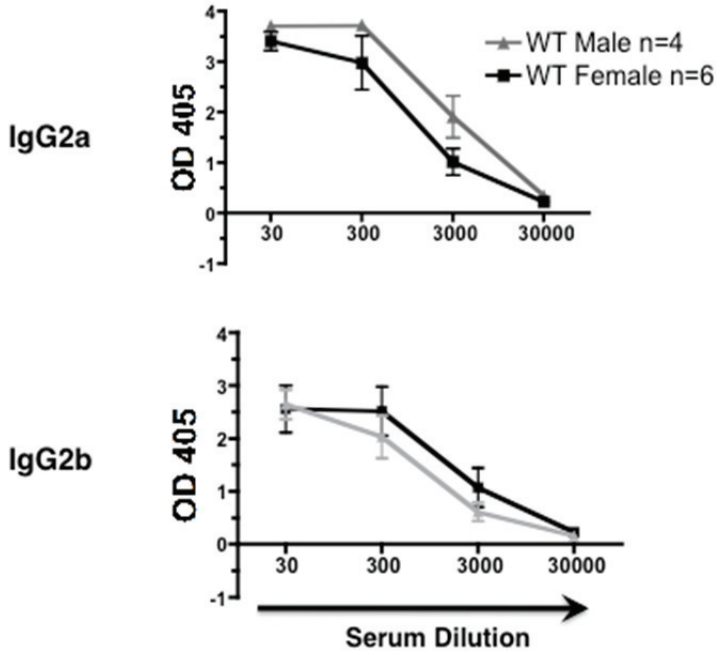

Figure 5: There is no significant difference in autoantibody production between male and female 564Igi WT mice; ELISA analysis of sera from indicated mice

The top panel shows IgG2a and the bottom is IgG2b. The ELISA was done as described in Figure 4. 564 WT female mice (n=4) and 564 WT Males mice (n=6). Error bars represent SEM. Significance was determined by the Student t Test **, <.1, *, <.05.

Sex disparity in autoantibody production between male and female 564Tlr7/Tlr9 DKO mice

I asked whether loss of both *Tlr7* and *Tlr9* in 564Igi completely eliminated autoantibody production as observed in 564Igi *Myd88* deficient mice (Figure 4B). Male and female 564Igi *Tlr7/Tlr9* double-deficient (DKO) mice were found to have markedly different levels of circulating autoantibodies. This sex disparity was not evident in the other 564Igi mouse lines tested (Figure 4C) including 564Igi WT mice (Figure 5). Male 564Igi *Tlr7/Tlr9* DKO mice had much less serum autoantibody than male 564Igi wild type (WT) mice (Figure 6B). Female 564Igi *Tlr7/Tlr9* DKO mice had levels of Id+ antibodies in their sera that were comparable to the female WT 564Igi mice (Figure 6B). Importantly, while serum from male and female *Tlr7/Tlr9* DKO mice had a lower titer of autoantibodies, there was no change in the RNA specificity of the autoantibodies produced as assessed by ANA analysis (Figure 6A and Figure 6B). This indicated that the remaining autoantibodies were created in response to RNA. Of the known mammalian toll like receptors, three *Tlr3*, *Tlr7* and *Tlr8* have this property. Of these, *Tlr3* does not utilize *Myd88* for downstream signaling, suggesting a role for *Tlr8*.

As the autoantibodies produced by 564Igi are against nucleic acids, it is unlikely but not impossible for another *Myd88* dependent TLR, outside of *Tlr7*, 8 and 9 to have a role in autoantibody production. To that end 564Igi *Tlr4* deficient mice were created and tested and no significant decrease in autoantibody production was observed. There was what appeared to be a difference in IgG2b

female mice however due to the fact that only one mouse was tested it cannot be quantified (Figure 7).

Another interesting difference between male and female 564Igi *Tlr7*/*Tlr9* DKO mice, is that there was a significant increase in the percentage of IgG/IgA bearing lymphocytes in the spleens of female mice that was not observed in 564Igi WT (Figure 6C) or in 564Igi *Tlr7* KO or *Tlr9* KO mice (data not shown). This sex disparity in the lymphocyte population provided a possible explanation for the increased autoantibody levels in the sera of female mice. It also indicates, that in the absence of *Tlr7* and *Tlr9*, *Tlr8* may lead to increased B-cell proliferation.

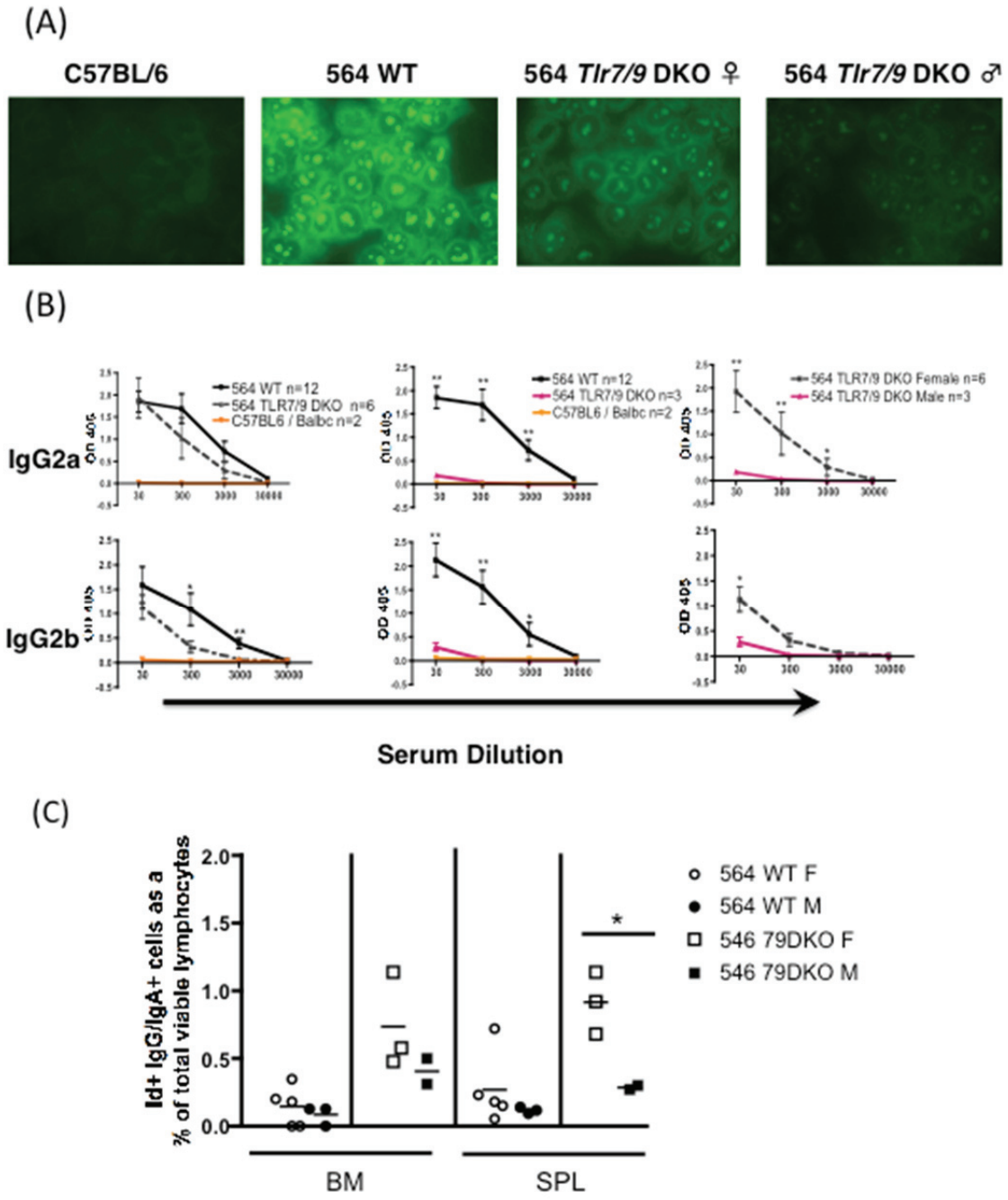

Figure 6: There is a sex disparity in autoantibody production between male and female 564 *Tlr7/Tlr9* DKO mice

(A) ANA testing was performed using sera from 3 month-old C57BL/6, Balb/c, 564Igi WT and 564Igi *Tlr7/Tlr9* DKO Male and female mice. (B) ELISA analysis

of sera from indicated mice. The top panel shows IgG2a and the bottom is IgG2b. The ELISA was done as described in Figure 4. Female *Tlr7/Tlr9* DKO mice (n= 6) vs. male *Tlr7/Tlr9* DKO mice (n=3), 564 WT female mice (n=12), 564 WT Males mice (n=12). Error bars represent SEM. (C) Flow cytometry analysis of the BM and SPL IgG or IgA bearing B lymphocytes. Significance was determined by the Student t Test **, <.1, *, <.05.

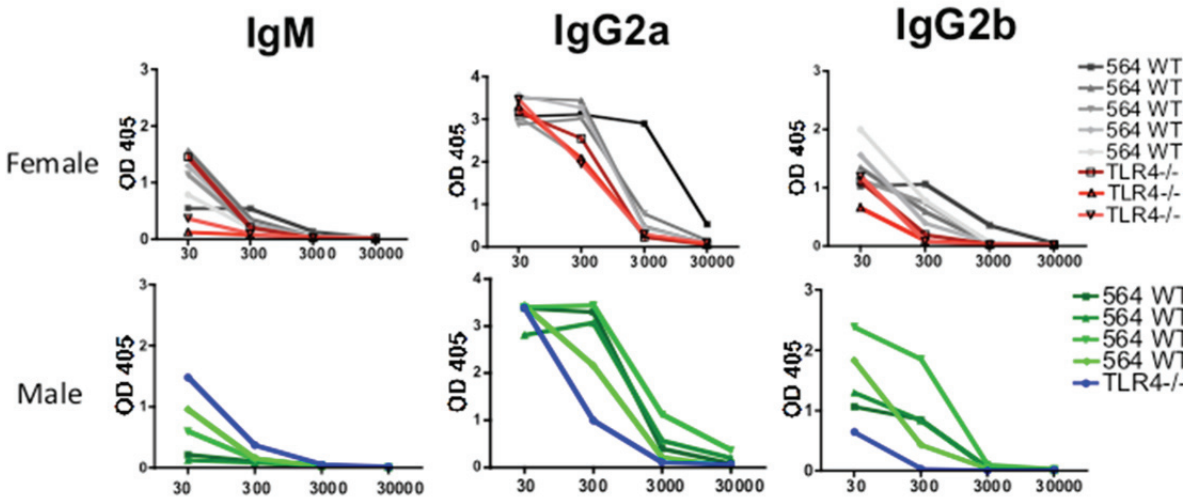

Figure 7: No Significant decrease in IgG2a, IgG2b, and IgM autoantibody production in 564Igi *Tlr4* deficient mice

ELISA analysis of 564Igi WT and 564Igi *Tlr4* deficient sera from male and female 5 month-old mice. . Significance determined by the Student t-test *,≤ .05 **, ≤ .01. 564Igi WT females n=4, 564Igi *Tlr4* -/- females n=1, 564Igi WT Males n=5, 564Igi *Tlr4*-/- Males n=3, C57BL/6, Balb/c =2

Increased gene expression of Tlr8 in the absence of Tlr7 and Tlr7/Tlr9 in the Id+ B lymphocytes of 564Igi.

Autoantibody production is a function of activated B-lymphocytes and it has previously been shown that *Tlr7* gene expression is increased in Idiotype positive 564Igi WT B cells (Han unpublished data, Figure 15). In complement 4 (C4) deficient 564Igi B cells this *Tlr7* gene expression increase was even more pronounced (Figure 15, Han unpublished data). Duplication of the *Tlr7* gene and therefore its gene products in the B6-FcgRIIB.Yaa mouse model has been shown to lead to accelerated autoimmunity (Pisitkun *et al.* 2006). Even moderate increases in *Tlr7* gene expression, as found in C57BL/6.*Tlr7* transgenic mice, were found to promote self-reactive lymphocytes specific for RNA and proliferation of myeloid cells (Deane *et al.* 2007).

Since TLR7 and TLR8 both recognize ssRNA, I wondered if *Tlr8* gene expression would be up-regulated in 564 Idiotype (Id) positive B cells in the absence of *Tlr7* or both *Tlr7* and *Tlr9* in 564Igi mice. In order to address these questions, quantitative taqman qPCR analysis was done to determine the relative gene expression of *Tlr8* in the 564 Idiotype positive B cells of the above mentioned 564Igi mouse lines. We determined that *Tlr8* gene expression was up-regulated in idiotype positive (ID+) B cells from 564Igi bone marrow (BM) and the spleen (SPL) (Figure 8A and B Supplemental figures). Further up-regulation of *Tlr8* gene expression was observed in 564Igi ID+ B cells in the absence of *Tlr7* and *Tlr7/Tlr9* in both the BM and SPL (Figure 8A and B). This indicates that *Tlr8*

gene expression is up-regulated in the BM and SPL of 564Igi mice and it indicates that *Tlr8* may be able to compensate for the loss of *Tlr7*.

