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The role of interferon regulatory factor five in systemic lupus erythematosus

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Thesis

THE ROLE OF INTERFERON REGULATORY FACTOR FIVE IN SYSTEMIC LUPUS ERYTHEMATOSUS

by

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DEDICATION

I dedicate this thesis to my family.

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RACHAEL ROWLEY

ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder associated with the development of immune complexes that deposit in multiple organ systems. The deposition of immune complexes is associated with tissue damage, most commonly seen in the form of kidney damage as lupus nephritis. IRF5 is an SLE susceptibility gene that is causal to type one interferon inflammatory cytokine production. We hypothesized that the peak luminescence of Donkey anti-mouse IgG Alexa-594 and for Goat anti-mouse C3-FITC in FcγRIIB-/- Yaa mice can show the disease state of SLE in murine models at which disease is most active. This thesis demonstrates that peak luminosity in FcγRIIB-/- Yaa mice is at 4.5 months of age. IHC staining was done to show the deposition of IgG and C3 protein in the glomeruli of FcγRIIB-/- Yaa at various ages. It also shows that the disease manifests between the ages of 2-4.5 months of age due to the differences in fluorescence intensity.

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LIST OF ABBREVIATIONS

ACR…………………………………………….………American College of Rheumatology

BAFF…………………………………………………………..B Cell Activating Factor

BU………………………………………………………………….Boston University

BSA……………………………………………….…………Bovine Serum Albumin

C3…………………………………………………………...Complement Protein 3

C4…………………………………………………………...Complement Protein 4

DHEA………………………………………………………….Dehydroepiandrosterone

DNA…………………………………………………………...Deoxyribonucleic acid

EULAR…………………………………………...European League Against Rheumatism

FcγRIIB…………………………………….…………………Fcγ Receptor II B

FITC……………………………………………………….....Fluorescein isothiocyanate

GWAS…………………………...……………...Genome Wide Association Study

IFN……………………………………………………………………….Interferon

IFNAR…………………………………………………………..Type I IFN Receptor

ISGF3……………………………………………………IFN Stimulated Gene Factor 3

ISRE…………………………..……………………IFN Stimulated Response Element

ITAM……………………………………Immunoreceptor tyrosine-based activating motif

ITIM……………………………………Immunoreceptor tyrosine based inhibitory motif

IgG…………………………………………………………………...Immunoglobulin G

IHC…………………………………………………………....Immunohistochemistry

IRAK………………………………………….Interleukin-1 receptor-associated kinase

IRF-5... Interferon Regulatory Factor 5

ISRE……………………………………………Interferon Sensitive Response Element

JAK1……………………………………………………………………..Janus Kinase 1

LPS………………………………………………………………Lipopolysaccharide

MHC……………………………………………..Major Histocompatibility Complex

MyD88…………………………………………………Myeloid Differentiation Factor 88

PAMP……………………………………………Pathogen Associated Molecular Pattern

pDCs……………………………………………………………..Plasma Dendritic Cells

PRR……………………………………………………Pathogen Recognition Receptor

RNA……………………………… ……………………………………...Ribonucleic acid

SLE……………………………………………………..Systemic Lupus Erythematosus

SNP……………………………………………………...Single Nucleotide Polymorphism

ssRNA…………………………………………………..Single stranded Ribonucleic Acid

STAT……………………………………...Signal Transducer and Activator of Transcription

SYK………………………………………………………Tyrosine Protein Kinase SYK

TBS…………………………………………………………........Tris Buffered Saline

TLR……………………………………………………………………..Toll Like Receptor

TRAF………………………………………………….......TNF associated receptor factor

TYK2…………………………………………………..Non-receptor Tyrosine Kinase 2

Yaa…………………………………Y chromosome linked autoimmune accelerator gene

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disorder that is characterized by the production of autoantibodies that bind autoantigens, forming immune complexes. These immune complexes may float freely in blood, but when deposited in organs, they may bind complement proteins. Together, the complement system may then be activated and cause inflammation and tissue damage. The classical presentation of lupus is in the skin through a facial "butterfly" malar rash on the nose and cheeks that is photosensitive, painful, and itchy (COJOCARU et al., 331). The immune complexes characteristic of SLE cause more than skin rashes; they also may deposit and cause systemic damage across the body.

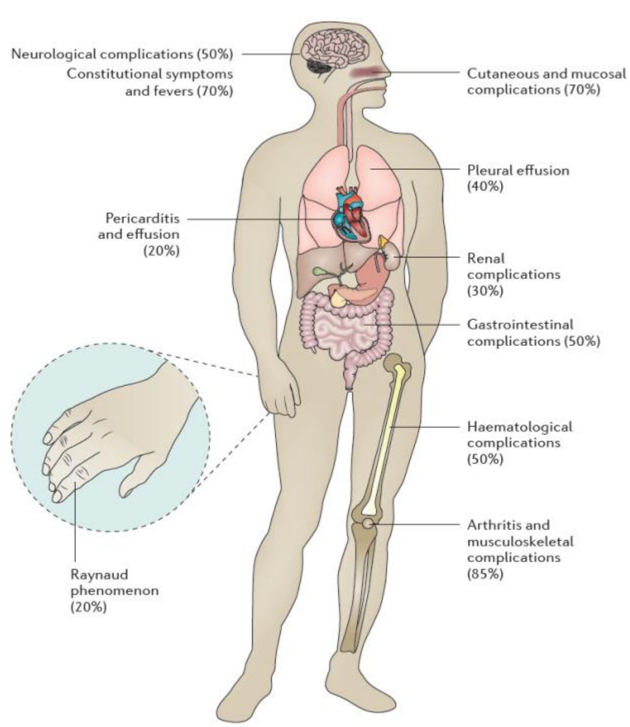


Figure 1: Manifestations of SLE (Kaul, Gordon, Crow, et al., 1)

With so many possible manifestations, SLE can be very difficult to diagnose. The most popular set of criteria is set by the American College of Rheumatology (ACR). A diagnosis using the ACR criteria is made based on several biomarkers, as seen below in Figure 2.

ACR/EULAR 2019 criteria for the diagnosis of SLE		
Domain	Manifestation	Points
Constitutional	Fever	2
Cutaneous	Alopecia	2
	Oral ulcers	2
	Subacute cutaneous/discoid	4
	Acute cutaneous lupus	6
Arthritis	Synovitis in 2+ joints & >30 min morning stiffness	6
Neurologic	Delirium	2
	Psychosis	3
	Seizure	5
Serositis	Pleural or pericardial effusion	5
	Active pericarditis	6
Hematologic	Leukopenia	3
	Thrombocytopenia	4
	Autoimmune hemolysis	4
Renal	Proteinuria >0.5 g/24 h	4
	Class II or V lupus nephritis	8
	Class III or IV lupus nephritis	10
Antiphospholipid antibody	Anticardiolipin IgG >40 GPL	2
	Anti-β2GP1 IgG > 40 U	2
	Lupus anticoagulant	2
Complement	Low C3 or C4	3
	Low C3 + C4	4
Highly specific antibodies	Anti-dsDNA	6
	Anti-Smith	6

Figure 2: ACR/EULAR criteria for the diagnosis of SLE. A score of 10 or higher is considered a diagnosis of SLE (Caza, 1).

However, even with this set criteria, it is still hard to diagnose SLE from an early onset due to the requirement for a score of 10. As an example, a patient could have low C3 and C4 but it would not necessarily indicate a definitive SLE diagnosis.

Currently, treatments for SLE are focused on reducing SLE complications and selective adjustments in the immune response. The anti-malarial drug hydroxychloroquine is a commonly used drug for treating SLE because it is able to decrease the amount of lupus flares and assist in the prevention of SLE-induced organ damage (Fava and Petri, 7). Other treatments for SLE are outlined in Table 1.

Current SLE Treatments

Drug Name	**Mechanism of Action**	**Use in SLE Treatment**
Hydroxychloroquine	-Inhibits BCR and TCR signaling -Inhibits TLR3 and TLR7 activation -Overall anti-IFN 1 effect	-Not immunosuppressive and increases survival -Reduces skin, arthritic, renal, and cardiovascular organ damage. -Reduces amount of SLE flares
Vitamin D	-Vitamin D3 receptor promotes an anti-inflammatory state in T cell differentiation. -Vitamin D has been correlated with better disease outcomes	-Many patients with SLE have Vitamin D deficiency. -Use has shown to reduce renal manifestations and reduce fibrosis.
Dehydroeipandrosterone (DHEA)	-Various studies have associated DHEA with the reduction of antibody production and pro-inflammatory cytokine activity.	-Females with SLE have lower androgens. Supplementation of DHEA has improved SLE symptoms.
Corticosteroids	-Reduce overall body inflammation and immune system activity.	-Corticosteroids are used sparingly; mostly used for manifestations of SLE that are potentially fatal.
Cyclophosphamide	-Suppresses antibody production by alkylating DNA. -The alkylating action reduces the amount of B and T cells.	-Rarely used, but was used in the past to treat severe lupus nephritis and CNS lupus

		manifestations.
Azathioprine	-Immunosuppressive medication that inhibits purine synthesis. This causes a decrease in DNA/RNA synthesis. This leads to a dramatic reduction in the number of immune cells.	-Most commonly given to pregnant SLE patients to diminish disease manifestations because the immunosuppressive effects do not affect the fetus.
Methotrexate	-Reduces purine synthesis by irreversibly inhibition of dihydrofolate reductase.	-Reduces anti-ds DNA and complement levels, especially for patients with joint and skin manifestations.
Mycophenolate	-Suppresses cell-mediated and humoral immune response.	-Drastically reduces lupus nephritis. It has been noted that some patients may go into remission for any SLE associated renal manifestations.
Calcineurin Inhibitors	-Blocks calcineurin and prevents T cells from being activated. This causes downstream inhibition of B cell activation.	-Treatment of lupus nephritis.
Rituximab	-A monoclonal antibody against CD20. When it binds CD20 on B cells, it triggers cell death. This depletes the production of autoantibodies.	-Treatment of lupus nephritis.
Belimumab	-A monoclonal antibody that helps stimulate apoptosis of autoreactive B cells.	-Treatment of both active renal and non-renal manifestations of SLE.

