PATHOGENIC AND PROTECTIVE POTENTIAL OF B CELL DYSREGULATION IN SYSTEMIC LUPUS ERYTHEMATOSUS

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PATHOGENIC AND PROTECIVE POTENTIAL OF B CELL DYSREGULATION IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by loss of tolerance to nuclear antigens. Hyperactive B cells are present in SLE patients and murine models of lupus, many of which have defects in inhibitors of B cell receptor (BCR) signaling or plasma cell differentiation. Autoantibodies against a wide range of self antigens contribute to the pathogenesis of SLE and are used to diagnose SLE, determine prognosis, and predict specific disease manifestations. Autoantibodies form immune complexes which deposit in the kidney and joints, resulting in glomerulonephritis and arthritis, respectively. Autoantibodies against antigens in the CNS can cause Neuropsychiatric SLE manifestations, such as psychosis, memory loss,

seizures, strokes, and mood disorders. Human SLE patients and murine models of lupus are used here to identify novel autoantibodies in SLE and to better understand the mechanisms by which autoantibodies accumulate in SLE.

Protein arrays can be used to identify autoantibodies and autoantigens that are targeted in SLE patients. Using this approach, we identified Stress Induced Phosphoprotein 1 (STIP1) as an autoantigen in a subset of SLE patients. Those patients with elevated levels of anti-STIP1 IgG autoantibodies in their serum were less likely to have parameters associated with more severe disease, suggesting a protective role for anti-STIP1 IgG. In addition, I defined a genetic interaction between the src tyrosine kinase, Lyn, and the Ets family transcription factor, Ets1, in autoimmunity. Lyn is both a positive and negative regulator of BCR signaling; however its net effect is inhibitory. Lyn deficiency results in hyperactive B cells and Lyn-/- mice serve as a murine model of SLE. Ets1 is a regulator of plasma cell differentiation, and Ets1-/- mice have a similar phenotype to Lyn-/- mice. Lyn and Ets1 are in a shared pathway in which Lyn maintains Ets1 levels, thus limiting plasma cell accumulation. Compound heterozygotes of Lyn and Ets1 were used to determine whether partial loss of Lyn and Ets1 results in accelerated autoimmunity. Lyn and Ets1 were found to synergize in limiting the accumulation of activated and memory T cells, myeloid dendritic cells, age associated B cells, and IgM, but not IgG autoantibodies.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Luo, W., Mayeux, J., Gutierrez, T., Russell, L., Getahun, A., Muller, J., Garrett-Sinha, L. A. (2014). A Balance between B Cell Receptor and Inhibitory Receptor Signaling Controls Plasma Cell Differentiation by Maintaining Optimal Ets1 Levels. J Immunol. doi: 10.4049/jimmunol.1400666

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LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ABC – age associated B cell

ANA – antinuclear antibody

APC – antigen presenting cell

Ab – antibody

Ag – antigen

Anti-Id – anti-idiotype antibodies target antigen binding site of another antibody

BAFF – B lymphocyte activating factor of the tumor necrosis factor family

BBB – blood brain barrier

BCR – B cell receptor

Btk – Bruton's tyrosine kinase

BUN – Blood Urea Nitrogen

CD – cluster of differentiation

CNS – central nervous system

CRP – C Reactive Protein

CSF – cerebral spinal fluid

DC – dendritic cell

dsDNA – double stranded deoxyribonucleic acid

EAE – experimental autoimmune encephalitis

ELISA – enzyme–linked immunosorbent assay

ELISPOT – enzyme-linked immunospot

ESR – Erythrocyte Sedimentation Rate

FcYRIIb – low affinity immunoglobulin gamma Fc Region receptor II-B

FITC – Fluorescein isothiocyanate

FO - follicular

GWAS- genome wide association studies

HC – healthy controls

HCO3 – serum bicarbonate

HSP – heat shock protein

IFN – interferon

IL – interleukin

mDC – myeloid dendritic cell

MHC – major histocompatibility complex

MDSC – myeloid derived suppressor cell

MS – Multiple Sclerosis

MZ – marginal zone

NAb – Natural IgM Antibodies

NFκB – nuclear factor-kappa B

NK – natural killer cells.