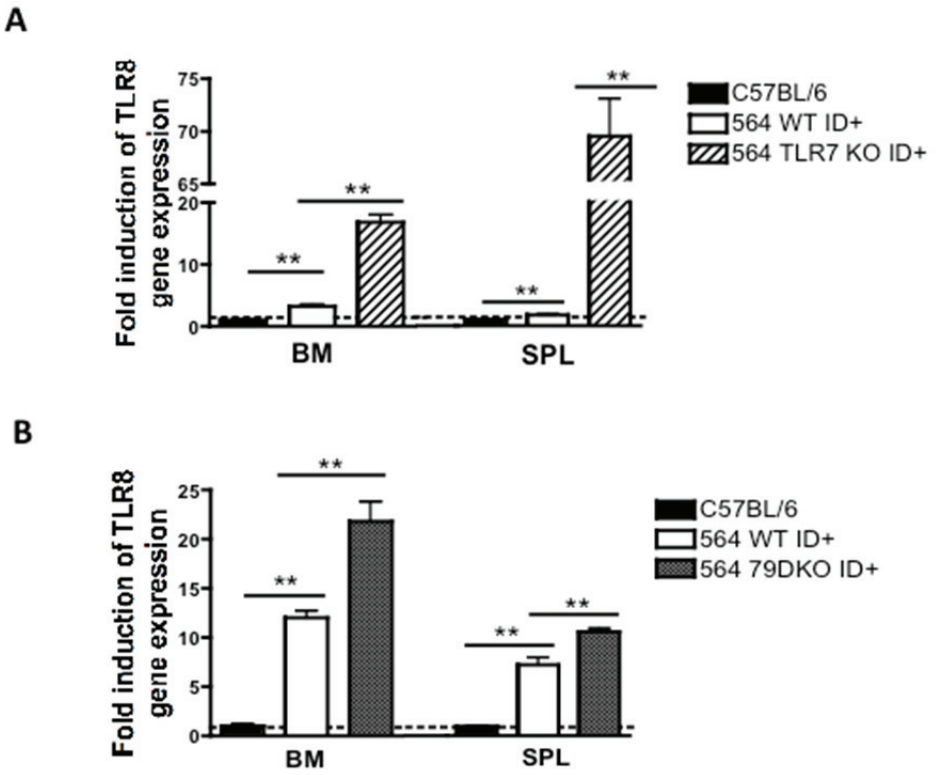

Figure 8: Increased gene expression of *Tlr8* in the absence of *Tlr7* and *Tlr7/Tlr9* in the Id+ B lymphocytes of 564Igi

Age matched 3 month-old female C57BL/6 and 564Igi mice with the indicated genetic backgrounds were sacrificed and the harvested BM and SPL cells were sorted. In the BM viable, double positive B220, AA4.1 cells (immature B) were sorted for 564 Id+ cells. In the SPL B220+ AA4.1- cells (mature B) were sorted for 564 Id+. B6 were sorted for B220+, AA4.1+(BM) and AA4.1-(SPL), κλ+. Real time Taqman reactions were done for with pre-designed primers and probes for *Tlr8*. The data were normalized to β-actin and expressed as a value relative to C57BL/ 6 (1). A) *Tlr8* gene expression in the BM and spleen of 564Igi *Tlr7* KO mice compared to 564Igi WT and C57BL/6 Two experiments with two different samples of 564Igi TLR7 KO compared with 564Igi WT and C57BL6 . B) *Tlr8*

expression in the BM and SPL of 564Igi *Tlr7/Tlr9* DKO mice compared to 564Igi WT and C57BL/6. Three different experiments with three different samples of 564 *Tlr7/Tlr9* DKO males, females and 564Igi WT and C57BL6 mice. Significance was determined by the student T-test **, < .01 *, < .05.

TLR8 protein expression was increased in female 564Igi Tlr7/Tlr9 DKO mice despite the significantly increased Tlr8 gene expression observed in males

To determine if *Tlr8* is responsible for the sex discrepancy observed with 564Igi *Tlr7*/*Tlr9* DKO mice I compared *Tlr8* gene and protein expression between male and female mice. My initial data obtained from qPCR showed that males had higher *Tlr8* gene expression than females (Figure 9A) This was observed in two of my three experiments. My experiments were repeated by a co-worker and the new results are more inline with the western blot analysis which showed that *Tlr8* gene expression and protein levels are higher in female *Tlr7/Tlr9* DKO than in males.

TLR8 immunoblot analysis was done of whole BM and whole spleen of males versus females C57BL/6, 564Igi WT, 564Igi *Tlr7/Tlr9* DKO, 564Igi *Tlr7* KO and 564Igi *Tlr9* KO mice. The results of this analysis showed that C57BL/6 and 564Igi WT males and females did not have significantly different protein expression of TLR8, but that 564Igi *Tlr7* KO and 564Igi *Tlr7/Tlr9* DKO females expressed higher amounts of TLR8 protein than the males (Figure 9B). In contrast, 564Igi *Tlr9* KO males were observed to have increased TLR8 protein expression in comparison to females (Figure 9B), perhaps in female mice lacking

Tlr9 but sufficient for *Tlr7,* TLR8 protein expression is not up-regulated. The levels of TLR8 protein expression was increased in C57BL6 and 564 WT mice compared to the various knockout mouse lineages. The explaination for these results is currently unknown; is as similar levels of lysate were analyzed as indicated by the control levels of beta actin. Additional experiments with the TLR8 antibody may clarify these results in the future.

Increased protein expression of TLR8 when TLR7 or both TLR7 and TLR9 are deficient is likely the reason why TLR7/TLR9 DKO females have a positive ANA compared to the TLR7/TLR9 DKO males and produce more autoantibodies by ELISA.

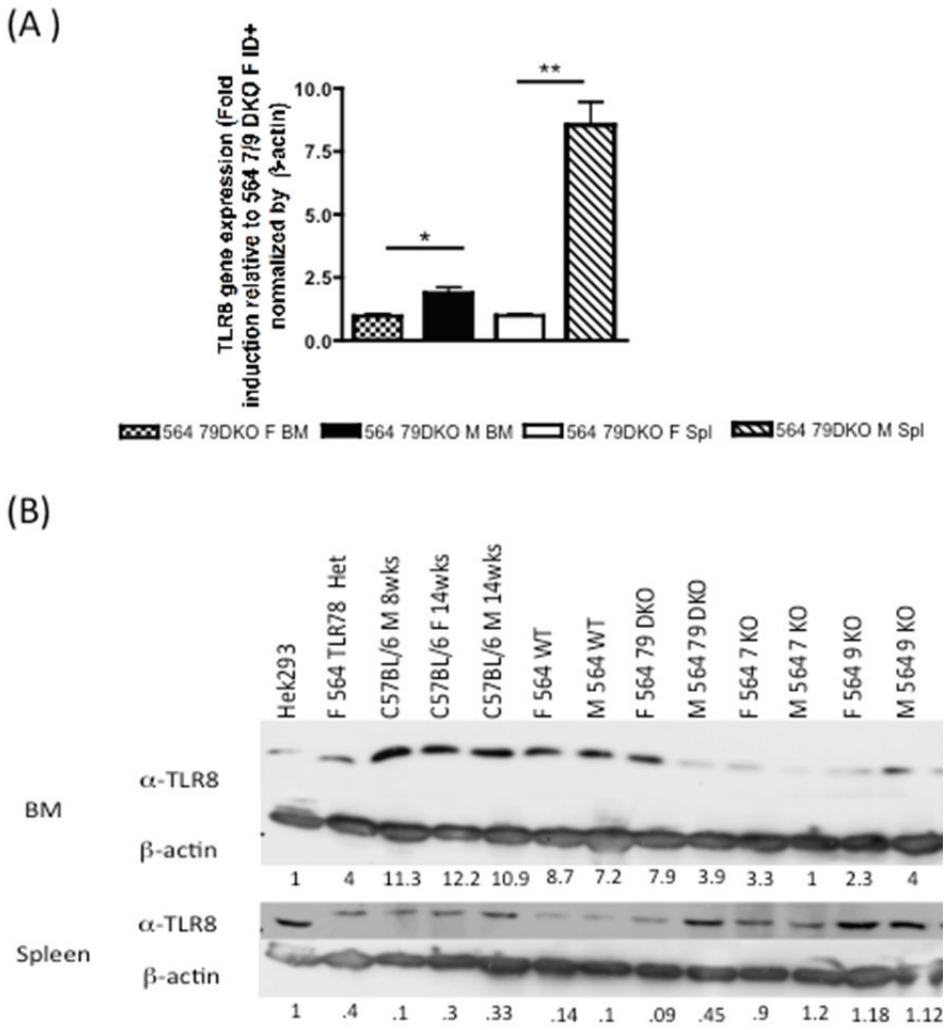

Figure 9: *Tlr8* gene expression is increased significantly in male mice but does not translate to increased TLR8 protein expression

A) 564 Idiotype + cells of 3 month-old age matched 564Igi *Tlr7/Tlr9* DKO female and male mice were sacrificed and BM and SPL cells were sorted. In the BM viable, B220+, AA4.1+ (immature) and Id- and Id+ cells. In the SPL B220+, AA4.1- (mature) cells were sorted for Id- and Id+. These populations were analyzed by Taqman real time qPCR for *Tlr8* expression. Data was normalized to β-actin and analyzed relative to (B220+, AA4.1+ κλ+, BM) or (B220+, AA4.1-,

κλ+, Spl) C57BL/6 cells. Significance was determined by the student T-test **, < .01 *, < .05. B) Western blot analysis of TLR8 protein expression in the BM and SPL of indicated mouse lineage. Comparison of protein expression was done by densitometry.

Determination of the pathogenesis of SLE in various mouse lineages

We performed mortality studies of several 564Igi mouse lineages to determine the effect of the various TLR deficiencies on the pathology of SLE. The longevity, disease progression, and the disposition of the BM, spleen and kidneys were compared between female 564Igi WT, 564Igi *Myd88* KO, 564Igi *Tlr7* KO, 564Igi *Tlr7*/*Tlr9* DKO, 564Igi *Tlr7/Tlr9* KO and male 564Igi Yaa mice by FACs and histological analysis. We observed that 564Igi *Tlr7*/*Tlr9* DKO mice die at a similar rate to *Tlr7* KO. This indicates that *Tlr8* expression seems to be having an effect on the mortality of the mice (Figure 10B). Two of six *Myd88* KO 564Igi mice died at the age of 14 months and 17 months respectively, however, four of them were still alive at 20 months of age when most of the other 564Igi mice were dead.

Histological analysis of kidney sections was done for deposition of antigen/antibody complexes and pathology of the glomeruli (Figure 10A). We have determined that in all 564Igi mouse lines, except *Myd88* KO mice, there were morphologic changes to the kidneys consistent with class 3 to 4 lupus. In 564Igi *Myd88* KO mice there are chronic glomeruli consistent with advanced age. Immunofluorescent examination however uncovered IC deposits that were IgG

positive but 564Id negative. 564Igi WT mice, had class 3 lupus nephritis with increased mesangial cellularity, segmental sclerosing lesions, vascular disease and capsular inflammatory changes. By immunoflourescent microscopy IC deposits were found in the glomeruli and were positive for IgG and 564 Idiotype. In 564Igi *Tlr7* KO mice, there were increased mesangial and endocapsular cellularity, diffuse global inflammatory changes with possible crescents. 564 Id and IgG IC deposits were identified in the glomeruli.

Both 564Igi *Tlr9* KO and 564Igi *Tlr7/Tlr9* DKO mice were classified as class 3 with increased mesangial cellularity, focal and segmental sclerosing lesions. Significant 564 Id positive IC deposits were found in both mice, however 564Igi *Tlr9* KO mice had severe disease with IC deposits found in the basement membrane, tubular epithelial cells and the cells surrounding the blood vessels of the kidneys. The IC deposits were strikingly positive for 564 Id, IgG and IgG2a. A similar staining pattern was observed in the case of 564Igi Yaa mice, however to a much milder degree. Immune complex deposits in the tubular epithelial cells and in the lining of blood vessels are associated with a poor prognosis in human lupus patients and presumably in mice as well (Figure 10A).

As expected, based on ELISA and ANA results 564 *Tlr9* KO mice had much more IC deposits in the glomeruli than 564Igi WT mice. Surprising however, 564 Tlr7/Tlr9 DKO mice had similar results to *Tlr9* KO mice when it came to anti 564 IC deposits. This IC deposition into the kidney and the resulting damage may indicate a mechanism for the increased mortality trend observed in 564Igi *Tlr7/Tr9* DKO mice (Figure 10B).