(*Adapted from info from Fava and Petri, 1)

General Review of Interferons

Interferons (IFN) are cytokines that have an essential role in the human immune system. Three groups of IFNs exist: type I IFN, type II IFN, and type III IFN. In order to trigger the production of IFNs, toll-like receptors (TLR) are triggered via the recognition of a pathogen associated molecular pattern (PAMP). A PAMP can be in the form of a nucleic acid associated with the pathogen, the peptidoglycan in the cell wall of bacteria, lipopolysaccharide (LPS) that is seen on the cell wall of gram negative bacteria, or other proteins. A TLR is a type of pathogen recognition receptor (PRR) that can trigger the transcription of genes that lead to the assembly of IFNs. Types of PAMPS and their respective TLR is shown in Figure 3.

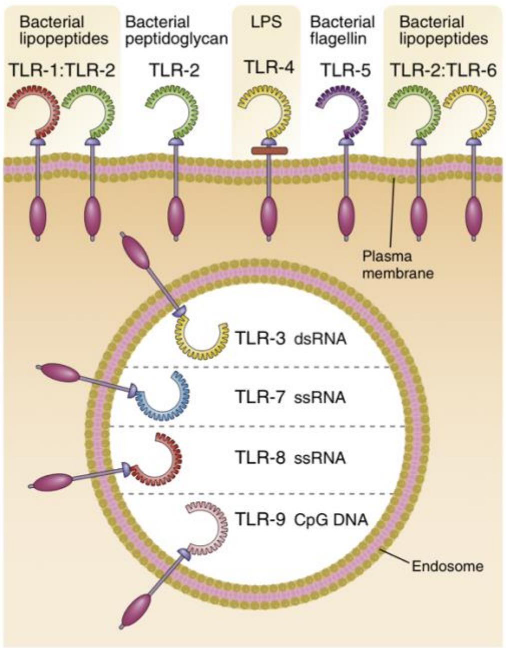


Figure 3: TLRs are PRRs that can respond to various properties of pathogens.

When the respective ligand binds to the respective TLR, TLRs stimulate the production of cytokines, such as IFNs. In regards to SLE, the activation of TLR-7 and TLR-9 has been associated with the sensing of immune complexes (Abbas et. al, 32).

Type I IFNs are a group of IFNs that are secreted mostly from plasma dendritic cells (pDC) (Chyuan, et al., 2). pDCs contain TLR7 and TLR9 in the membranes of their endosomes, which have the ability to sense single stranded (ssRNA) and CpG DNA. If pDCs sense a pathogen containing ssRNA or CpG DNA, pDCs phagocytose the pathogen via their FcγRIIA receptor and the nucleic acids bind their respective TLR (Chasset and Arnaud, 45). When initiated, TLR7 and TLR9 will activate a protein called myeloid differentiation factor 88 (MyD88) (Chyuan, et al., 2). MyD88 will in turn initiate the transcription of interferon regulatory factors, such as IRF5, and the creation of type I IFNs will occur. The produced type I IFN proteins will then engage with the type I IFN receptor (IFNAR), a transmembrane receptor that specifically binds type I IFN proteins. IFNAR activation then causes Janus Kinase 1 (JAK1) and non-receptor tyrosine kinase 2 (TYK2) to phosphorylate two proteins: signal transducer and activator of transcription (STAT) transcription factors 1 and 2, (Chasset and Arnaud, 45). Phosphorylation of STAT 1 and 2 induce it's dimerization, and the dimer is then translocated to the nucleus. In the nucleus, the STAT dimer forms a complex with interferon regulatory factor 9 (IRF-9) to form a unit called the IFN-stimulated gene factor 3 (ISGF3) (Chyuan et. al, 3). ISGF3 then begins the process of transcribing IFN regulated genes (IRGS), as well as binding IFN-stimulated response elements (ISREs) that cause the cell to operate in an antiviral manner (Chyuan et. al, 3).

To aid in viral defense, type I IFN (namely IFN-α) enhances the adaptive immune response by instigating dendritic cells (DCs) to release CSC chemokine ligands 9 and 10 to attract T cells (Chyuan et. al, 6). The humoral response is also potentiated as type I IFNs cause (DCs) to make B-cell activating factor (BAFF) and a type of proliferation-inducing ligand to induce class switching in antibodies (Chyuan et. al, 6).

While there are many types of type I IFNs, there is only one type of type II IFN. IFN-γ can be made by a variety of immune cell types, such as CD4+ and CD8+ T lymphocytes, B cells, and Natural Killer (NK) cells. IFN-γ may lead to not only the to the synthesis of pro-inflammatory and anti-inflammatory cytokines, but also the synthesis of anti-inflammatory cytokines such as IL-10 (Chasset and Arnaud, 4).

Type III IFNs (also referred to as IFN-) share many similarities with type I IFNs. Type III may also detect molecular PAMPs via PRRs such as TLRs, and their primary producer is also pDCs (Chyuan et. al, 5). They may also be produced via the action of type I IFNs through the action of the JAK-STAT signaling pathway that type I IFNs also use. A summary of all types of IFN can be seen in table 2.

With regards to SLE, immune complexes containing self-nucleic acids are thought to lead to repeated and sustained type I IFN signaling. The nucleic acids are then taken up by the pDC, activate TLRs 7 and 9, and release type I IFNs that cause an inflammatory response. IFN-α has been highlighted to having a major role in this dysregulation of inflammatory response. In healthy individuals, there is little to no production of type I IFNs. In fact, healthy patients have no detectable IFN-α (Chasset and Arnaud, 45). In various studies, IFN-α has been reported in augmenting the autoimmune response. IFN-α has the ability to increase expression of both MHC classes I and II on DCs, help develop CD8+ T lymphocytes, and quell the actions of regulatory T lymphocytes (Chyuan et. al, 5).

Table 2: IFN Types and Members

Interferon Family	**Cytokines**	**Receptors**	**Producers**
Type I	-IFN-α -IFN-β -IFN-δ -IFN-ε -IFN-κ -IFN-τ -IFN-ω -IFN-ζ	-IFNAR1 -IFNAR2	-pDCs -Fibroblasts -Keratinocytes -Macrophages
Type II	- IFN-γ	-IFNGR1 -IFNGR2	-NK cells -T lymphocytes
Type III	-IFN-λ-1 -IFN-λ-2 -IFN-λ-3 -IFN-λ-4	IFN- λ R1 -IL-10R2	-DCs -Epithelial cells

*Adapted from (Chasset and Arnaud, 46)

Hence, the production of IFN-α production is a key component of SLE manifestations. For example, the cutaneous symptoms of SLE are thought to be linked to IFN-α production related to the self-DNA and RNA left from the apoptosis of Ultraviolet ray exposed keratinocytes (Chasset and Arnaud, 46). The many other roles of IFN-α are summarized in Table 3.

Table 3: Actions of IFN-α in SLE

1. Differentiation of monocytes to myeloid DCs to increase antigen presenting function.
2. Decreases maturation of natural T regulatory cells.
3. Decreases maturation of Th2 and Th17 cells.
4. Possibly promotes podocyte loss in lupus nephritis.
5. Increases NETosis caused by anti-ribonuclear antibodies.
6. Aids development of CD4+ and CD8+ T cells.
7. Intensifies action of NLRP3 inflammasome.
8. Possibly disrupts marginal zone macrophages that clear nuclear antigen leftover from apoptosed cells.
9. May induce apoptosis of keratinocytes.
10. Increases B cell production of autoantibodies via B-lymphocyte stimulator.
11. Increases killing ability of NK cells.

*Adapted from (Chasset and Arnaud, 47).

The role of other type I interferons, like IFN-β and IFN-κ, is inconclusive. Although IFN-α and IFN-β both use the same IFNAR1 and IFNAR 2 receptors, IFN-β has actually been linked to possible anti-inflammatory production of IL-10 (Chasset and Arnaud, 47). Studies have suggested that IFN-β may also decrease the activity of the inflammasome and reduce production of inflammatory cytokine IL-1 (Chasset and Arnaud, 47). At the same time, IFN-β has also been linked to SLE-related tissue injury (Chasset and Arnaud, 47). IFN-κ is linked to the cutaneous symptoms of SLE because it is prevalent in keratinocytes, but further study is needed to elucidate its role in extracutaneous manifestations of SLE (Chasset and Arnaud, 47).