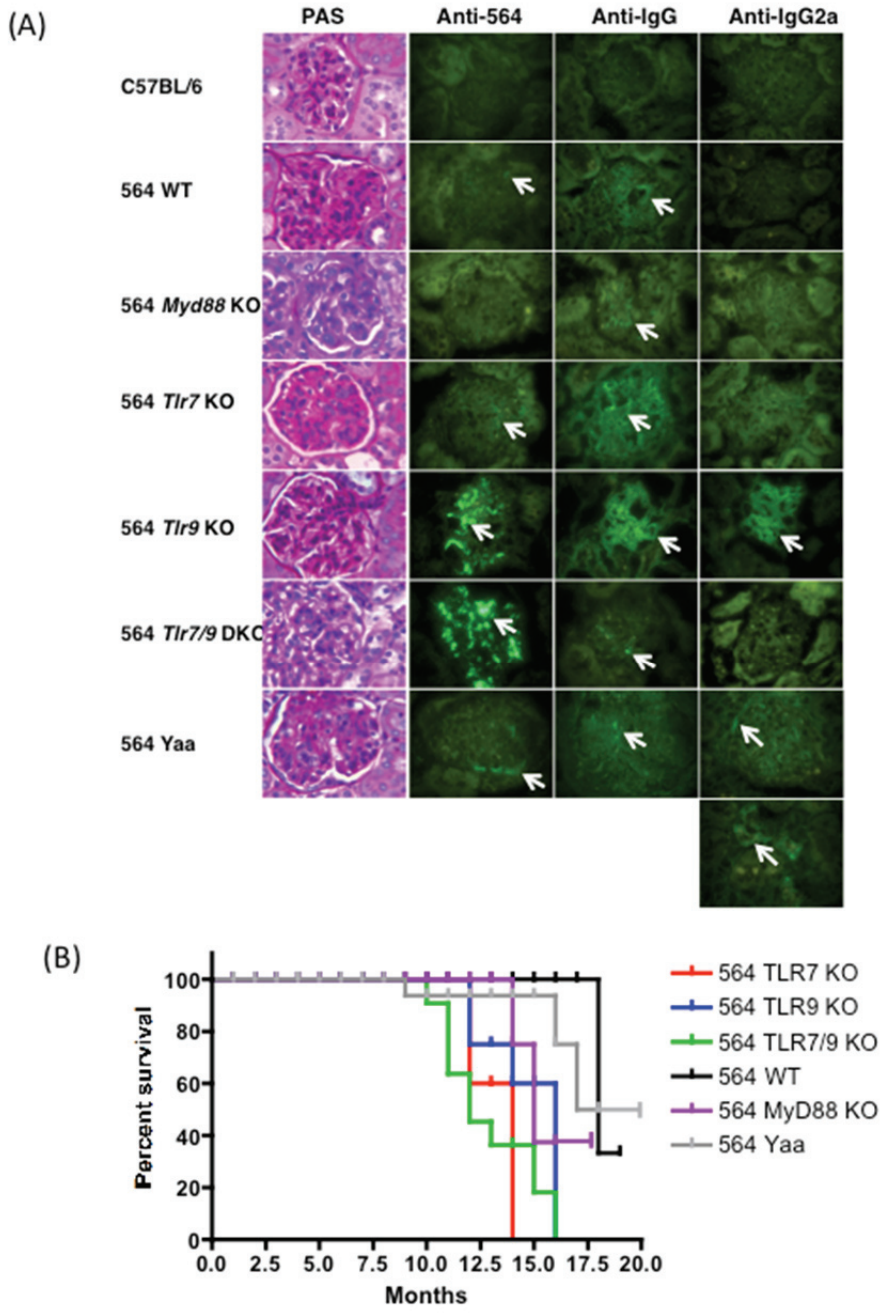

Figure 10: Determination of the natural course of SLE in various 564Igi mouse lineages

A) Kidneys were harvested from moribund mice and frozen in OTC solution or fixed in formulin. Tissue samples were sectioned and stained with PAS, Alexa

488 conjugated anti-564 Idiotype antibody (B6.256), FITC conjugated anti IgG and FITC conjugated anti-IgG2a. In order to determine that a sample was negative for Immune complex deposits at least 10 glomeruli were examined. B) 564Igi Yaa male mice and female mice of the various 564Igi lineages were set aside and aged to determine the natural course of the SLE disease progression as well as to determine the average age of mortality of these mice. 564Igi WT (females) n=3, 564 TLR 9 KO (females) n=4, 564 *Tlr7*/ *Tlr9* DKO (females) n=10, 564 MyD88 KO (females) n=6, 564 *TLR7* KO (females) n=4, 564Igi Yaa (males) n=5.

Loss of both Tlr7 and Tlr8 results in a drastic reduction of IgG2a and IgG2b RNA autoantibodies in 564Igi

Based on all the evidence collected, it seemed likely that TLR8 was playing an important role in autoantibody production. In order to directly assess the influence and function of *Tlr8* in autoantibody production in the 564Igi mouse model, a *Tlr8* KO animal was necessary. Unfortunately such an animal was not readily available so I bred 564Igi to *Tlr7*/*Tlr8* DKO mice obtained from the Flavell laboratory (Figure 11A). These mice allowed us to ascertain the effect of loss of both known TLRs responsible for sensing ssRNA.

Analysis of sera from 564Igi *Tlr7/Tlr8* double deficient mice indicated that anti-564 autoantibodies are almost abolished, similar to that found in the sera of 564Igi *Myd88* deficient mice (Figure 4A, B and Figure 11B). ANA analysis using HEp2 cells indicated that there were no detectable autoantibodies in the sera of

these mice (Figure 11C). There was also no difference in autoantibody production between male and female 564Igi *Tlr7/Tlr8* DKO, indicating that the factor that originally was responsible for the sex discrepancy observed in 564 *Tlr7/Tlr9* DKO mice was no longer effective (Figure 11A, B, C and Figure 6). Due to a lack of any staining in the ANA analysis, as well as the significant reduction in the levels of anti-RNA antibodies in the sera as assessed by RNA ELISA (Figure 11C and D) we conclude that autoantibody production in 564Igi is dependent of both *Tlr7* and *Tlr8.*

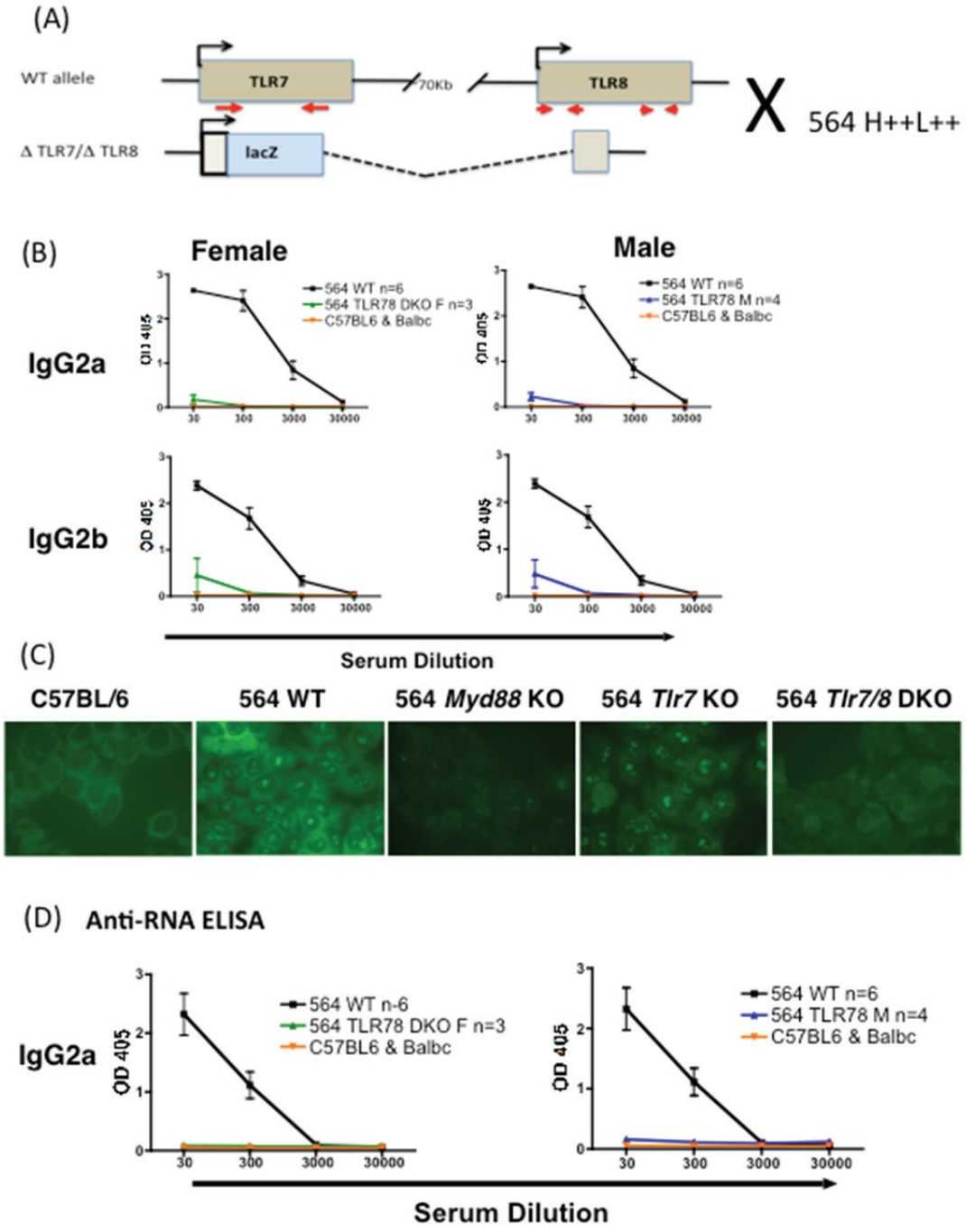

Figure 11: Loss of both *Tlr7* and *Tlr8* result in a drastic reduction of IgG2a and IgG2b autoantibodies in 564Igi

(A) Schematic of *Tlr7* and *Tlr8* deletion from the X chromosome and the basic breeding plan to create 564Igi *Tlr7/Tlr8* DKO mice. (B) ELISA analysis of Sera

from 564Igi WT, C57BL/6, Balb/c and 564 *Tlr7/Tlr8* DKO mice. The top panel shows IgG2a and the Bottom is IgG2b. The ELISA was done as described in Figure 4 except data collected 70min post PnP substrate addition. **C**) HEp2 cell staining of 564Igi *Tlr7/Tlr8* DKO mice D) RNA ELISA using sera from 564Igi WT versus 564Igi *Tlr7/Tlr8* DKO male and female mice developed with AP conjugated IgG2a and IgG2b. 564 WT (n=6), 564 *Tlr7/Tlr8* DKO Female (n=3), 564 *Tlr7/Tlr8* DKO male (n=4) and C57BL/6 and Balb/c (n=2). Error Bars represent SEM.

Autoantibody deficiency in 564Igi TLR7/8DKO mice is not due to a generalized deficiency in IgG2a or IgG2b antibodies.

One possible explanation for the lack of autoantibody production in 564Igi Tlr7/Tlr8 DKO mice is that antibody production on a whole is inhibited. This hypothesis is refuted by the fact that there is no difference in total IgG2a or IgG2b antibody production between 564 Tlr7/Tlr8 DKO mice and 564 WT mice (Figure 12).

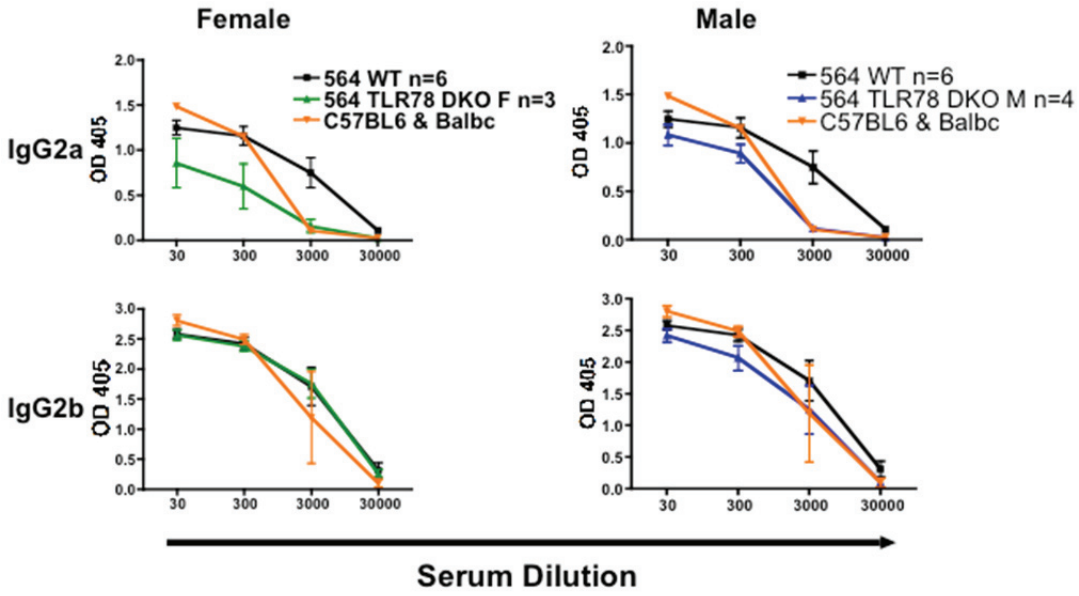

Figure 12: Loss of both *Tlr7* and *Tlr8* has no effect on total IgG2a and IgG2b antibody production in 564Igi

ELISA analysis of Sera from 564Igi WT, 564Igi *Tlr7/Tlr8* DKO, C57BL/6, Balb/c mice. The top panel shows IgG2a and the Bottom is IgG2b. The ELISA was done as described in Figure 4 except plates were coated with total IgG2a or IgG2b. 564 WT (n=6), 564 *Tlr7/Tlr8* DKO Female (n=3), 564 *Tlr7/Tlr8* DKO male (n=4) and C57BL/6 and Balb/c (n=2). Error Bars represent SEM.

Chapter III: 564Igi granulopoiesis is MyD88/TLR dependent

Introduction

SLE development and pathogenesis is thought to be mediated by the deregulation of the adaptive immune system. Recent work by our lab and others has elucidated the role that innate immune receptors such as TLR7 and TLR9 play in disease development (Leadbetter et al. 2002; Deane et al., 2007;Pisitkun *et al.* 2006; Subramanian *et al.,* 2006; Pisithun *et al.,* 2006; Fairhurst *et al.* 2008; Berland *et al.* 2006; Christensen *et al.* 2006; Christensen *et al.* 2005; Santiago-Raber *et al.* 2009; Nickelson *et al.* 2010). Another indicator of the involvement of the innate immune system in autoimmunity was the discovery of elevated levels of INF-α in the sera of SLE patients (Shi et al. 1987).

Type 1 interferon (INF-1) is a family of pro-inflammatory cytokines produced and secreted by a variety of cells including plasmacytoid dendritic cells (pDC) (Ronnblom and Alm, 2002), granulocytes and monocytes (Han et al. unpublished data, Figure 16, Figure 17 and Figure 18) and many other innate immune cells in response to activation by endogenous ligands. It was found that 19% of patients with viral Hepatitis or carcinoid tumors that were treated with INFα developed autoimmunity.

Recent work has uncovered an “IFN signature” as well as a “granulopoiesis signature” of gene expression that correlated to the presence of

SLE disease in human children (Peripheral blood leukocytes from SLE patients (both child and adult) were found to express both IFN-I and interferon signature genes (Krug 2008; Baechler et al. 2003; Bennett et al. 2003). Determination of which cell types produced INF-I became a highly important area of research. It is generally accepted that a subtype of DC called pDCs are the major producers of INF-I in both human and murine SLE (Farkas et al. 2001; Jahnsen et al. 2002; Fitzgerald-Bocarsly 2002). Recent studies have however, implicated neutrophils in INF-I production and SLE development (Han unpublished data, Figure 17 and Figure 18).

One interesting aspect of human SLE was the discovery of low buoyant density neutrophils in "contaminated" Ficoll-Hypaque density gradient preparations of peripheral blood mononuclear cells (Hacbarth and Kajdacs-Balla 1986). Bennet et al. also found by microarray analysis, up-regulation of granulopoiesis specific genes such as defensin DEFA-3, which is a major product of immature granulocytes. Additionally they found that a large fraction of their pediatric SLE test subjects had increased immature neutrophils in their blood implicating neutrophils in SLE pathogenesis (Bennett et al. 2003).

Neutrophils are a key initial innate immune response to host infection. They destroy bacteria by generating reactive oxygen species, secretion of granules with bactericidal proteins and the recognition of PAMPs leading to phagocytosis (Malech 2007). These cells also produce cytokines, chemokines and regulate vascular permeability, ensuring the recruitment of additional immune effector cells (Weiss *et al.* 1981). In essence these cells hold the fort

until the cavalry (in the form of adaptive immunity) arrives. Dysregulation of the function of these cells on the other hand can lead to severe tissue damage such as what is seen in SLE.

In a study published recently on adult SLE patients, a distinct subset of pro-inflammatory neutrophils were isolated and they were found to have impaired phagocytic potential and an activated phenotype. They induced significant endothelial cell cytotoxicity and secreted increased levels of IFN-I which was sufficient to inhibit endothelial cell differentiation. It was suggested that these neutrophils may be important for the development of vascular damage in lupus, as they both damage endothelial cells and prevent their repair (Denny *et al.* 2010). Expansion of these pro-inflammatory neutrophils in SLE can be quite dangerous.

Granulopoiesis in 564Igi

Studies from our laboratory, using the 564Igi SLE mouse model, have also shown that these mice, similar to humans, have in the BM and periphery granulopoiesis that can be attributed to increased numbers of neutrophils. This expansion of neutrophils was further enhanced when 564Igi was breed to C4 deficient mice (Han Unpublished data, Figure 18). It has been well documented that IFN-I is important for SLE development in humans and mice, and INF-1 production was found to be increased in 564Igi neutrophils compared to C57BL/6 mice (Han Unpublished data, Figure 18).

Neutrophils are the largest fraction of cells in the granulocyte compartment as well as in peripheral blood (Janke *et al.* 2009), and as it was found that myeloid cells including neutrophils were the major producers of IFN-α in the bone marrow (Han unpublished data, Figure 17), we wondered if the role of granulocytes such as neutrophils in SLE might be more prominent than previously believed.

IL-1 has also been implicated in human disease such as autoimmune rheumatoid arthritis (O'Neill *et al.* 2000). Due to the intricate relationship between the IL-1R and TLR signaling pathways, I questioned if there may be a role for IL-1 in SLE. Evidence that IL-1 might be involved in the pathogenesis of SLE came from a report by Ueda *et al.* where they found that the absence of IL-1R type 1 had a significant impact on the neutrophil and granulocyte population in an alum induced inflammatory mouse model (Ueda *et al.* 2009). This result suggests that the IL-1R signaling pathway may also play a role in the autoimmune granulopoiesis observed in 564Igi mice.

Results

Granulopoiesis is *Tlr7/Tlr8* and *Myd88*- dependent and is suppressed by *Tlr9* in 564Igi Mice

Previously, we found that 564Igi mice have a significantly increased neutrophil populations in the BM, blood and spleen (Han et al. unpublished, Figure 18). I therefore, wanted to determine the effect of TLR deficiency on granulopoiesis. Surprisingly we found that loss of *Tlr7* or *Tlr7*/9 in 564Igi did not lead to a decrease in percentage of granulocytes in the BM or spleens of these mice compared to 564Igi WT. However, absence of *Myd88* in 564Igi mice led to a significant reduction of granulocytes to a level comparable to that seen in C57BL/6 mice (figure 13A and 13B). Similar to the change observed in autoantibody production, *Tlr9* deficiency resulted in significantly increased granulopoiesis as compared to 564Igi WT. Lastly double deficiency of *Tlr7/Tlr8* resulted in a significant decrease in the percentage of BM granulocytes in comparison to 564Igi WT mice (Figure 13B).

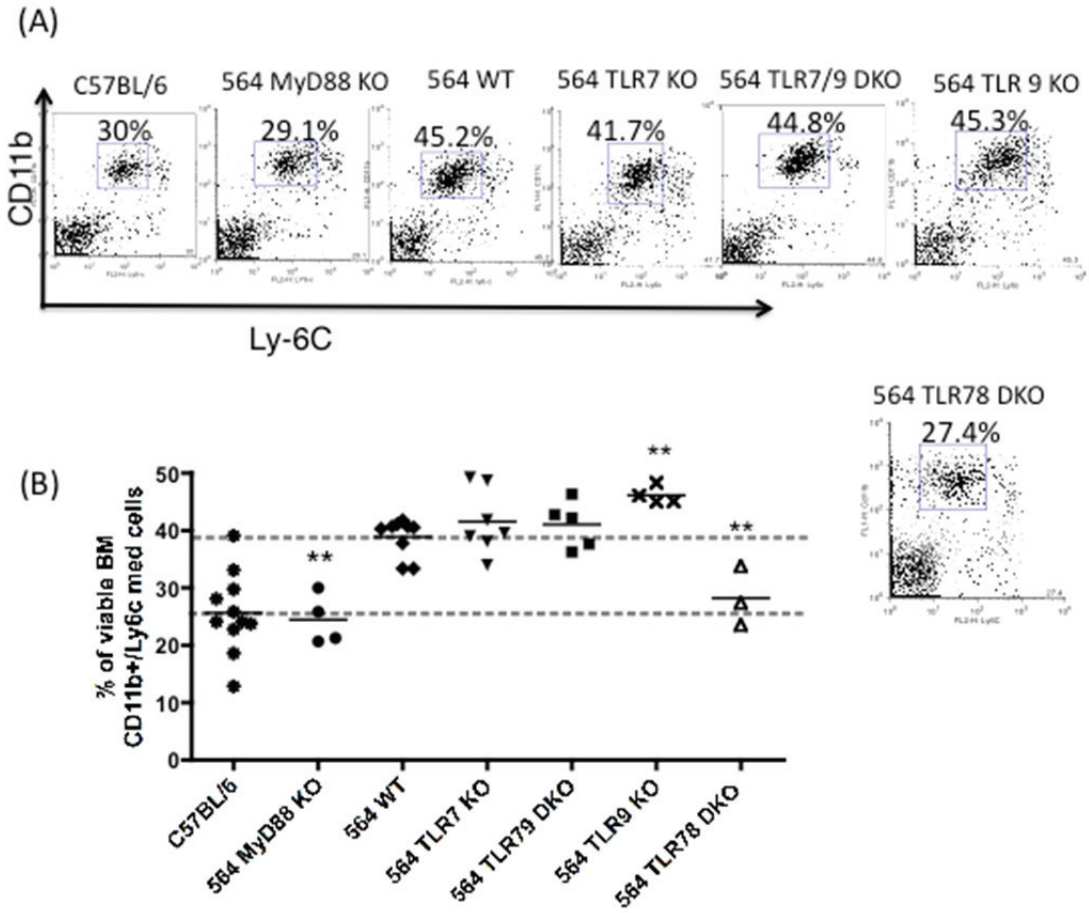

Figure 13: Granulopoiesis is *Tlr7/Tlr8* and *Myd88* dependent and suppressed by *Tlr9* in 564Igi Mice

A) Three month old 564 WT females, 564Igi *Tlr7* KO, 564 *Tlr7*/*Tlr9* DKO, 564Igi *Myd88* KO, 564Igi *Tlr9* KO and C57BL/6 mice were sacrificed and their BM was harvested and analyzed by FACS. B) Quantification of the data from all test subjects. Significance was determined by the one way ANOVA test P value= **, <.01 *, <.05. C57BL/6 n=6, 564 WT mice n=7, 564Igi *Tlr7* KO mice n=7, 564Igi *Tlr7*/*Tlr9* DKO mice n=5, 564Igi *Myd88* KO mice n=4, 564Igi *Tlr9* KO mice n=4 and 564Igi *Tlr7/Tlr8* DKO mice n=3.

IL-1 does not contribute to autoantibody production or granulopoiesis in 564Igi mice

Since *Myd88* lies in the IL-1 receptor type 1 signaling pathway (Akira and Takeda 2004, Sun D. and Ding A. 2006) and inflammatory granulopoiesis and reactive neutropenia was found to be IL-1R type 1 dependent, we investigated the potential role of IL-1 in autoantibody production and granulopoiesis by using IL-1R type 1 deficient 564Igi mice (figure 14). This analysis showed that autoantibody production and granulopoiesis in 564Igi is IL-1R independent and solely *Tlr7/Tlr8* dependent. In the flow cytometry done during this experiment staining of the granulocytes with CDIlb and Ly-6C was reduced over all compared to previous experiments using these antibodies. While it is impossible to compare these experiments to other experiments done to examine granulopeoisis we can compare the mice listed to each other as they were done in triplicate using the same antibody cocktail.

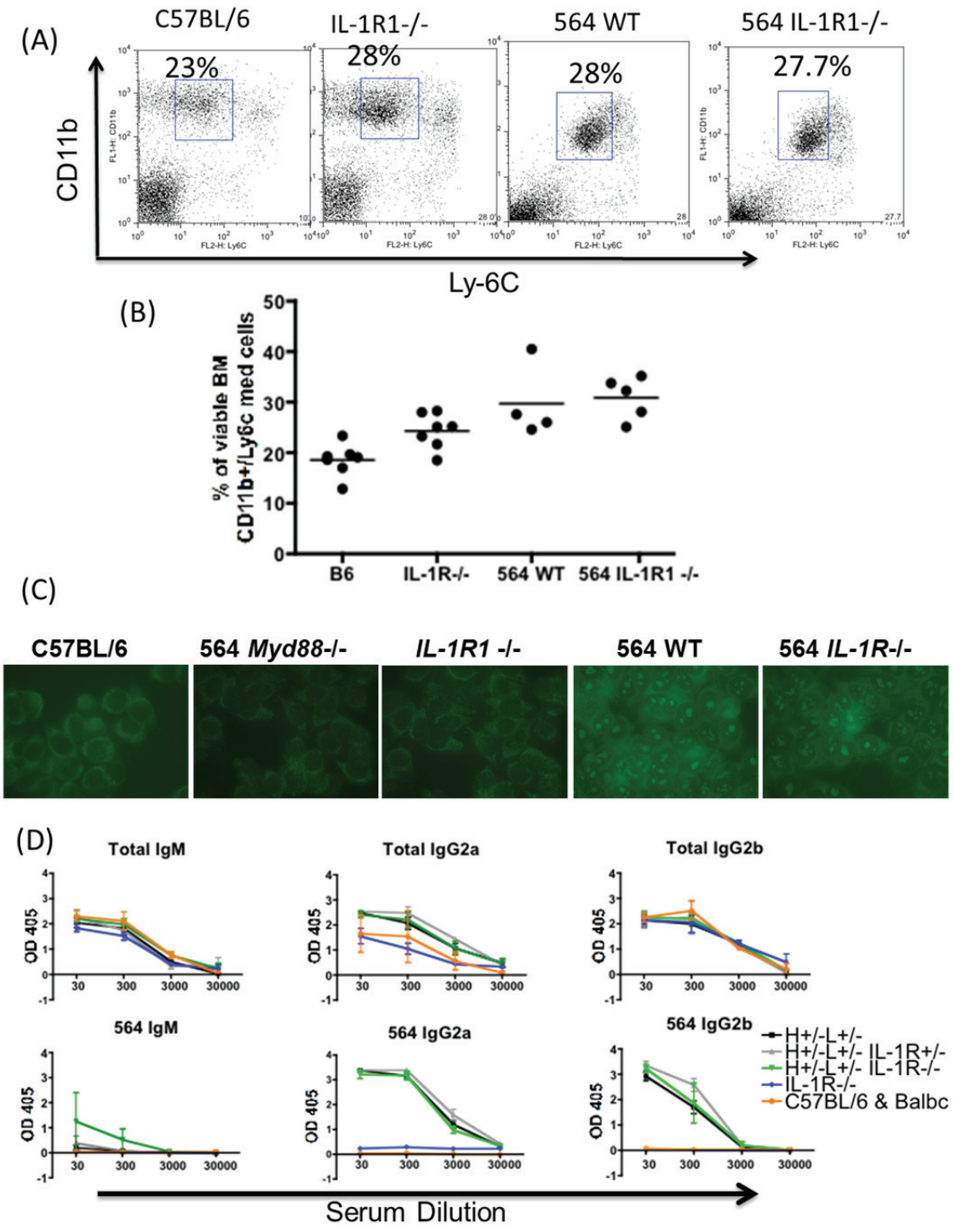

Figure 14: IL-1R type 1 signaling is not responsible for MyD88 dependent autoantibody production and granulopoiesis in 564Igi mice

A) Three month old 564 WT females, 564 IL-1R1 KO, IL-1R1 KO, and C57BL/6 mice were sacrificed and their BM was harvested and analyzed by FACs. B) Quantification of the data from all test subjects. Significance was determined by the one way ANOVA test P value= **, <.01 *, <.05. (C) ANA testing was performed using sera from 3 month old C57BL/6, 564 WT, MYD88 KO, 564 IL-1R1 KO and IL-1R1 KO mice. Slides containing HEp2 cells were incubated with mouse sera at different dilutions for 30 min and then treated with mouse IgG (Fab') Shown 1:25 dilution (D). Sera from the same 3-month-old female mice detailed in (A), in addition to 564 IL-1R1 heterozygotes, were tested by ELISA on plates coated with total IgG2a and IgG2b. Serial dilutions were done as indicated and the plates were developed using alkaline phosphatase (AP) labeled anti-IgG2a and IgG2b. Upper panel represents IgG2a and the lower panel is IgG2b. Significance was determined by the Student t-test. **, < .01, *, <. 05. B6 (n=2), 564 WT mice (n=3), 564 IL-1R1 heterozygote, mice (n=2), 564 IL-1R1 KO (n=3), IL-1R1 KO (n=3). Error bars represent the SEM.