General Review of Fc Receptors

Fc receptors are receptors located on several types of immune cells that can recognize the heavy chain region (Fc) of the immunoglobulin IgG. There are 6 types of

Fc receptors and they can be classified as activating or inhibitory. When an activating Fc receptor interacts with the Fc region of an antibody for a pathogen, it allows for phagocytosis and apoptosis of an infected cell that is covered with antibodies (Smith and Clatworthy, 328).

While activating Fc receptors are useful in the defense against invading pathogens, activating Fc receptors also have the ability to incite an immune response to self antigens. If activating Fc receptors are cross linked via an immune complex, phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) occurs and this stimulates a molecule called signaling molecule SYK, (Smith and Clatworthy, 328). SYK then initiates an inflammatory reaction against the self-antigen in the immune complex (Smith and Clatworthy, 328).

The only inhibitory Fc receptor is FcγRIIB. When an immune complex containing self antigen interacts with an FcγRIIB, the activating signal will be diminished. FcγRIIB will cross link with an activating Fc receptor and the immunoreceptor tyrosine-based inhibitory motif (ITIM) will be phosphorylated (Smith and Clatworthy, 328). With ITIM phosphorylation, FcγRIIB is able to prevent the activating Fc receptor from engaging in phagocytosis and release of inflammatory cytokines (Smith and Clatworthy, 328). FcγRIIB receptors affect the immune response in four domains: humoral immunity, antigen presentation by DCs, innate immunity, and hypersensitivity reactions.

On B cells, FcγRIIB receptors have the ability to prevent B cells making autoantibodies from a self-antigen in an immune complex. To accomplish this goal, FcγRIIB prevents B cells from presenting self-antigens to T cells by lessening the activation of the B cell receptor (Smith and Clatworthy, 329). Follicular DCs also express FcγRIIB and prevent self-antigen from being presented to germinal center B cells (Smith and Clatworthy, 329). FcγRIIB is able to influence antigen presentation because it prohibits DCs from uptaking self-antigen and cross presentation between CD4+ and CD8+ T Cells (Smith and Clatworthy, 330).

Within the innate immune response, FcγRIIB can cross link and inhibit TLR 4 activation and cytokine release by cross linking with an activating Fc receptor A similar mechanism takes place in neutrophils, where FcγRIIB is able to inhibit rolling, adhesion, and migration of neutrophils (Smith and Clatworthy, 330). FcγRIIB also has the ability to inhibit the release of IgE from mast cells during a hypersensitivity reaction. By inhibiting the release of IgE, FcγRIIB is able to limit the amount of IL-4 and histamine produced from a hypersensitivity reaction (Smith and Clatworthy, 330).

Due to all of these effects on the immune response, it has hypothesized that the deficiency of FcγRIIB may be a factor in the pathogenesis of SLE. In murine models of mice that are deficient in FcγRIIB and backcrossed with a C57Bl/6 background tend to develop an SLE like disease that is similar to SLE (Smith and Clatworthy, 334). In another type of mouse model, MRL-Ipr mice (an established model of glomerulonephritis) exhibited drastic reduction in SLE symptoms once FcγRIIB was reinstated (Smith and Clatworthy, 334). A visual depiction of potential mechanisms in which FcγRIIB may influence SLE pathogenesis are in Figure 4.

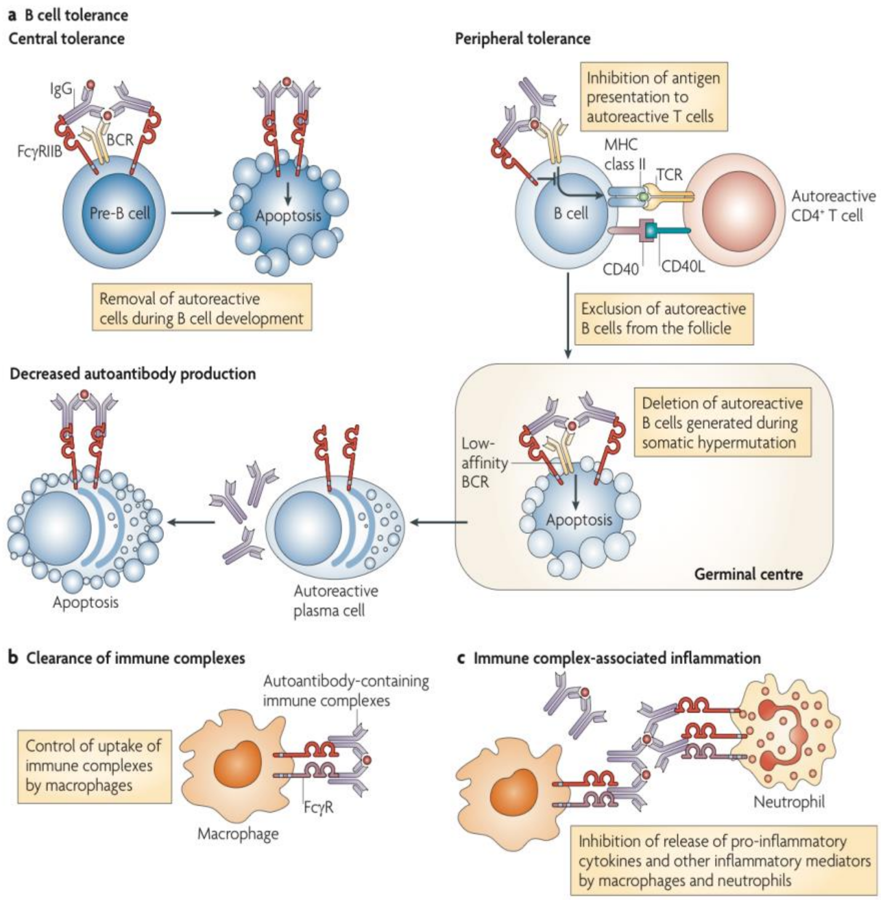


Figure 4: Three possible mechanisms for how FcγRIIB may contribute to SLE.

Panel a depicts the impact on FcγRIIB in immunological tolerance. It is thought that FcγRIIB may impact B cell tolerance because of its involvement with apoptosis of B cells reactive to self-antigen. It is theorized that a deficiency in FcγRIIB cross ligation with B cells can lead to apoptosis that leaves behind exposed self-antigen. These remnants may then be acted on by immune cells and result in an autoimmune response. This has not been demonstrated in FcγRIIB deficient murine models, however, so further study is needed to confirm this possible FcγRIIB mechanism. Panel B demonstrates that the presence of autoantibodies and destruction of B cell tolerance do not yield autoimmune disease. The final trigger in developing autoimmune disease is the lack of immune complex clearance by activating FcγRs. Therefore, the upregulation or downregulation FcγRIIB actions on activating FcγRs controls the amount of immune complexes that can circulate and deposit in organ systems. Panel C illustrates how immune complexes that do deposit in tissue have the ability to cause an inflammatory response with FcγRs on resident immune cells. The capacity for FcγRIIB to mitigate the release of cytokines determines the amount of tissue damage and inflammation. Thus, defective FcγRIIB action may contribute to SLE in this way (Smith and Clatworthy, 336).

General Review of the *Yaa* Gene

The Y chromosome-linked autoimmune acceleration gene (*Yaa*) is a gene that is associated with the development of SLE in murine models. The *Yaa* mutation is most known for affecting the gene for TLR7. In the *Yaa* mutation, TLR7 activation and the creation of type I IFN is sustained, creating the inflammatory symptoms associated with SLE in murine models. However, the effects of the Yaa locus are minimal with healthy male mice on a B6 background (Fairhurst et. al, 2). Instead, the combination of FcγRIIB with the Yaa locus is necessary; the increased exposure to the TLR7 activation due to the *Yaa* mutation and the lack of inhibitory action by FcγRIIB creates a quintessential environment for the development of SLE symptoms (Horvei et. al, 2). The SLE symptoms evident in the FcyRIIB -/- Yaa model include the presence of anti-nuclear antibodies, immune complex deposition in the renal glomerulus, and splenomegaly (Horvei et. al, 2). In a study done with FcyRIIB -/- Yaa mice by Horvei et. al, the renal levels of TLR7, TLR8, and TLR9 mRNA were upregulated in both murine models of moderate and severe SLE glomerulonephritis. Most notably, it was a statistically significant finding that TLR7 and TLR9 were upregulated in moderate models. In the severe model, only TLR7 was statistically significantly upregulated.

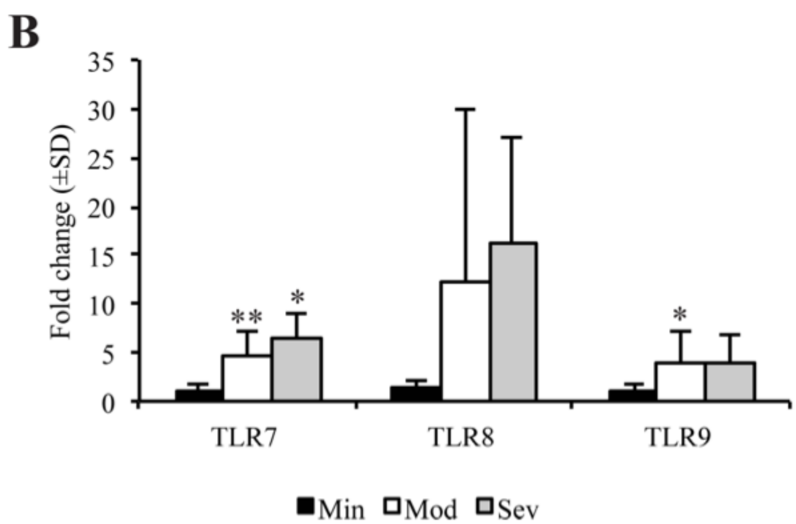


Figure 5: In FcyRIIB -/- Yaa, TLR7 and TLR9 had significant changes in renal expression. In comparison to the minimal glomerulonephritis model, the p values in the moderate glomerulonephritis model for TLR7 was 0.006 and 0.027 for TLR9 (Horvei et. al, 11). For the severe glomerulonephritis model, the p value was 0.006 for TLR9 (Horvei et. al, 11).

Molecular Biology of SLE

It is thought that autoantibody production may have roots in microbial infection. Though infection itself does not render autoantibodies, molecular mimicry techniques by infectious microorganisms have been cited to contribute to their development (Wallace and Hahn 107). The infecting microbe may exhibit an antigen that is similar to the human self-antigen, causing B cells to mark the self-antigen for destruction. When the body does not resolve this autoimmune process caused by molecular mimicry, a loss of tolerance to self-antigen occurs. The innate immune response is also involved the formation of autoantibodies due to faulty cell debris clearance mechanisms (Liu and Davidson, 874). Autoantibodies may also arise from microbial nucleic acids. When pre autoimmune lupus-prone mice were injected with bacterial DNA containing unmethylated CpG motifs, it was found that the antibodies produced by the injection were also cross-reactive with mammalian double stranded DNA (Wallace and Hahn, 130).

Mammalian DNA contains some CpG motifs and can activate TLR9. TLR3 may be activated by DNA containing immune complexes. Mammalian single stranded mRNA may also activate TLR7 and TLR8.

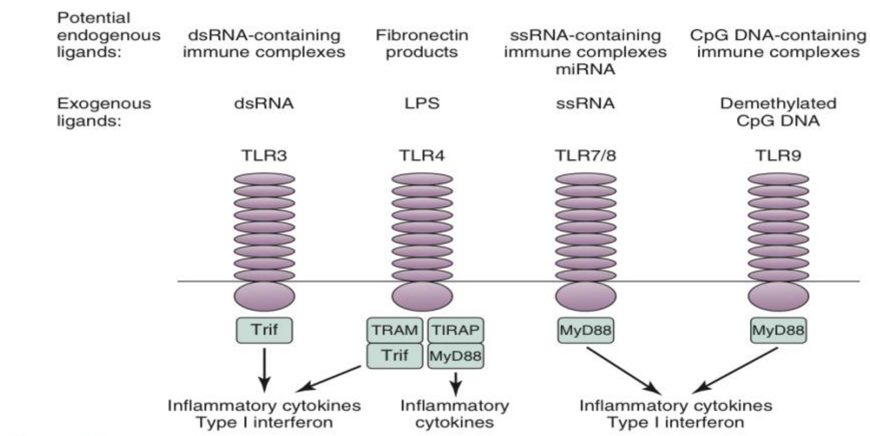


Figure 6: Potential ligands that could cause the formation of autoantibodies. Binding of potential ligands occurs through pictured respective TLR (Wallace and Hahn, 140).

Apoptosis has also been theorized to lead to the creation of autoantigens in SLE. In normal cellular debris clearance, phagocytes engulf apoptotic cells and cellular debris. In SLE, phagocytes do not engulf apoptotic cells and thus leave behind self-RNA and DNA that can lead to the activation of autoreactive B cells (Wallace and Hahn, 218). Once antibodies specific to these nucleic acids bind, they form immune complexes. Complement proteins then opsonize the immune complexes. The opsonized immune complexes can be taken up by dendritic cell Fc gamma receptors(FcγR). Later on, the RNA and DNA taken up by the dendritic cell FcγR's causes activation of TLR7 and 9. This prolonged activation of TLRs causes type 1 IFN signaling and advances the inflammatory state, (Wallace and Hahn, 218). SLE patients also have trouble often with opsonized immune complex removal via FcγR's. (Wallace and Hahn, 218) The reduced removal of these opsonized immune complexes leads to lower levels of C3 and C4 levels in serum and greater levels of and tissue injury as they deposit in various organ systems throughout the body.

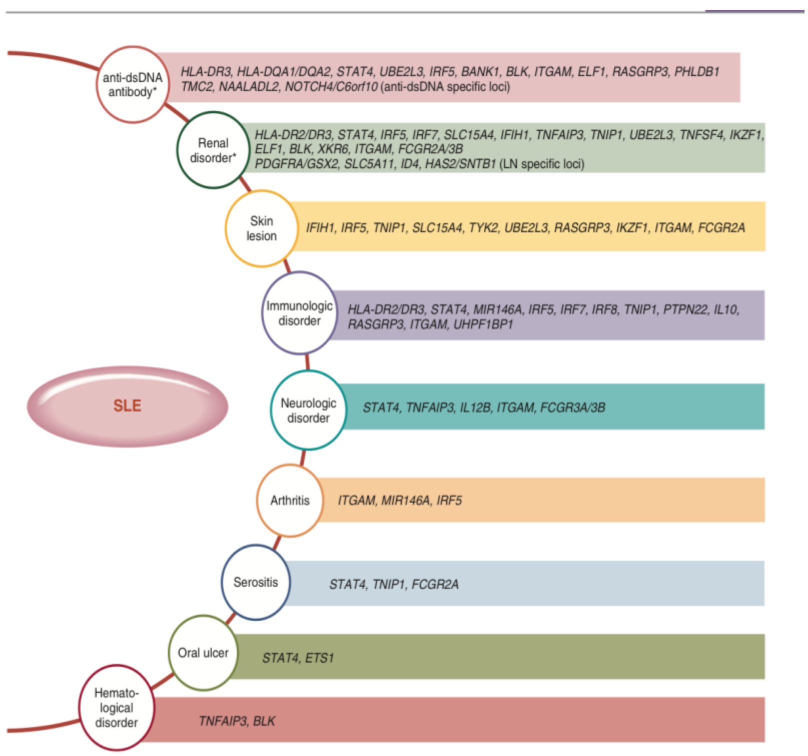


Figure 7: List of Susceptibility Genes Associated with SLE. IRF5 is implicated in various manifestations of SLE, including renal disorder, immunologic disorder, Arthritis, and skin lesions (Wallace and Hahn, 63).

Though there is no definitive root cause of SLE, it is thought that it arises from interactions between one's genetics and environment. Several Genome Wide Association studies (GWAS) have been performed and identified various genes that may predispose individuals to SLE. Coupled with environmental influences like UV light, lifestyle factors, and infections, the interactions with the associated genes may trigger the irreversible loss of immunologic self-tolerance (Goulielmos et. al, 1).

Lupus Nephritis

The kidney is an organ involved in several vital homeostatic processes in the human body. It is involved in "removal of wastes; acid-base balance; regulation of electrolytes, extracellular fluid volume, and blood pressure; production of erythropoietin; and conversion of vitamin D to its active form as a contribution to calcium homeostasis," (Wallace and Hahn, 270). The kidney is made up of units called nephrons. Within nephrons are tufts of capillaries known as the glomerulus. The function of the glomerulus is to filter blood plasma and produce a filtrate that will pass through the latter parts of the nephron to become urine. In SLE, it is common for immune complexes to deposit in the basement membrane of the glomerulus. The glomerulus has a layer called the mesangium, which is involved in the removal of the immune complexes from the kidney (Wallace and Hahn, 272). However, the clearance of the complexes by the mesangial cells lead to production of cytokines that lead to lesions in the glomerular tissue (Wallace and Hahn, 272). Using immunofluorescent staining, the damage caused by the interaction between the mesangial cells and immune complexes may be visualized and measured using antibodies against IgG and C3 complement protein. Renal damage can have grave consequences and may lead to renal failure, dialysis, or renal transplantation.

Epidemiology of SLE

In 2014, The Michigan Lupus Epidemiology and Surveillance Program published that the estimated total incidence and prevalence of SLE was 5.5 and 72.8 per 100,000 people respectively (Somers et al., 2). While SLE may affect anyone at any age, SLE disproportionately affects women. Among women enrolled in a study by the Michigan Lupus Epidemiology and Surveillance Program, SLE prevalence was 10 fold higher in females than in males. Broader epidemiological analysis has suggested that there is a 9:1 female to male incidence difference in SLE diagnosis (Goulielmos et al., 1).

Lupus also disproportionately afflicts people of color. In the Michigan Lupus Epidemiology and Surveillance Program, SLE prevalence was 2.3 fold higher in black patients than white patients (Somers et al., 2). Several other epidemiological studies have confirmed that SLE prevalence is higher in African-American, Asian, and Hispanic populations than in populations of European ancestry (Goulielmos et al., 60). Black patients involved in the Michigan Lupus and Epidemiology Surveillance Program study also had a greater chance of developing renal disease and were diagnosed with SLE at a younger age than white patients (Somers et al., 2). A higher elevated incidence of Lupus Nephritis has also been indicated in African American, Asian, and Hispanic populations (Goulielmos et al., 60). Five and half years after receiving a SLE diagnosis, European patients only had an 29.1% incidence of lupus nephritis while the incidence was 68.9% of African Americans and 60.6% of Hispanics respectively (Goulielmos et al., 60).