Chapter IV: Discussion

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by elevated levels of nuclear antigen specific autoantibodies in the sera of patients. Autoantibodies are produced by B cells leading to antigen/antibody complexes (IC) that are deposited into vital organs. These ICs eventually lead to inflammation, tissue and organ damage such as lupus nephritis. Understanding how autoantibody production is regulated is important for elucidating the pathogenesis of SLE.

The results presented in this thesis clearly show that there is regulation of autoantibody production by the MyD88 dependent nucleic acid sensing toll-like receptors 7, 8 and 9. Utilizing Rag deficient animals incapable of producing T cells, our laboratory has previously reported that autoantibody production in 564Igi mice is T-cell independent ((Berland *et al.*, 2006). We have also shown that *Tlr7*-deficient mice have significantly decreased, but not complete abrogation of autoantibody production (Berland *et al.*, 2006). Studies initiated by other laboratories have confirmed that TLR7 is important in the pathogenesis of SLE (Deane et al., 2007; Fairhurst et al., 2008; Pisitkun et al., 2006; Subramanian *et al.*, 2006). This result raised the question, is there another TLR involved in autoantibody production? Or is it another factor altogether?

To address this question I bred *Myd88*-deficient mice to 564Igi, as MyD88 is the adapter protein for all TLRs except TLR3. I found that 564Igi *Myd88*-deficient mice were depleted of autoantibodies indicating that the relevant factor

is essential for autoantibody production and must be MyD88 dependent (Figure 4A, B, and C). Despite the fact that TLR3 is not MyD88 dependent, it is a nucleic acid sensing TLR, therefore it is a possible candidate. This concern was addressed by Christensen et al. in the MRL/*lpr* mouse model that showed that ablation of TLR3 did not effect either RNA or DNA autoantibody production (Christensen *et al*., 2005).

There are non-TLR nucleic acid sensing receptors such as RIG-1 like receptors which can be involved in the recognition of self-nucleic acids and the initiation of an autoimmune response. A role for these receptors in 564Igi autoimmunity is diminished by the finding that MyD88 is necessary for the production of auto-antibodies in 564Igi mice. While it is possible that the downstream cascades of one or more of these non-TLR's might utilize MyD88 based on the information that we currently have available it is more likely that a known utilizer of MyD88 is the unknown factor.

IL-1 receptor type 1 utilizes MyD88 in its signaling cascade, however, I found that there was no effect of loss of IL-1R1 on autoantibody production in 564Igi mice (Figure 14D). This implicated TLRs in the regulation of autoantibody production. One important question was whether all MyD88 dependent TLRs were involved in autoantibody production or if it was restricted to nucleic acid sensing TLRs. In an attempt to address this question I breed 564Igi mice to *Tlr4* deficient mice. I found that autoantibody production was TLR4 independent, suggesting that nucleic acid sensing TLR are the main mechanism of

autoantibody production in 564Igi (figure 7). To determine if this was the case I tested another nucleic acid sensing TLR, TLR9.

Consistent with previously published results (Christensen *et al.*, 2005; Christensen *et al*., 2006), *Tlr9* deficient 564Igi mice had increased titers of autoantibodies (figures 4A and 4B). This increase in autoantibody titers suggested that TLR9 might play a suppressive role in the regulation of autoantibodies in 564Igi mice.

It has previously been reported in other mouse models that TLR7 and TLR9 may play opposing roles in autoantibody production (Christensen et al., 2006). As both TLR7 and TLR9 have been shown to be involved in autoantibody production, determining the effect of dual deficiency of *Tlr7* and *Tlr9* in 564Igi mice was important. 564Igi *Tlr7/Tlr9* DKO mice had an interesting discrepancy in antibody titers that was sex dependent (figure 6A and B). Female 564Igi *Tlr7/Tlr9* DKO mice had near WT levels of autoantibody, while male 564Igi *Tlr7/Tlr9 DKO* mice had diminished levels, implicating another factor in autoantibody production in the female 564Igi *Tlr7/Tlr9* DKO mice, possibly *Tlr8*. This sex discrepancy was not observed in 564Igi *Myd88* KO, 564Igi *Tlr7* KO, *and 564Igi Tlr9* KO or in 564Igi WT mice (Figure 4C and Figure 5).

It was important to determine whether *Tlr8* gene expression was up-regulated in 564Igi mice, potentially compensating for *Tlr7* deficiency or the double deficiency of *Tlr7* and *Tlr9*. I found that *Tlr8* gene expression was up-regulated in 564Igi and this up-regulation was enhanced when either *Tlr7* or

Tlr7/Tlr9 was deficient (Figure 8A and B). This suggests that TLR8 may be able to compensate for TLR7 deficiency in 564Igi mice.

The sex discrepancy observed in 564Igi *Tlr7/Tlr9* DKO mice could be explained by the increased TLR8 protein expression in females (figure 6 and Figure 9B). This was to be expected, considering TLR8 is located on the X-chromosome. Surprisingly, TLR8 gene expression was found to be significantly increased in 564Igi *Tlr7/Tlr9* DKO male mice in comparison to female mice (figure 9A), however, this gene up-regulation did not translate at the protein level (figure 9B). This disagreement of the mRNA and protein expression can be explained by post-transcriptional or post-translational regulation that is hormonally mediated. It is known that male and female sex hormones can differentially regulate the gene expression of interferon responsive genes such as Ifi202, which lies in the Nba2 locus (Panchanathan et al., 2009). It is therefore conceivable that estrogen and androgen can also regulate TLR8 protein expression.

Understanding the function of *Tlr8* was hampered due to the notion that TLR8 was non-functional in mice (Heil. *et al.* 2004). However, the ligand R848 known to activate hTLR8 did not stimulate *mTlr8.* When another *TLR8* ligand was used mouse and human TLR8 were clearly activated *(*Gorden *et al., 2006).*

To determine the end result of TLR deficiency on morbidity and mortality of 564Igi mice I aged a number of mice until they were moribund, sacrificed them and harvested their kidneys for histological examination (figure 10A and 10B).

The mouse lineages aged were C57BL/6, 564Igi WT, 564Igi *Tlr7* KO, 564Igi *Tlr9* KO, 564Igi *Tlr7/Tlr9* DKO, 564Igi *Myd88* KO and 564Igi Yaa mice.

It is clear that *Tlr8* expression is important in SLE pathogenesis, as 564Igi *Tlr7*/*Tlr9* deficient animals have increased mortality and the highest gradable level of anti-564 IC deposition in the glomeruli of the kidney (figure 10B). These IC deposits, were only rivaled by those found in the kidneys of 564Igi *Tlr9* KO mice. Not only were there IC deposition in the kidneys, there was also significant inflammatory infiltrates, basement membrane damage and generalized enlargement of the glomeruli when compared to C57BL/6 or 564Igi Myd88 deficient mice (Figure 10B).

The most direct method to determine the function of TLR8 and the role it might play in autoantibody production is to study a *Tlr8* knockout animal. Unfortunately a *Tlr8* knockout mouse was not available so I investigated the effect of loss of both *Tlr7* and *Tlr8* in 564Igi mice. *Tlr7/Tlr8* double deficient mice were obtained from the Flavell laboratory and bred to 564Igi (Figure 11A). The resulting animals were found to have completely abrogated 564 idiotype and RNA specific autoantibody production (Figure 11B and D). These mice also had undetectable antibody titers as determined by ANA testing (Figure 11C). This abrogation of autoantibody production was symmetrical between the sexes and was not due to a complete deficiency of antibody production as total IgG2a and IgG2b were not significantly different between 564WT and 564Igi *Tlr7/Tlr8* DKO mice (Figure 11 and Figure 12). The conclusion that I found to this line of inquiry

is that autoantibody production in 564Igi mice is TLR7/TLR8 and MYD88 dependent (Figure 11B, C, D, Figure 4A and B).

Upon TLR recognition of its ligand, a signaling cascade is initiated which initially utilized the adapter protein MyD88 and ultimately results in the initiation of an immune response by initiating the transcription of INF-I cytokine. As previously described IFN-I, promotes; cell proliferation, B-cell differentiation into plasma cells and activation of DCs. Treatment of patients with viral Hepatitis or carcinoid tumors with INF-α was found to cause autoimmunity in 19% of patients highlighting the role of INF-α in disease development (Kalkner et al. 1998; Ronnblom et al. 1991).

Elevated levels of INF-α were found in the sera of SLE patients (Shi et al. 1987). In addition, several groups have reported an "IFN and granulopoiesis signature" of gene expression that correlated to the presence of autoimmune disease (Bechler et al. 2003; Bennet et al. 2003; Crow et al. 2003). There has been a strong belief that the major producers of INF-I in SLE patients were pDCs (Farkas et al. 2001; Jahnsen et al. 2002; Fitzgerald-Bocarsly 2002). However, work done in our laboratory by Jin-Hwan Han revealed that myeloid cells and granulocytes are in fact the largest producers of IFN-I (Han unpublished data, Figure 17).

Up-regulation of serum INF-I is not the only similarity between human and murine SLE. Both humans and mice also have increased granulopoiesis (Figure 18 and Bennett et al. 2003; Denny *et al.* 2010). Granulocytes are a group of different cell types the larges fraction of which, are neutrophils. Neutrophils are

the key initial innate immune response to host infection. In a study done on SLE patients, a distinct subset of pro-inflammatory neutrophils were isolated that had an activated phenotype; they induced significant endothelial cell cytotoxicity and secreted increased levels of IFN-I (Denny *et al.* 2010). In 564Igi mice the neutrophil population was shown to be increased compared to C57BL/6 mice. The Neutrophil population was defined as CD11b+ GR-1 hi. The neutrophils was increased in the bone marrow, spleen and in the peripheral blood. They were found to secrete increased levels of INF-β when compared to wildtype C57BL6 mice. (Han unpublished data, Figure 18).

Neutrophils are activated by stimulation of TLRs by their ligands and Fcγ receptor crosslinking (FcγRIII and FcγRIV) upon preferred recognition of the IgG2a or IgG2b component of immune complexes (Nimmerjahn *et al*. 2005; Nemeth *et al* 2008). This results in the activation of the effector function of these cells including phagocytosis and the release of cytokines and chemokines. Since we have shown TLRs specifically TLR7 and TLR8 are important for autoantibody production I wondered if deficiencies in TLR signaling would have an effect on granulopoiesis in 564Igi mice. It has been shown that TLR8 but not TLR7 induces selective and direct activation of human neutrophils (Janke *et al*., 2009), so perhaps TLR8 plays a similar role in the activation of murine neutrophils.

I found that like autoantibody production, granulopoeisis was significantly reduced by MyD88 deficiency and increased by TLR9deficiency (figure 13A and 13B). Surprisingly, deficiencies in TLR7 did not have an effect on granulopoiesis (figure 13A and 13B). An explanation for this phenotype is that since TLR7

deficiency did not deplete all of the autoantibodies immune complexes were still being made and granulocytes were activated and stimulated to proliferate. This is an important finding as it might provide a link between autoantibody production and the percentage of granulocytes in 564Igi mice.

This link was not due to MyD88's role in the IL-1 receptor type 1 signaling pathway (Figure 14A and B), nor is it due to the role that IL-1 receptor plays in inflammatory granulopoeisis (Ueda et al., 2009 and Figure 14). We can surmise that granulopoiesis in 564Igi mice is a function of autoantibody production which is dependent on MyD88, *Tlr7*/*Tlr8* and suppressed by *Tlr9*.

As I previously described the role of TLR7 in autoimmunity is well described. TLR7 is involved in the major percentage of autoantibody production however; there has been no report that links TLR7 to the increase in granulocytes. The goal of this thesis was to elucidate what role TLR8 played in autoimmunity. Utilizing our 564Igi *Tlr7/Tlr8* DKO mice we have seen that the remaining percentage of autoantibodies can be accounted for by TLR8. However while 564Igi *Tlr7* KO and *Tlr7/Tlr9* DKO mice did not have a decrease in the percentage of granulocytes, The 564Igi *Tlr7/Tlr8* DKO mice did. This implies that TLR8 is the factor likely most responsible for this portion of the immune response.

In conclusion I have shown that TLR8 is important in autoantibody production and granulopoiesis, two significant aspects of SLE in humans and 564Igi mice. I believe that due to the many similarities between murine and human SLE described in this thesis and the role for murine TLR8 in autoantibody

production and granulopoeisis, TLR8 should be explored as a critical factor for SLE disease development in humans.

Future Directions

It is clear that TLR8 expression is important in the pathogenesis of murine SLE in 564Igi. Despite this interesting and positive result there are still unanswered questions. Such as, what would be the outcome of deficiency of all the MyD88 dependent nucleic acid sensing TLRs without affect the activity of other TLRs? One way to address this question is to breed Unc93b1 KO mice to 564Igi. UNC93B is an endoplasmic reticulum transmembrane protein expressed in B cells and DCs and is essential for TLR 3, 7, 8 and 9 signaling (Fukui et al 2009; Brinkmann *et al.* 2007). The protein product from the Unc93B gene interacts with these TLRs and allows them to reach the endolysosomes. Patients deficient in Unc93B were found to accumulate self-reactive B cells but did not develop autoimmune disease (Isnardi et al. 2008). Patients with active SLE were found to have significant up-regulation of UNC93B mRNA and protein in their B cells, which correlated with the production of anti-dsDNA antibody correlating with the established role that TLR up-regulation plays in SLE pathogenesis (Nakano et al. 2010).