Molecular Biology of Interferon Regulatory Factor 5

Interferon Regulatory Factor 5 (IRF5) is a transcription factor involved in the signaling of type 1 interferon (type 1 IFN) production (Lazzari and Jefferies, 346). In normal immunologic function, IRF 5 is involved in viral defense because type 1 IFN "drives the recognition and degradation of viral RNA/DNA, inhibits protein synthesis to prevent viral replication and particle assembly, and finally drives apoptosis of infected cells to contain the viral infection," (Eames et al., 167). IRF 5 is also "crucial for driving macrophages toward a proinflammatory phenotype by regulating cytokine and chemokine expression and modulating B cell maturity and antibody production," (Eames et al., 167). Furthermore, it has been established that murine dendritic cells exposed to human SLE immune complexes must express IRF 5 in order to initiate the type 1 IFN response (Eames et al., 167). The mechanism of IRF 5 activation is indicated in Figure 5.

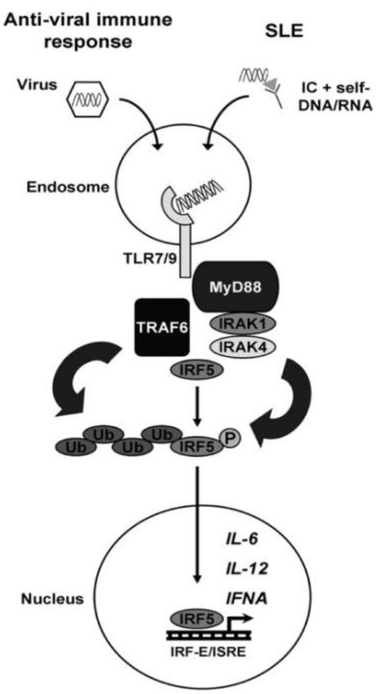


Figure 8: IRF 5 activation by foreign viral nucleic acids or self-nucleic acids. It's activation proceeds through TLR 7 or 9 and several phosphorylation events occur through MyD88 adaptor protein, TRAF, IRAK kinases 1 and 4. TRAF and IRAK kinases 1 and 4 ubiquitinate and phosphorylate IRF 5 to allow it to translocate to the nucleus and bind IRF-E/ISRE and stimulates transcription of type 1 IFN genes (Lazzari and Jeffries, 346).

Regarding SLE, many single nucleotide polymorphisms (SNPs) have been identified that are associated with either a subtractive or additive effect in SLE pathogenesis.

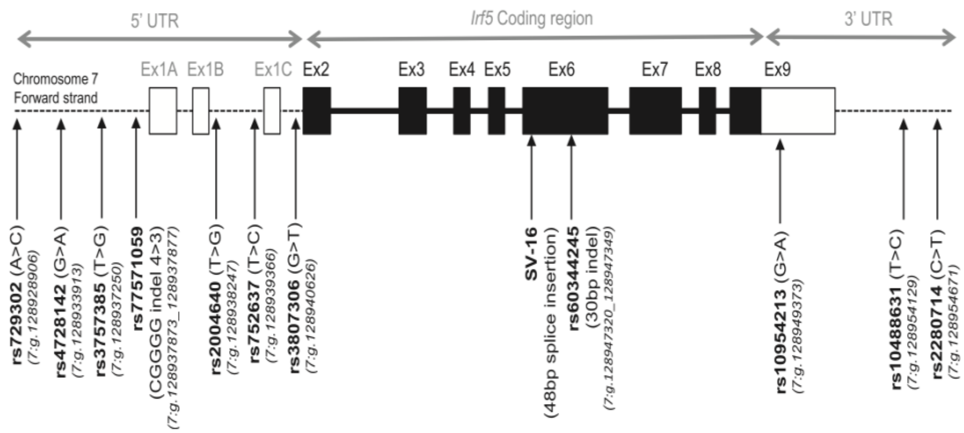


Figure 9: Identified IRF 5 SNPs. The parentheses indicate the specific base changes that indicate increased risk or protective factors for SLE (risk>protective factor) (Eames et.al, 170).

IRF 5 risk alleles have been associated with increasing IFN alpha levels (a type 1 IFN) in European Americans and Hispanic Americans (Goulielmos et al., 66). IRF 5 also has unmethylated CpG island in its promoter, which “suggests either basal levels of expression or the opportunity for rapid IRF 5 expression on induction, which is in line with the known roles of IRF5 in innate immunity,” (Eames et al., 169).

Murine models of SLE have shown that blockage and/or deficiency of IRF 5 decreases the severity of SLE symptoms (Barnes et al., 1).

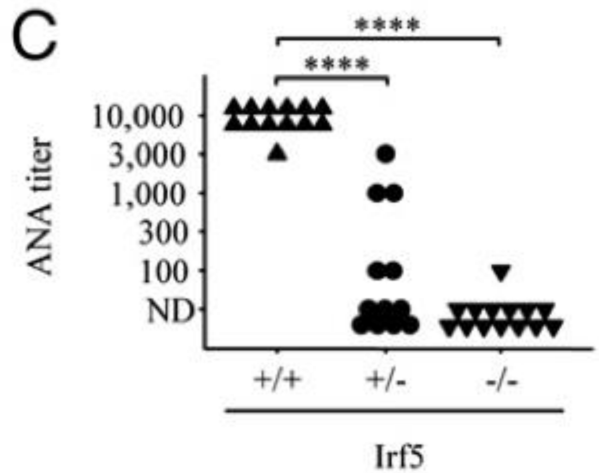


Figure 10: IRF5 blockage and autoantibody production. Deficiency or blockage of IRF 5 in mice decreases autoantibody production (Richez, et. al, 802).

It was a statistically significant finding that mice that were deficient or completely missing IRF-5 had decreased autoantibodies against the nucleus and decreased autoantibodies against dsDNA (Richez, et al, 802).

In terms of decreasing the severity of SLE, notable improvement has been found in reducing lupus nephritis in mouse models of SLE.

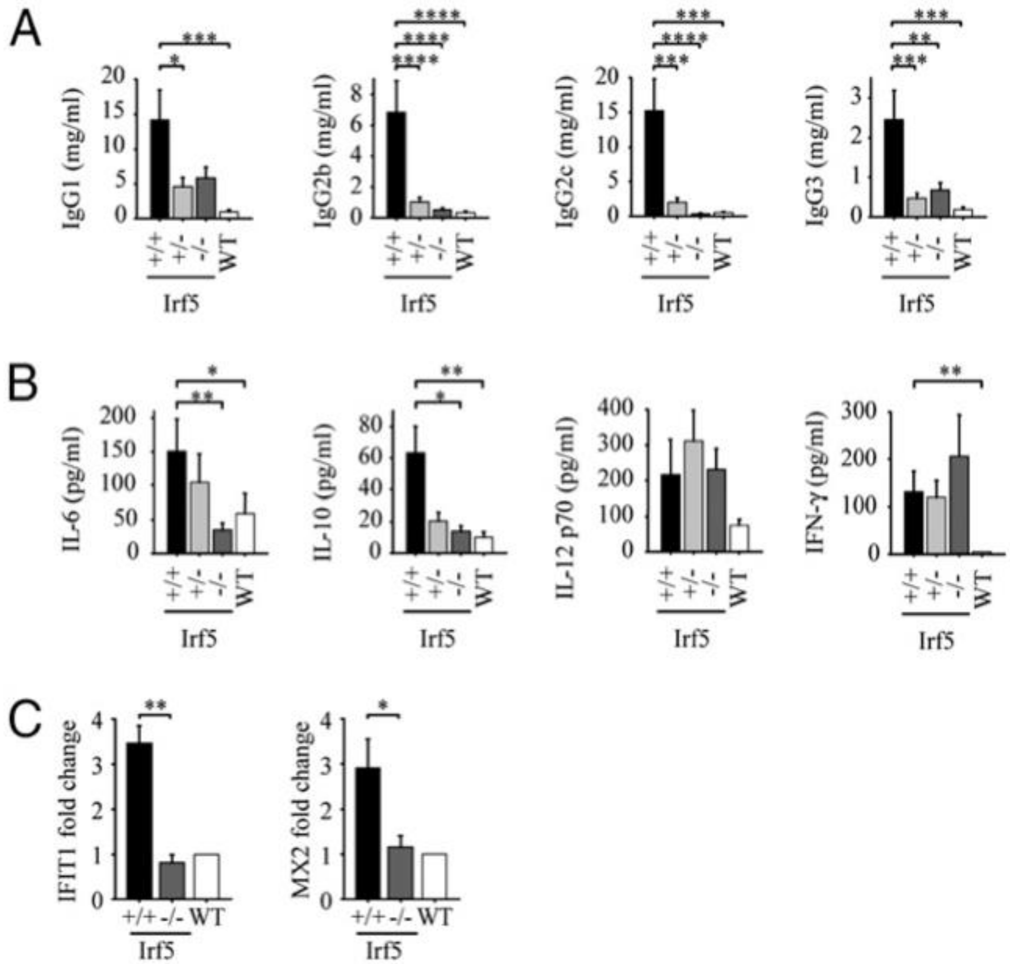


Figure 11: Serum cytokine levels in IRF5 deficient mice. Statistically significant differences were found in serum inflammatory cytokine and IgG levels of IRF 5 deficient mice (Richez et. al, 799).