It will be interesting to see what differences if any exist between 564Igi *Tlr7/Tlr8* DKO and 564Igi *Unc93b1* KO mice. This would include ELISA analysis, HEp2 cell analysis and FACS analysis. I would also be interested to know what

effect this would have on interferon expression in 564Igi mice that was deficient in UNC93B.

Another question to be explored is whether interferon gene or protein expression is specifically affected by the lack of *Tlr7* and *Tlr8* in neutrophils and plasmacytoid dendritic cells. I would repeat experiments done by Jin Hwan Han looking at INF-α and INF-β expression in neutrophils identified by CD11-b and Gr-1 in C57BL/6 mice and 564 WT mice and I would expand the experiment to also look at interferon expression in 564Igi *Myd88* KO, *Tlr7* KO, *Tlr9* KO, *Tlr7/Tlr9* DKO and *Tlr7/Tlr8* DKO. I would posit that expression of interferon would track along the same trajectory as the percentage of granulocytes in each individual mouse lineage. For example, 564Igi *Myd88* deficient and *Tlr7/Tlr9* DKO mice would have less interferon expression when compared to 564Igi WT and the other 564Igi TLR deficient lineages.

Further experimentations with the aging population of mice are necessary. For example 564Igi *Tlr7/Tlr8* DKO mice should be aged to compare their mortality rate and the rate of autoimmunity if any in these mice against the other 564Igi TLR deficient mouse lineages. In addition the aging population of 564Igi WT and TLR deficient mice were examined for autoimmune disease in their kidneys and spleens, however it is well know that lupus can affect multiple organ systems and that each individuals' lupus behaves differently. There for it would be interesting to examine the skin, hearts and lungs of these animals looking for other end-organ damage from being on an autoimmune prone background such as 564Igi.

Chapter V: 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). 564Igi WT mice were breed in house to create homozygous *Tlr4* deficient obtained from A. Poltorak (Tufts University School of Medicine, Boston, MA), *Tlr7* deficient, TLR9 deficient, TLR7/TLR9 double deficient and *Yaa* mice. MyD88 deficient mice were obtained from A. Marshak-Rothstein (Boston University School of Medicine, Boston, MA) and L. Hu (Tufts Medical Center, Boston, MA) and TLR7/TLR8 (obtained from R. Flavell Yale University School of Medicine, New Haven, CT) double deficient mice. C57BL/6 mice and B6; 129S-*Tnfrsf1a*^{tm1Imx} *Il1r1*^{tm1Imx}/J (IL-1 receptor deficient) were purchased from Jackson laboratories.

ELISA

To determine the level of 564 IgG2a, IgG2b, IgM and in some cases IgG3 and IgA antibody in the sera of various mouse lines. 96 well plates were coated with 5mg/well of B6-256 anti-idiotype (id) antibody O/N. Plates were washed and blocked in borate buffer with 1% BSA and then mouse sera from different mouse lines was added from several different lineages of mice at serial dilutions from 1:30 to 1:30,000. The plates were developed using alkaline phosphatase (AP)

labeled anti-IgG2a (southern biotech), IgG2b and IgM and treated with 4-nitrophenyl phosphate disodium salt hexahydrate (pNP; Sigma-Aldrich). The data was acquired in a Spectra Max 340 ELISA plate reader (Molecular Devices) at the optical density of 405 nm (OD_{405}).

RNA ELISA

To determine the level of RNA binding antibodies in the Sera of 564Igi WT, 564 *Tlr7*/*Tlr8* double deficient mice, BALB/c and C57BL/6 mice. ELISA plates were coated with 50mg/ml poly-L-Lysine diluted in dd$H_2$0 overnight at 4 degrees Celsius. After washing plates 3 times with 1xPBS/ 0.05%tween coat with 10mg of yeast RNA in PBS overnight at 4 degrees Celsius. ELISA plates were blocked in 1xPBS/ 0.05%tween/ 5% goat serum. At this point the RNA ELISA is conducted as described above.

Flow Cytometry and Cell Sorting

Except where noted, three month old 564Igi WT males and females, 564Igi *Tlr7* KO, 564Igi *Tlr7*/*Tlr9* DKO, 564Igi *Tlr9* KO, 564Igi *Tlr7*/*Tlr8* DKO, 564Igi MyD88 KO, C57BL/6 and 564Igi Yaa mice were sacrificed and their BM and spleens were harvested and analyzed by FACs analysis. Cells were stained for flow cytometry via standard procedures. Propidium iodide (PI) was added just prior to sorting on a MoFlo instrument (Dako Cytomation), and FACS analysis was done with FACSCalibur (BD Biosciences) acquired data was analyzed by FlowJo

software. In order to sort pre-B, immature B (both from BM) and mature B cells (from spleen) from wild type C57BL/6 mice, cells were stained with goat anti-mouse λ and κ polyclonal antibodies labeled with FITC (SouthernBiotech). Subsequently PE-labeled rat anti-mouse AA4.1- and Alexa647-labeled rat anti-mouse B220 monoclonal antibodies (both from Pharmingen) were used. To sort pre-B, immature B (both from BM) and mature B cells (from spleen) from 564Igi and the mice deficient in TLRs they were stained with FITC labeled rat anti-mouse B220 monoclonal antibodies and PE-labeled rat anti-mouse AA4.1 (both from Pharmingen) and Alex647 conjugated B6.256 anti-564-idiotypic antibody. To assess the percentage of B-lymphocytes in the BM or spleen of mouse lineages by FACS analysis, cells were stained with a mixture of FITC labeled anti-IgG and anti IgA, PE-labeled rat anti-mouse AA4.1 and Alex647 conjugated B6.256 anti-564 idiotype antibody. For the identification and visualization of granulocytes in the BM or spleen of mouse lineages cells were stained with FITC labeled anti-CD11b (cat # 11-0112-85) and PE labeled anti-Ly6-c. To determine the percent of plasmacytoid dendritic cells (pDC) in the BM and spleen, cells were stained with FITC labeled rat anti-mouse B220 monoclonal antibodies, PE labeled anti-mouse Siglec-H antibody and finally with Alexa647 labeled anti-mouse CD11c antibody. Viable cells were gated by PI-exclusion.

Antinuclear antibody (ANA) testing

HEp2 cells were probed using sera from 3-month old C57BL/6, 564 WT, MYD88 KO, *Tlr7* KO, *Tlr9* KO and *Tlr7*/*Tlr9* DKO and *Tlr7*/*Tlr8* DKO mice. Slides containing Hep2a cells were incubated with mouse sera serially diluted 5-fold at concentrations from 1:25 to 1:300 for 30 minutes in a humidity chamber. Slides were then treated with mouse IgG (Fab') for an additional 30 minutes prior to washing, mounting and visualization using a fluorescent microscope at 100X magnification.

Genotyping

In order to determine the genotype of mice in our colony, we first cut 3-5mm of 21 day old newly weaned mice tails and incubated them overnight in 180µl of Viagen Direct PCR (Tail) lysis buffer (Cat no. 102-T) and 20µl of Qiagen Proteinase K overnight shaking at 55 Celsius (^{o}C). The next day the crude lysates were heat inactivated at 85 ^{o}C for 45 min and cooled prior to use. I utilized several PCR strategies and primer sets to determine the genotypes of the mice in my colony. For all genotyping reactions, I utilized invitrogen reagents. The taq polymerase, $MgCl_2$and the 10X PCR reaction buffer come as a part of a set (cat no. 18038-042). The 10mM dNTPs were made by diluting 100mM ATP, CTP, GTP and TTP in ddH_20 (cat. no.). PCR products are run out on 1.5% agarose at 90 volts for 35 minutes except where noted.

564Igi

In order to determine whether the mice were 564Igi four primer pairs were used. For determination of the genotype of the heavy chain I used the forward primer 564V_H–F 5' TGGAGCTATATCATCCTCTTT 3' and the reverse primer JH4Eμ-R 5' CACAGATTCTTACTTTTTCAA 3'. To identify the endogenous heavy chain I used the forward primer JH25-F 5' TCAGGTCATGAAGGACTAGGGACAC 3' and the same reverse primer JH4Eμ-R. Typically I used 1μl of crude genomic DNA, 14.8 μl of ddH_20 (Purchased RNAse and DNAse free), 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .8μl of 50mM $MgCl_2$, .4μl of each primer pair and .2 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. To determine the genotype of the light chain I used the forward primer 564Vκ-F 5' CAAGTGCAGATTTTCAGCTTC 3' and the reverse primer Jκ5-R 5' CAGCTTGGTCCCAGCACCGAA 3'. To identify mice with the endogenous light chain, I used forward primer Jκ23-F 5' AGTAACTAAACTAGGGGAAGAGGG 3' and the same reverse primer Jκ5-R. Similar to the protocol for the heavy chain, I typically used 1μl of crude genomic DNA, 15.3 μl of ddH_20 (Purchased RNAse and DNAase free), 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .5μl of 50mM $MgCl_2$, .4μl of each primer pair and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. Both 564 heavy, light and endogenous heavy and light chain should be run at 94 ^oC for 3 min, 40 cycles of 94 ^oC for 30 sec, 55 ^oC for 30 sec, 72^oC for 2 min and 72 ^oC for 15min holding at 4^oC. The controls for these PCRs were a known sample of 564 homozygote and C57BL/6. Expected product size for each PCR reaction was

~700bp for 564 heavy chain, ~1100bp for endogenous heavy chain, ~550bp for 564 light chain and lastly ~800bp for the endogenous light chain. If the mouse has no 564Igi band and one endogenous band this indicates that the mouse is not 564Igi. If the mouse has one 564Igi band and one endogenous this indicates that the mouse is a heterozygote and lastly if there is only a 564Igi band the mouse is a 564Igi homozygote.

Tlr4

For our experiments we needed 564Igi *Tlr4* deficient mice. In order to determine the status of the *Tlr4* gene, primers to detect the endogenous gene and the Knockout gene were used. For the endogenous gene forward primer Mouse exon2_up 5' TTATTCATCTTTGGAGAGGAGTGG 3' and reverse primer Mouse exon2_dn 5' AAGGAAGTTTAGTTAGAACCACCTTG 3' were used. To detect the knockout gene forward primer TLR4-/- up 5' GCAAGTTTCTATATGCATTCT 3' and reverse primer TLR4-/- down 5' CCTCCATTTCCAATAGGTAG 3' were used. Both primer sets utilized 1μl of crude genomic DNA, 12.7 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, 1μl of 10mM dNTP, .5μl of 50mM $MgCl_2$, .4μl of each primer pair and 1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. Both TLR4 primer sets were run at 94 ^oC for 3 min, 40 cycles of 94 ^oC for 15 sec, 50 ^oC for 45 sec, 68^oC for 30 sec and 68 ^oC for 7 min holding at 4^oC. Expected product size for each PCR reaction was ~200bp for the endogenous *Tlr4* gene and ~139bp for the knockout *Tlr4* gene.

The controls for these PCRs were a known sample of Tlr4 deficient genomic tail DNA and C57BL/6 genomic tail DNA.

Tlr7

One if the more important mouse lines needed for my experiments were 564Igi *Tlr7* deficient mice. In order to determine the status of the *Tlr7* gene, primers to detect the endogenous gene and the knockout gene were used. For the endogenous gene forward primer Tlr7WT 5' ACGTGATTGTGGCGGTCAGAGGATAAC 3' and reverse primer Tlr7XT 5' CCAGATACATCGCCTACCTACTAGACC 3' were used. To detect the knockout gene forward primer Tlr7Neo 5' ATCGCCTTCTATCGCCTTCTTGACGAG 3' and reverse primer Tlr7XT were used. For Tlr7 WT gene detection I used 1μl of crude genomic DNA, 14.8 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .8μl of 50mM $MgCl_2$, .4μl of each primer pair and .2 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. For *Tlr7* knockout gene detection I used 1μl of crude genomic DNA, 15.2 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .5μl of 50mM $MgCl_2$, .4μl of each primer pair and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. Both *Tlr7* primer sets were run at 94 ^oC for 3 min, 40 cycles of 94 ^oC for 30 sec, 60 ^oC for 30 sec, 72 ^oC for 2 min and 72 ^oC for 15 min holding at 4^oC. The controls for these PCRs were known samples of *Tlr7* deficient genomic tail DNA, *Tlr7* heterozygote and C57BL/6 genomic tail DNA.

Expected product size for each PCR reactions was ~1.6 bp for both the endogenous *Tlr7* gene and the knockout *Tlr7* gene.

Tlr9

Another important mouse line needed for my experiments, were 564Igi*Tlr9* deficient mice. In order to determine the status of the *Tlr9* gene, primers to detect the endogenous gene and the knockout gene were used. To detect both the endogenous and the knockout Tlr9 gene forward primer WT1 5' CTCCTGTGCAGGGGTTCTTGTAGTAG 3' and reverse primer XT 5' GCAATGGAAAGGACTGTCCACTTTGTG 3' was used in addition to the forward primer Neo 5' ATCGCCTTCTATCGCCTTCTTGACGAG 3'. For Tlr9 gene detection I used 1μl of crude genomic DNA, 14.8 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .5μl of 50mM $MgCl_2$, .4μl of each primer and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. The mixture was run at 94 ^oC for 3 min, 40 cycles of 94 ^oC for 30 sec, 60 ^oC for 30 sec, 72 ^oC for 1 min and 72 ^oC for 15 min holding at 4^oC. The controls for these PCRs were known samples of *Tlr9* deficient genomic tail DNA and C57BL/6 genomic tail DNA. Expected product size for each PCR reaction was ~2-1.65 bp and 1.05-850bp for the endogenous *Tlr9* gene and the knockout *Tlr9* gene respectively. I run out the PCR products from this reaction on 3% agarose at 90-100 volts for 45 min to 1 hr in order to have proper separation of PCR products.