OBJECTIVES

The objective of this thesis is to:

I. Analyze disease manifestations in murine models of SLE.

II. Determine the extent of immune complex deposition in the kidney by measuring the differences of IgG and C3 protein deposition by immunofluorescence in a murine lupus model.

III. Evaluate how immune complex deposition in the kidney changes during the course of the disease in a murine lupus model.

METHODS

FcyRIIB -/- Yaa and B6 Mice

FcyRIIB -/- Yaa male mice were used at different ages as demonstrated in Table 2.

FcyRIIB -/- Yaa mice on a C57BL/6 background were obtained from S. Bolland (National Institute of Allergy and Infectious Diseases, Bethesda, MD). C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME) were used as a negative control for the experiment.

Table 4: FcyRIIB -/- Yaa Mice Kidneys Used

Gender	Age
Male	5 months old
Male	4.5 months old
Male	8 months old
Male	2 months old

Table 5: B6 Mice Used

Mouse ID	Gender	Age
B6	Male	2 months old

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was completed on the mice to confirm the genetic identity of FcyRIIB -/- Yaa and B6 model mice used. A master mix of sterile water, GoTaq Flexi (5U/ ƛ), 5X GoTax Flexi Buffer (green), 25 mM of $MgCl_2$, 2.5 mM dNTP mix, 10 µM IRF5-KI-F02, 10 µM of IRF5-KI-R02. Once the master mix was added, the solution was added to all tubes containing mouse DNA. The PCR program is shown in the table below.

Table 6: PCR Program

Cycles	Temperature in degrees Celsius	Time (minutes)
1	95	3:00
40	95 59 72	0:30 0:30 1:00
1	72	1:00
1	25	1:00

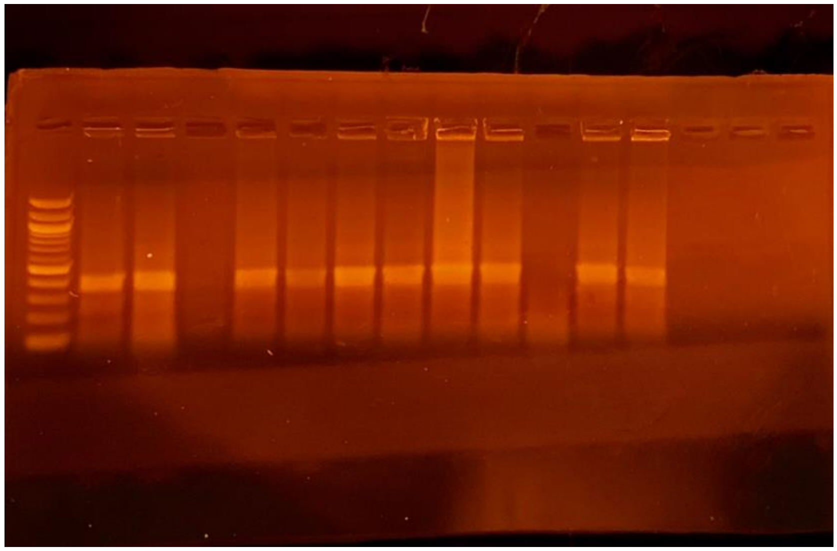


Figure 12: PCR to confirm the genetic identify of FcyRIIB -/- Yaa mice used in experiment. The PCR product was run on a 1% Agarose gel at 90 volts for 1.5 hours. A 100 base pair ladder was used in the farthest left lane. In this image, 15 µL of PCR product was used for each lane.

Immunohistochemistry

Immunohistochemistry (IHC staining) was performed on kidney sections that were snap-frozen in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA). Sections were fixed in a 1:1 solution of acetone and methanol for 10 minutes at -20 degrees Celsius. This was followed by 3 washes for 5 minutes each using a 1% Tris-Buffered Saline (TBS) solution (Boston Bioproducts Inc., Cat #BM-301, Lot # H18M102). Excess TBS was tapped off of the slides and a PAP pen was used to create a hydrophobic barrier around the kidney tissue. The kidney tissue was then blocked using a 1% Bovine Serum Albumin in TBS solution in a humidified chamber at room temperature for one hour. Donkey anti-mouse

IgG Alexa-594 (Invitrogen, A21203, Lot #1820027) was diluted 1:500 in 1% BSA in TBS. Goat anti-mouse C3-FITC (Cappel, Cat #55500) was diluted 1:200 in 1% BSA in TBS. 100 µl of antibody was applied to each section and incubated in a humidified chamber at the +4 ℃ overnight. After the incubation, the slides were rinsed 3 times with 1X TBS in 5 minute increments on a shaker. The slides were protected with foil to prevent exposure of the antibody to light. Excess TBS liquid was tapped off on a paper towel. The slides were then mounted with ProLong Gold Antifade Mountant with DAPI (Life Technologies, Cat# P36931) and the mounting media was allowed to curd in the dark at room temperature overnight.

Nikon deconvolution wide-field Epifluorescence Microscope Scanning

Images were captured from the IHC stained slides via a Nikon deconvolution wide-field Epifluorescence Microscope. The parameters for scanning were 2.9 ms for Donkey anti-mouse IgG Alexa-594 and 19 ms for Goat anti-mouse C3-FITC. These parameters were set by using the "oversaturation" tool in the microscope software. Oversaturation would have provided misleading data about the fluorescence intensity of Donkey anti-mouse IgG Alexa and Goat anti-mouse C3 FITC.

Quantitation of Immunofluorescence

The fluorescence intensity representing IgG and C3 deposition was measured as the mean fluorescence intensity (luminosity) in 10-15 glomeruli per mouse using the software ImageJ. Using the "Analyze" feature, glomeruli were outlined and then the mean fluorescence intensity was calculated for both Donkey anti-mouse IgG Alexa-594 and Goat anti-mouse C3 FITC.

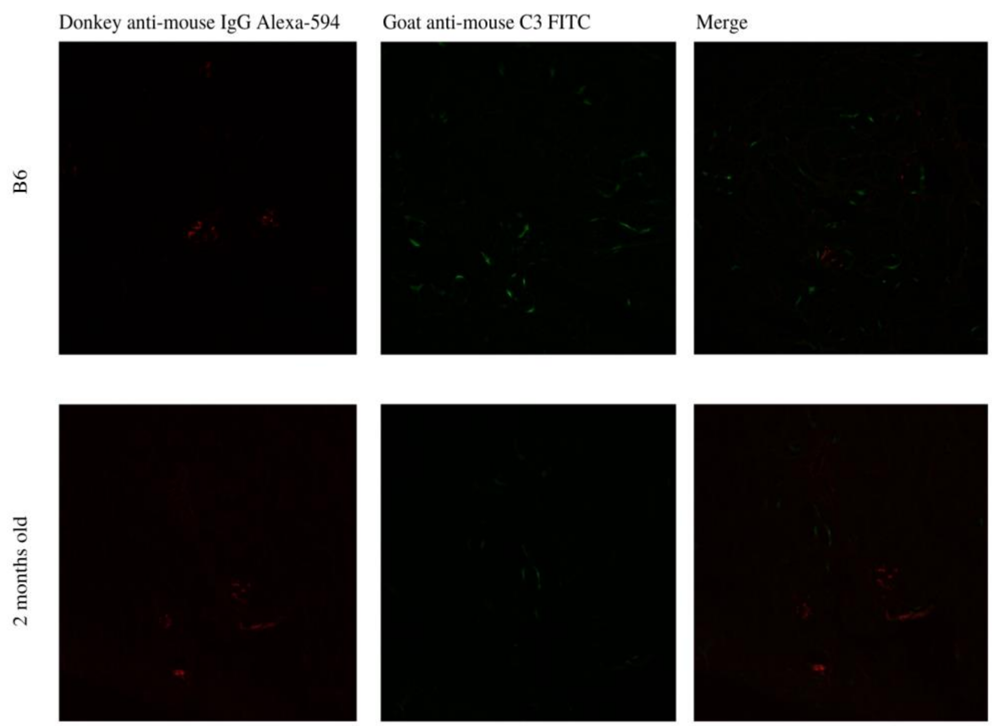
RESULTS

I. Determining the extent of immune complex deposition in murine lupus models

A total number of four FcγRIIB-/- Yaa mice at four different ages were used to analyze the immune complex deposition in the kidney. FcγRIIB-/- Yaa mice were used because previous research has used these models to mimic SLE in murine models (Horvei, et al., 1). 2 month old C57BL/6 mice kidney sections were used as a negative control in the study. To measure the deposition of IgG and C3 complement protein, immunostaining sections were cut and stained with Donkey anti-mouse IgG Alexa-594 (Invitrogen, A21203, Lot #1820027) and Goat anti-mouse C3-FITC (Cappel, Cat #55500). Donkey-anti-mouse IgG Alexa 594 stains red while Goat anti-mouse C3 FITC stains green. When Donkey-anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC come together in the form of an immune complex, the immune complex depositions appears yellow.