Tlr7/Tlr9

The 564Igi *Tlr7/Tlr9* double deficient mice are a combination of the *Tlr7* deficient and the *Tlr9* deficient mice and a genotyped for both genes as described above.

Myd88

In order to determine the status of the *Myd88* gene, primers to detect the endogenous gene and the knockout gene were used. To detect both the endogenous and the knockout *Myd88* gene, forward primer *Myd88* F 5' TGGCATGCCTCCATCATAGTTAACC 3' and reverse primer *Myd88 R* 5' GTCAGAAACAACCACCACCATGC 3' were used in addition to the forward primer *Myd88* N 5' ATCGCCTTCTATCGCCTTCTTGACG 3'. For *Myd88* gene detection I used 1μl of crude genomic DNA, 15.8 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .5μl of 50mM $MgCl_2$, .4μl of each primer and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. The mixture was run at 94 ^oC for 3 min, 40 cycles of 94 ^oC for 30 sec, 55 ^oC for 30 sec, 72 ^oC for 1 min and 72 ^oC for 15 min holding at 4^oC. The controls for these PCRs were known samples of *Myd88* deficient genomic tail DNA and C57BL/6 genomic tail DNA. Expected product size for each PCR reaction was ~550 bp for the endogenous *Myd88* gene and ~650 bp for the knockout *Myd88* gene. I run out the PCR products from this reaction on 3% agarose at 90-100 volts for 45 min to 1 hr in order to have proper separation of PCR products.

IL-1R type 1

To rule out the involvement of IL-1R type 1 in granulopoiesis and autoantibody production I breed 564Igi to *Il1r1*$^{tm1/mx}$ deficient mice. To determine the status of the *Il1r1* gene, primers to detect the endogenous gene and the knockout gene were used. For the endogenous gene forward primer oIMR7898 5' GGTTTGAATGTTGGGGTTTG 3' and reverse primer oIMR7899 5' CACCACCACCTGGCTACTTT 3' were used. To detect the knockout gene forward primer oIMR0160 5' TCTGGACGAAGAGCATCAGGG 3' and reverse primer oIMR0161 CAAGCAGGCATCGCCATG were used. For *Il1r1* WT gene detection I used 2μl of crude genomic DNA, 13 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .5μl of 10mM dNTP, .8μl of 50mM $MgCl_2$, .8μl of each primer pair and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. For *Il1r1* knockout gene detection I used 2μl of crude genomic DNA, 14 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .5μl of 10mM dNTP, .8μl of 50mM $MgCl_2$, .3μl of each primer pair and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. Both *Il1r1* primer sets were run at 94 ^oC for 3 min, 35 cycles of 94 ^oC for 30 sec, 63 ^oC for 1 min, 72 ^oC for 1.5 min and 72 ^oC for 2 min holding at 10^oC. The controls for these PCRs were known samples of *Il1r1* deficient genomic tail DNA, and C57BL/6 genomic tail DNA. Expected product size for each PCR reactions was 353 bp for both the endogenous *Il1r1* gene and ~106bp for the knockout *Il1r1* gene.

***Tlr7/Tlr8* DKO**

Tlr7 and Tlr8 are located adjacent to each other on the X chromosome. To create the double deficient mice both genes were knocked out simultaneously (Valenzuela et al. 2003). In order to determine the status of *Tlr7* and *Tlr8* genes, several different primer pairs were used. The methodology used was to look for the absence of the *Tlr7* gene and the *Tlr8* gene and the presence of the neomycin gene. Primers to detect the *Tlr7* gene are, forward TLR7_F 5' AGG GTA TGC CGC CAA ATC TAA AG 3' and reverse TLR7_R 5' ACC TTT GTG TGC TCC TGG AC 3'. To determine whether Tlr8 is also absent I utilized forward primer TLR8-TIKF 5' ATG GAA AAC ATG CCC CCT CAG TC 3' and reverse primer 5' AATGCCATTACACATATAACGAAAGAG 3'. To detect the neomycin gene I used forward primer Neo sense: 5' TTC GGC TAT GAC TGG GCA CAA CAG 3' and reverse primer Neo antisense 5' TAC TTT CTC GGC AGG AGC AAG GTG 3'. For Tlr7 gene detection and neomycine gene detection I used 1μl of crude genomic DNA, 11.6 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, 1μl of 10mM dNTP, 1μl of 50mM $MgCl_2$, .5μl of each primer pair and .4 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. For *Tlr8* gene detection I used 1μl of crude genomic DNA, 14.8 μl of ddH_20 (Purchased RNAse and DNAse free) 2μl of 10x PCR buffer, .4μl of 10mM dNTP, .5μl of 50mM $MgCl_2$, .4μl of each primer pair and .1 μl of 5U/μl Taq polymerase per reaction in a total of 20μls. Both the *Tlr7* primer set and the neomycin primer set were run at 94 ^oC for 3 min, 40 cycles of 94 ^oC for 30 sec, 53 ^oC for 30 sec, 72 ^oC for 30 sec and 72 ^oC for 5 min holding at 4^oC. The *Tlr8*

primer set were run at 94 ^{o}C for 3 min, with 40 cycles of 94 ^{o}C for 30 sec, 60 ^{o}C for 30 sec, 72 ^{o}C for 1 min and 72 ^{o}C for 15 min holding at 4^{o}C. The controls for these PCRs were known samples of *Tlr7* /Tlr8 double deficient genomic tail DNA, and C57BL/6 genomic tail DNA.

Histology

In order to determine the extent of disease pathology in aging 564igi mouse lineages mice were sacrificed when moribund and kidneys were harvested and preserved in a 15% formalin solution and embedded in paraffin or embedded into Tissue-Tek ® O.C.T. compound (Cat. # 62550-01,4583) and frozen using a mixture of dry ice and acetone. Tissue blocks and formalin preserved kidneys were then processed at the Tufts Medical center Histology laboratories. Frozen kidney samples were cut using a Leica cryostat CM1900 into 4-5 μm sections and stained using hematoxylin and eosin (HE), Periodic acid Schiff (PAS), Alexa 488 conjugated anti-564Idiotype antibody (B6.256), FITC conjugated anti IgG and FITC conjugated anti-IgG2a. The stained sections were photographed in a Zeiss fluorescence microscope. Kidney samples preserved in 15% formalin and embedded in paraffin, were cut using a Leica RM2155 microtome into .5 μm paraffin-embedded sections were stained with PAS and analyzed by light microscopy in a blind manner. When scoring the PAS sections for morphologic evidence of lupus the clinical scale of the international Society of Nephrology/Renal pathology Society (ISN/RPS-2003) Classification of Lupus Nephritis was used. The scale is ordered into classes from 1 through 6. Class 1 indicated minimal mesangial lupus, indicative of normal glomeruli by light microscopy but mesangial immune deposits by Immunofluorescence, class 2 was mesangial proliferative lupus nephritis, class 3 was focal lupus nephritis with active, sclerosing and chronic inactive lesions class four is diffuse lupus nephritis with active, sclerosing and chronic inactive lesions, 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 2005). Immune complex deposits were evaluated by immunofluorescence. Immune complex deposits in the glomeruli of 564Igi mouse lines were rated on a scale of 1 to 3, 3 being the highest degree of deposits. In order to determine that a sample was negative for Immune complex deposits at least 10 glomeruli were examined.

RNA purification, DNase I treatment, and generation of first-strand cDNA

Less than $1x10^7$ cells were lysed in 1 ml of Trizol reagent (Invitrogen) for 5 min at room temperature (RT). 200 μl of chloroform was added and mixed by a vigorous shake and then incubated for 3 min at RT. Subsequently, the samples were spun at 12,000x *g* for 15 min. at 4°C. The top aqueous layer was transferred to fresh tubes after which 500 μl of 100% isopropanol was added and mixed gently by inverting the sample. After 10-min. incubation at room temperature, the sample was spun at 12,000x *g* for 10 min. at 4°C. The supernatant was discarded carefully, and 1 ml of 75% ethanol mixed with RNase-free water was added. The sample was spun again at 7,500x *g* for 5 min. at 4°C after mixing with a vortexer. The supernatant was decanted again carefully and briefly dried at the benchtop. 1 U/μl concentration of an amplification grade DNase I (Invitrogen) was added to less than 1 μg of purified RNA. After 15-min. of the DNA digestion reaction at

room temperature, 1 μl of 25 mM EDTA was added to the sample, and it was incubated at 65°C for 15 min. to inactivate DNase I.

Less than 1 μg of DNase I-treated purified RNA was mixed with 1 μl of 50 μM oligo$(dT)_{20}$ primer and 1 μl of 10 mM dNTP. The mix was incubated at 65°C for 5 min in a Robocycler PCR machine. After the incubation, the mix was cooled down on ice for more than 1 min. The RNA sample with dNTP and oligo$(dT)_{20}$ was mixed with a reverse transcriptase, 1 μl of 200 U/μl SuperScript III (Invitrogen). In a PCR machine, reverse transcription took place by incubating the sample at 50°C for 50 min. followed by heat-inactivation of the enzyme at 85°C for 5 min. RNA-cDNA mixture was mixed with 1μl of RNaseH per sample and incubated at 37°C for 20 min. Store purified cDNA at -20°C or use for qPCR.

Quantitative Real Time Taqman PCR

564 Idiotype $^{+}$ cells of the BM and Spleen of 3 month-old age matched 564Igi WT, 564Igi *Tlr7* deficient and 564Igi *Tlr7*/*Tlr9* double deficient female (and in certain cases male) mice were sacrificed and the harvested BM and Spleen were sorted. In the BM viable, double positive B220, AA4.1 and Id- and Id+ cells. In the Spleen B220+, AA4.1- cells were sorted for Id- and Id+. Experiments were analyzed relative to (B220+, AA4.1+ κλ+, BM) or (B220+, AA4.1-, κλ+, Spl) C57BL/6 cells. All qPCR experiments were carried out and analyzed as follows: first-strand cDNA synthesis was performed on 4-fold serial dilutions of purified RNA. Triplicate for each cDNA reaction was used for amplification with a

predesigned mouse *Tlr8*- or β-actin (endogenous control)-specific TaqMan primer/probe set (Applied Biosystems) in a Biorad IQ5 quantitative PCR system (Biorad). Relative quantification was determined after establishing standard curves for mouse *Tlr8* and β-actin expression using serially diluted Raw cell cDNA. qPCR data represent means that were obtained from triplicates of each 4-fold dilution of first-strand cDNA reaction ± SD. Significance was determined by the student T-test **, < .01 *, < .05.

Western Blot

Whole BM and spleen were harvested from 564Igi mouse lines and C57BL/6 males and females. Red blood cells from cell suspensions were lysed with cell lysis buffer (Sigma Cat. #R7757) 2ml/ per organ for 5min at room temperature. Cells were then washed with RPMI-1640 media with 10% fetal bovine serum (Atlantic Biologics), 100mM MEM non-essential amino acids (Gibco Cat. # 11140-050), 1 mM sodium pyruvate (Gibco Cat. # 11360-070), 500ng/ml of Amphotericine B (Cellgro Cat. #30-003-CF), 2mM L-glutamine (Lonza BioWhittaker Cat.# BW17-605E), 1x penicillin-streptomycin (Lonza BioWhittaker Cat.# BW13-115E) and 5mM 2-Mercaptoethanol (Sigma Cat.# M-7522). Bone marrow and spleen cells were re-suspended in 5 times more lysis buffer (20.mM Tris HCl ph 8.0, 100mM KCL, 1mM DTT, .2%Triton-X-100, 1.2% NP40, 10% glycerol and 1x Halt protease and phosphatase inhibitor cocktail, (Thermo Scientific Cat. #78440) than the volume of the cell pellet. 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 supernates 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 (200mM Tris.HCl pH 6.9, 8% SDS, .4% bromophenol blue, 40%glycerol and .04% β-ME) 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 is 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 cut at the 64 kD mark on the pre-stained protein ladder and incubated for 1-2 hrs at room temperature with polyclonal *Tlr8* antibody 1:400 (ANASpec Cat. #54163) above 64 kD and with mouse anti-β-actin monoclonal antibody (Sigma #A3441) 1:10,000 below the 64 kD mark. 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 #sc-) 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 (Deville Scientific #E3018) was placed in a cassette with the membrane for less than 1min and developed on a Kodak M35A X-OMAT processor.

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Appendix

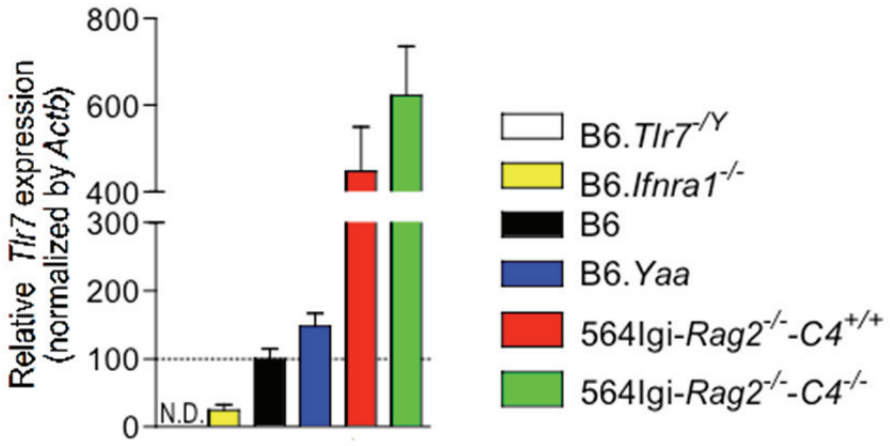

Figure 15: Relative TLR7 and AID expression in bone marrow immature B cells from various mutant mice

Bone marrow immature B cells ($B220^+AA4.1^+\kappa^+\lambda^+$ from B6.*Tlr7*$^{-/Y}$, B6.*Ifnar1*$^{-/-}$, B6, or B6.*Yaa* mice; $B220^+AA4.1^+\kappa^+$ from 564Igi-*Rag2*$^{-/-}$-*C4*$^{+/+}$ or 564Igi-*Rag2*$^{-/-}$-*C4*$^{-/-}$ mice) were purified by cell sorting. Real-time TaqMan reactions were done with pre-designed primer/probe sets for mouse *Tlr7*. The relative level of TLR7 gene expression to that of B6 immature B cells (100) was calculated by normalizing the expression of endogenous control, β-actin.