We first aimed to establish what the kidneys looked like at an early time point. For this, we chose the two month time point because other studies have shown that there are not many circulating autoantibodies at this time. Very weak staining of both Donkey-anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC was seen, as demonstrated in Figure 10. In the 2 month old FcγRIIB-/- Yaa mouse, the mean fluorescence intensity was 111.627 for Donkey-anti-mouse IgG Alexa 594 and 116.723 for Goat anti-mouse C3 FITC (Figures 14 and 15). The 2 month old FcγRIIB-/- Yaa staining appeared slightly brighter than the C57BL/6 mice. The values for the C57BL/6 mice were 106.059 and 153.224 for Donkey anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC respectively.

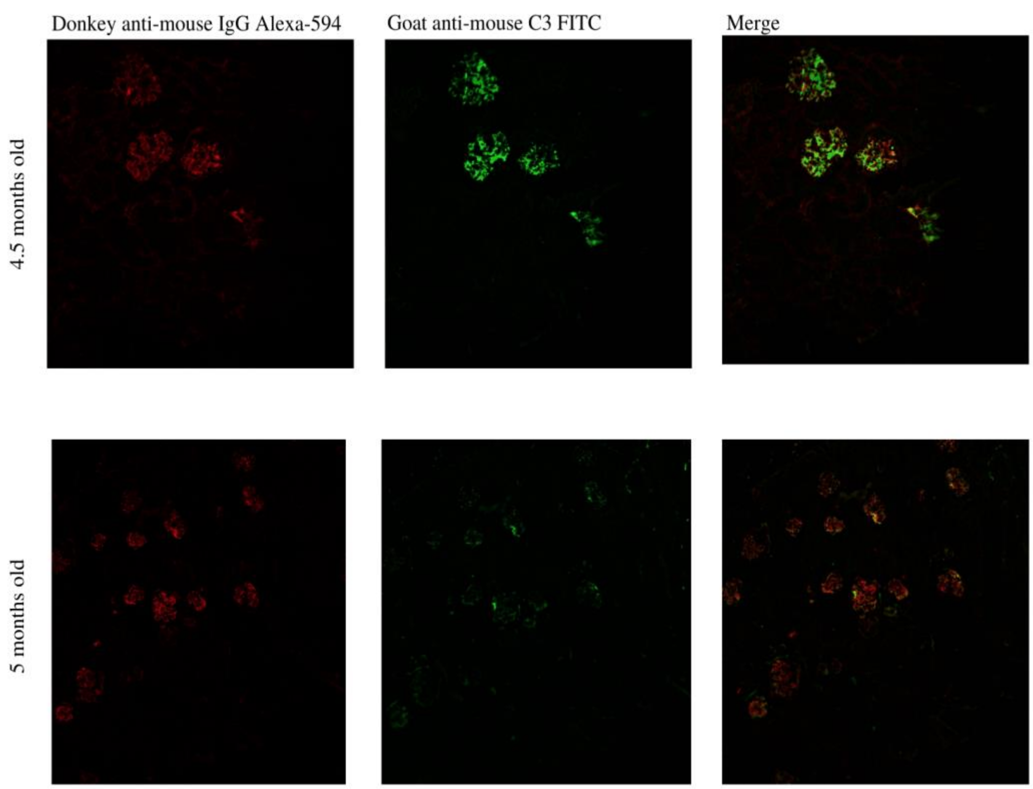
Figure 13: B6 and 2 month old FcγRIIB-/- Yaa mice kidney sections stained with both Donkey anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC. Individual channels and merge shown.



To assess middle age disease state, we stained 4.5 and 5 month old FcγRIIB-/- Yaa mouse kidney sections with Donkey-anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC. Strong staining was seen for both the 4.5 month and 5 month old age group (Figure

10). It is notable however that the 4.5 month old age group had the greatest fluorescence intensity, with the values being 1307.241 for Donkey-anti-mouse IgG Alexa 594 and 903.492 for Goat anti-mouse C3 FITC, as seen in Figures 12 and 13.

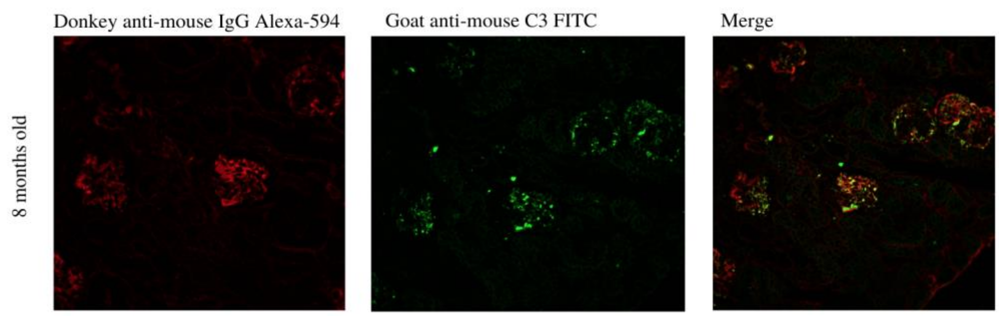
Figure 14: 4.5 and 5 month old FcγRIIB-/- Yaa mice kidney sections stained with Donkey anti-mouse IgG Alexa 594, Goat anti-mouse C3 FITC, and both Donkey anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC. Individual channels and merge shown.



We also wanted to assess how long immune complex deposition could be detected. For this, we used an 8 month old FcγRIIB-/- Yaa mouse kidney section. After staining with

Donkey-anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC, the kidneys appeared to have similar but less saturated staining compared to the 4.5 or 5 month old FcγRIIB-/- Yaa mouse kidneys. The mean fluorescence intensity was 1239.222 for Donkey-anti-mouse IgG Alexa 594 and 639.769 for Goat anti-mouse C3 FITC in the 8 month mouse kidney (Figures 12 and 13).

Figure 15: 8 month old FcγRIIB-/- Yaa mice kidney sections stained with both Donkey anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC. Individual channels and merge shown.



II. Evaluate how immune complex deposition in the kidney changes during the course of the disease in a murine lupus model.

In order to evaluate how immune complex deposition in the kidney changes during the course of disease, we used the software Image J to quantitate the mean fluorescence intensity. In each mouse, 10-15 glomeruli were selected and outlined using the polygon selection tool. The polygon selection tool was used to most accurately outline the glomeruli because the stained glomeruli may appear irregularly shaped. In the analysis features of Image J, we used the "Measure" function to quantitate the mean fluorescence (luminosity) of both Donkey anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC for each glomerulus (n=10-15). After collecting this data, the mean for each age group was calculated using Excel, as shown in Figures 12 and 13.

Figure 16: IgG-Alexa 594 Mean Luminescence in FcγRIIB-/- Yaa mice. Image J was used to quantitate the mean luminescence of Donkey anti-mouse IgG Alexa 594 in FcγRIIB-/- Yaa mice.

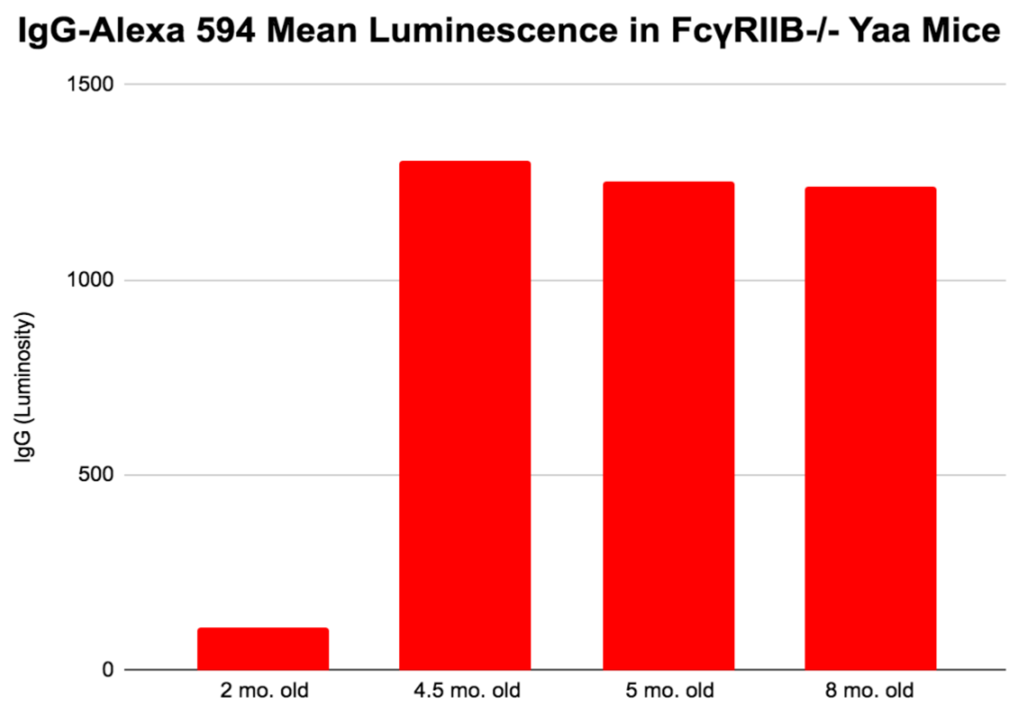


Figure 17: C3-FITC Mean Luminescence in FcγRIIB-/- Yaa mice. Image J was used to quantitate the mean luminescence of Goat anti-mouse C3 FITC in FcγRIIB-/- Yaa mice.