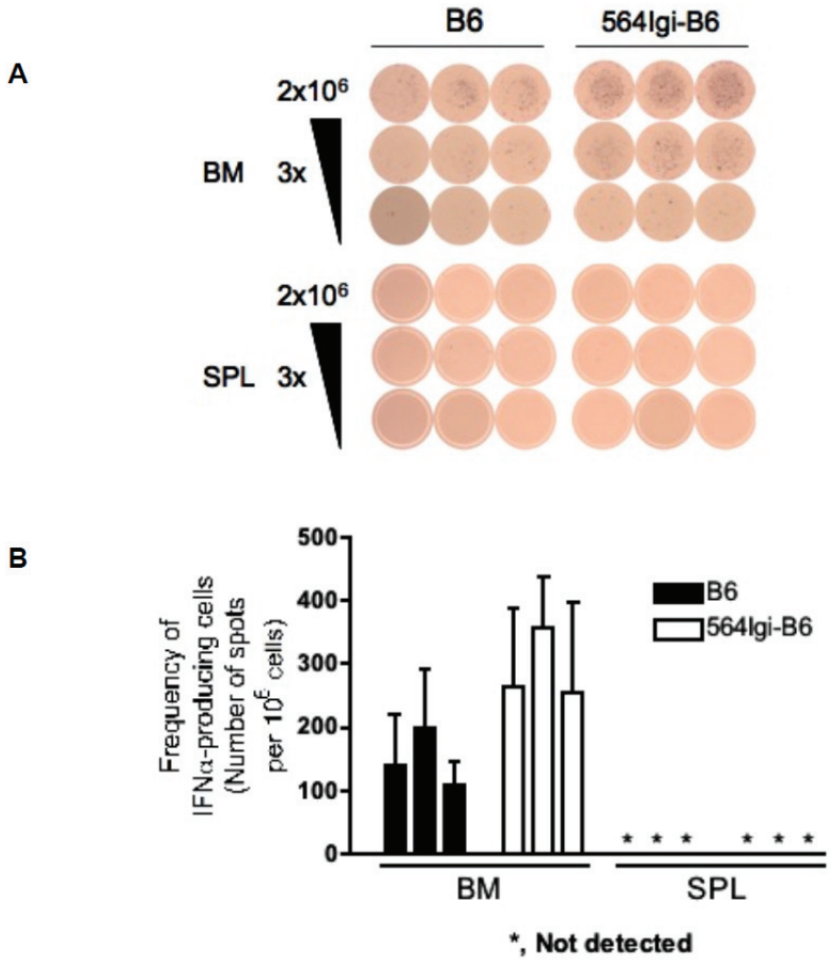

Figure 16: Increased IFN-α production in the BM of 564Igi mice

The frequency of IFN-α-producing cells was measured by ELISPOT in the BM or spleen from 3 month-old normal B6 or 564Igi-B6 mice. (A) A representative triplicate set of serial dilution from one mouse of each group on the ELISPOT plates is shown. (B) The frequency of IFN-α production from each mouse is depicted. Each error bar represents standard deviation.

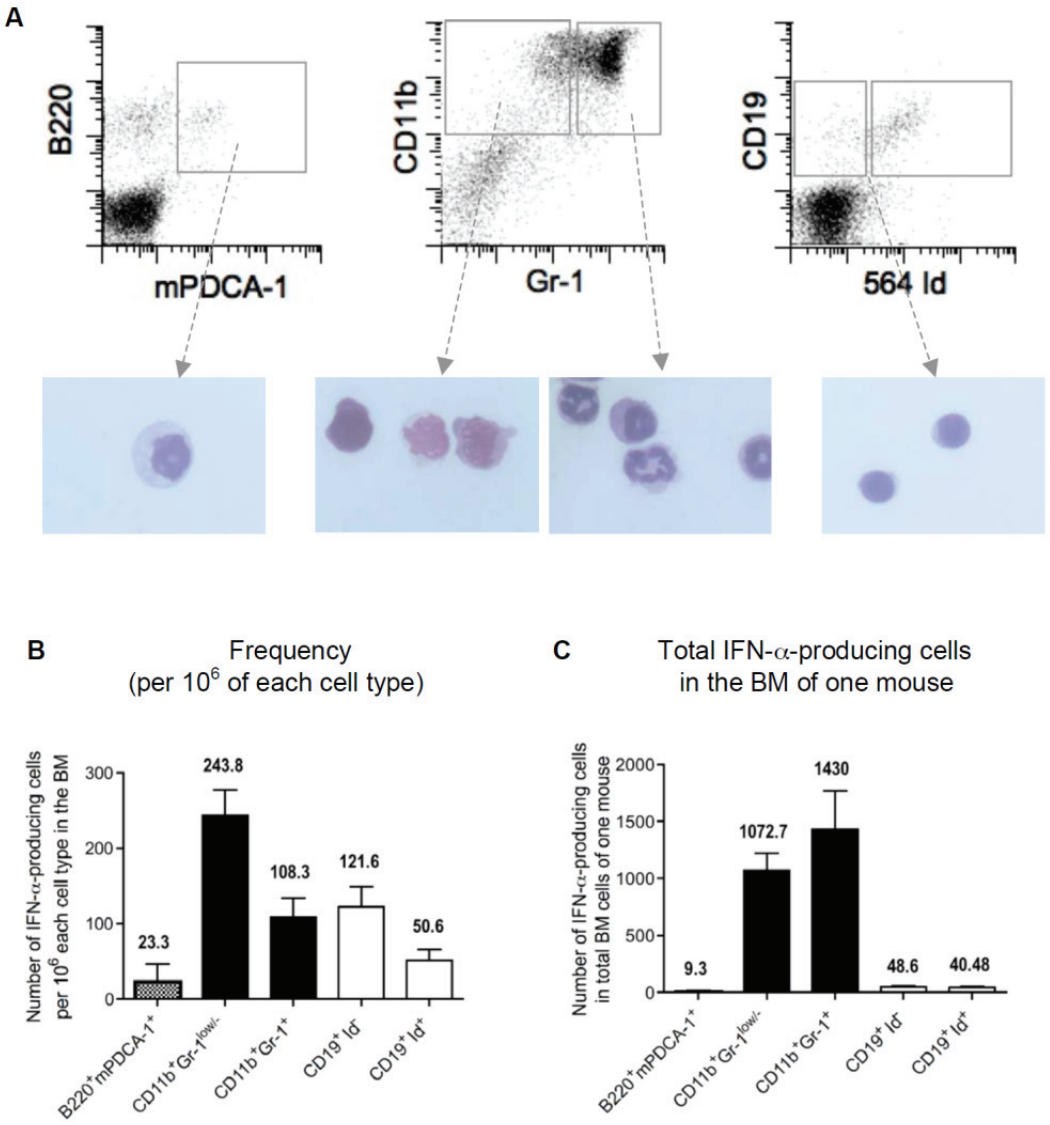

Figure 17: Bone marrow myeloid cells are the major IFN-α producers

BM cells of 564Igi mice were sorted using the gating schematic shown in (A) and purified cells were analyzed for the production of IFN-α. (B) Total number of IFN-α-producing cells was calculated by multiplying the frequency by the total number of viable sorted cell numbers in each subset.

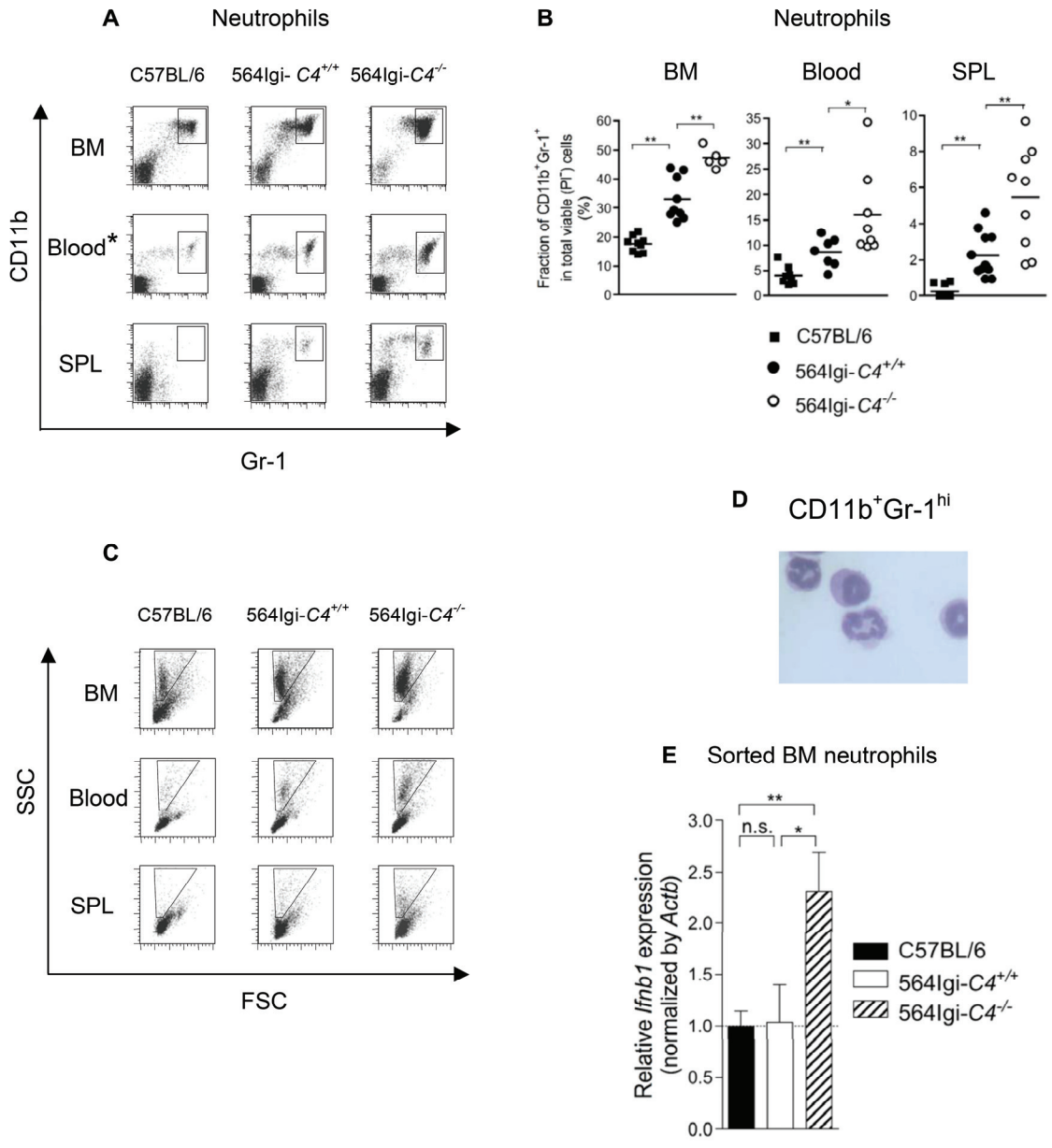

Figure 18: Increased expansion and IFN-β production by neutrophils in 564Igi-*C4*$^{-/-}$ mice

(A) Flow cytometric analysis of granulocyte populations in BM and spleen of representative age-matched C57BL/6, 564Igi-*C4*$^{+/+}$ and 564Igi-*C4*$^{-/-}$ mice. The fraction of viable (PI$^-$) CD11b$^+$Gr-1^{hi} cells from the indicated tissues of the indicated mice was determined by flow cytometry. In order to be able to analyze

CD11b expression in the blood, anti-CD11b antibody was 10 times more diluted because CD11b expression in the blood cells was much higher than that expressed by BM and spleen cells. (B) The fraction of $CD11b^{+}Gr\text{-}1^{hi}$ cells in BM and spleen of multiple age-matched mice analyzed as shown in (A). (C) The FSC/SSC profile of the gated, viable $CD11b^{+}Gr\text{-}1^{hi}$ population is shown among the entire population, including un-gated populations, in order to demonstrate the relative expansion of $CD11b^{+}Gr\text{-}1^{hi}$ in the entire populations. (D) Morphology of sorted $CD11b^{+}Gr\text{-}1^{hi}$ cells. Sorted cells were spun onto microscope slides by cytospin and stained with HEMA3. (E) Expression of *Ifnb1* gene relative to endogenous control, *Actb*, measured by real-time PCR (TaqMan). Total RNA was purified from sorted BM developing 564Igi B cells (564 $Id^{+}B220^{+}AA4.1^{+}$) from 564Igi-$C4^{+/+}$or 564Igi-$C4^{-/-}$ mice as well as from sorted immature B ($B220^{+}AA4.1^{+}\kappa^{+}\lambda^{+}$) cells from normal C57BL/6 mice. All the mice used for this figure were age-matched (3 months old). **, $p< 0.01$; *, $p< 0.05$; n.s., not significant ($p>0.05$). Each p value was calculated by a two-tailed Student's t-test.