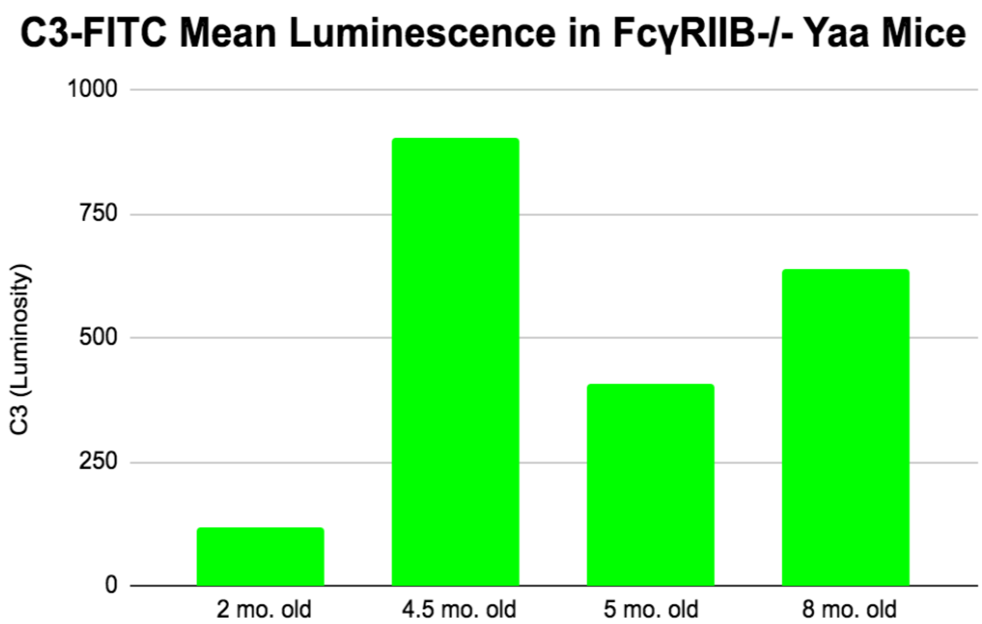
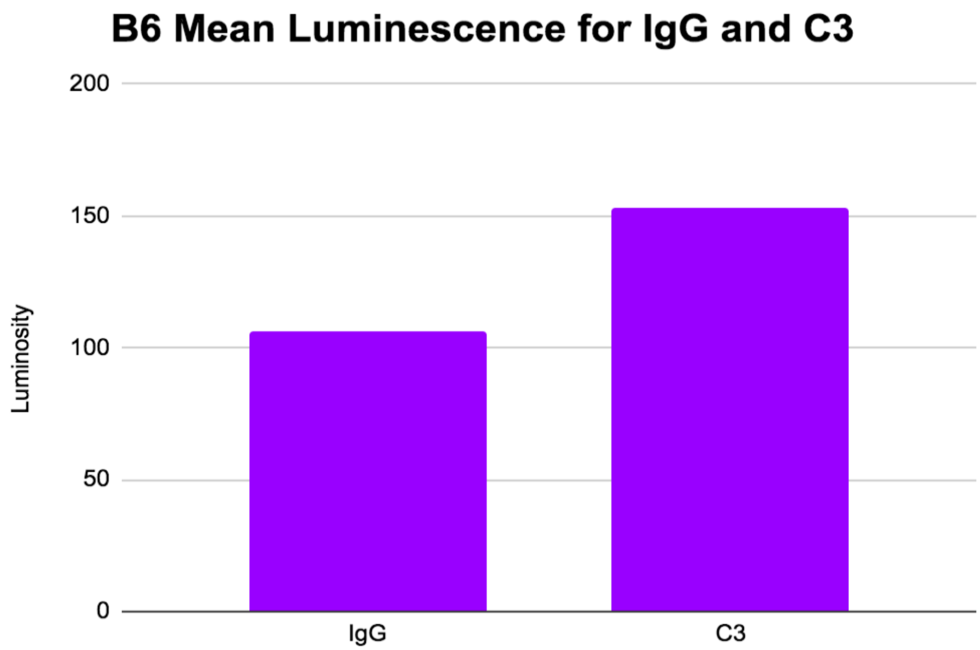


Figure 18: B6 Mean Luminescence. Image J was used to quantitate the mean luminescence for Donkey anti-mouse IgG Alexa 594 and Goat anti-mouse C3 FITC for the 2 month old C57BL/6 mouse.



DISCUSSION

The quantitation of immunofluorescence of FcγRIIB-/- Yaa mice at the ages of 2 months, 4.5 months, 5 months, and 8 months of age allowed for the evaluation of changes in the deposition of immune complexes during the course of SLE in a murine lupus model. The goal of this thesis was to evaluate how immune complex deposition correlates with the other aspects of disease severity in SLE. From the results, the following observations may be made:

I. Peak Luminescence for IgG-Alexa-594 and C3 Complement Protein was at 4.5 months of age in FcγRIIB-/- Yaa mice

This thesis demonstrates that the highest mean IgG-Alexa-594 and C3-FITC complement protein luminosity was shown in mouse models of 4.5 months of age. The higher the fluorescence intensity (luminosity), the greater the deposition of immune complexes, as seen in Figures 12 and 13.

II. FITC- C3 and IgG-Alexa-594 Luminescence is similar to B6 models at 2 months of age

The mean levels of C3 and IgG luminescence was similar in the B6 model . This suggests that the renal damage associated with lupus nephritis primarily occurs between the ages of 2-4.5 months in Yaa R2 KO mice.

III. C3 levels decrease in 5 months then increase again at 8 months

The mean fluorescence intensity of the Goat anti-mouse C3 FITC in the 5 month old FcγRIIB-/- Yaa mouse was 407.042. In the 8 month old FcγRIIB-/- Yaa mouse the mean fluorescence intensity of Goat anti-mouse C3 FITC was 639.769.

IV. Limitations

Based on the analysis to this point, our results are not definitive because we only used one mouse (n=1) per age group. Therefore, all of the observations made in this thesis could be seen as a stochastic outcome. One outcome in particular, could be seen as stochastic. Normally, the levels of C3 in late stage SLE are very low due to the fibrosis and necrosis caused by immune complex deposition (Wallace and Hahn, 218). When the kidneys are so damaged, it is theorized that further immune complex deposition occurs. Therefore, more mice need to be studied at the 8 month age mark in order to support the idea that C3 deposition increases again at the 8 month age mark in FcγRIIB-/- Yaa mice.

Another limitation that should be considered is the variability in SLE disease representation from mouse to mouse. Although all the mice are genetically similar and inbred, the presentation of the deposition of IgG and C3 may vary between mice of the same age group. Further study should include a greater sample size of mice per each age group in order to gain statistical power and confirm the results of this thesis.

Although immune complexes have been correlated with the severity of SLE disease manifestations (Toong, et al., 2011) other explanations for the glomerulonephritis in these mice must also be considered. Glomerulonephritis and overall renal inflammation may be caused by other processes in the kidney. In recent years, neutrophils have been implicated in renal inflammation. When neutrophils are activated via a surface FcγR, they degranulate and release reactive oxygen species and lytic enzymes. These reactive oxygen species and lytic enzymes may damage the glomerulus (Frangou et al., 2020). Furthermore, neutrophils have the ability to form extracellular traps (NETS) that capture pathogens. Released NETS may trigger other immune cells, such as plasmacytoid dendritic cells (pDCs) to stimulate type I interferon release (Frangou et al., 2020). The release of type I interferon may then activate other branches of the immune response and contribute to the renal damage associated with immune complex deposition.

The involvement of resident kidney cells must also be further investigated in order to evaluate their role in the pathogenesis of renal damage. Podocytes are cells on the glomerular surface that aid in the filtration barrier of the glomerulus. Recent literature has suggested that podocytes may be directly targeted by immune complex deposits (Sakhi, Hamza et al., 2019). The injury of the podocyte cells may lead to further inflammation and damage to the glomerulus.

LIST OF JOURNAL ABBREVIATIONS

ARTHRITIS RHEUMATOL…………………………….…...Arthritis and Rheumatology

CLIN IMMUNOL……………………………….……….…............Clinical Immunology

FEBS J……………………...…………………………………………The Febs Journal

INT J NEPHROL RENOVASC DIS…….……...International Journal of Nephrology and renovascular disease

J AUTOIMMUN…………………………………….……………..Journal of Autoimmunity

J CLIN MED…………………………….………………...Journal of Clinical Medicine

J IMMUNOL……………………………………………………....Journal of Immunology

J LAB CLIN MED………………………….Journal of Laboratory and Clinical Medicine

MAEDICA…………….………………………....Mædica Journal of Clinical Medicine

NAT IMMUNOL………………………………………………..Nature Immunology

NAT MED………………………………………………………………..Nature Medicine

NAT REV DIS PRIMERS…………………………....Nature Reviews Disease Primers

PLOS ONE…………………….……………………………………………PLoS ONE

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