NMDAR - N-methyl-D-aspartate receptor

NP - neuropsyciatric

NSAIDS – non–steroidal anti–inflammatory drugs

PBMC – peripheral blood mononuclear cell

PE – phycoerythin

PerCP – peridinin chlorophyll protein complex

PC – plasma cell

pDC – plasmacytoid dendritic cell

PIR-B – paired immunoglobulin-like receptor B

PrPc – cellular prion protein

PRR – pattern recognition receptor

RA – rheumatoid arthritis

RBCs – red blood cells

SHIP-1 – SH2-containing inositol-5'-phosphatase 1

SHP-1 – SH2-containing phosphatase 1

SLE – Systemic Lupus Erythematosus

SLEDAI – SLE disease activity index

STIP1 – stress induced phosphoprotein 1

Tfh – T follicular helper

Th – T helper

TLR – toll-like receptors

Tregs – T regulatory cells

UProtein/UCr – Ratio of urine protein levels to urine creatinine levels

WBC – white blood cells

wt – wildtype

XLA – X-linked agammaglobulinemia

CHAPTER ONE
Introduction

AUTOIMMUNITY

Central and Peripheral Tolerance

The role of the immune system is to recognize and mount a response against potentially harmful foreign material. Regulation of the immune response is key to preventing the immune system from attacking innocuous or self antigens and causing damage to the host. Autoimmunity results from a loss of tolerance to self antigen. Central and peripheral tolerance are mechanisms by which the immune system ensures that immune responses are only mounted against foreign, but not self, material. If self reactive lymphocytes escape central tolerance within the primary lymphoid tissue, and encounter their antigen in secondary lymphoid tissue, peripheral tolerance is in place to prevent an autoimmune response. Dysfunction of either central or peripheral tolerance can lead to autoimmunity.

Central Tolerance

During central tolerance, the cells of the adaptive immune response, lymphocytes, are educated against self antigen in the primary lymphoid tissue (Hogquist, Baldwin et al. 2005, Pelanda and Torres 2012). B and T lymphocytes arise in the bone marrow from a common lymphoid progenitor. B cells continue to develop in the bone marrow where they go through negative selection at the immature B cell stage. During negative selection, if a B cell receptor (BCR) binds too strongly to a self antigen, it will undergo receptor editing. This process involves the expression of Rag1 and Rag2, which allows for the secondary rearrangement of the immunoglobulin (Ig) light chain genes, first kappa then lambda, to achieve a BCR which has a different specificity and no longer binds self antigen. The BCR will be tested again and if it does not bind self antigen it is released into the periphery as a mature B cell where it will become activated upon subsequent encounter with its foreign antigen. If the edited BCR still reacts with self antigen and has gone through all possible gene rearrangements then the B cell is deleted by apoptosis so that strongly autoreactive cells do not escape into the periphery. However if a BCR reacts weakly with self antigen, or with a soluble self antigen which does not crosslink BCRs, the B cell will be released into the periphery in a state of anergy, or unresponsiveness. BCR and costimulatory molecule expression is decreased in anergic B cells and they fail to become activated upon BCR crosslinking.

While both B and T cells arise from a common lymphoid progenitor in the bone marrow, T cell progenitors migrate from the bone marrow to the thymus to continue their development into T cells and undergo central tolerance. T cells expressingT cell receptors (TCRs) interact with major histocompatibility complexes (MHCs) on cortical epithelial cells in the thymus. Positive selection is the process of selecting T cells containing a TCR that can bind to MHCs. Negative selection occurs in T cells expressing TCRs that bind MHCs to determine if the TCR is reacting too strongly to ensure that only functional, but not self reactive T cells will be released to the periphery. Unlike B cells, if a TCR reacts too strongly with an MHC presenting a self peptide, there is no receptor editing, and the T cell will be deleted by apoptosis. However if the TCR has low affinity for the self peptide, then that T cell will survive negative selection and be released into the periphery as a mature T cell to become activated. The purpose of positive and negative selection together is to ensure that the TCR will be able to bind MHCs but does not react strongly with self peptides.

Peripheral Tolerance

Peripheral tolerance is in place to ensure that autoreactive cells that have escaped selection or that become autoreactive in the periphery do not mount a response against

self antigen. A non-autoreactive B cell encountering its antigen can undergo somatic hypermutation, during which mutations are introduced which allow the BCR to bind to antigen more or less efficiently. During this process, the BCR can lose its affinity for the foreign antigen it once recognized and gain affinity for a self antigen, becoming autoreactive. Various cells in the periphery are responsible for maintaining tolerance to self antigen in the periphery. T regulatory cells (Tregs), B regulatory cells (Bregs), and Myeloid derived suppressor cells (MDSCs) have different mechanisms to maintain tolerance in the periphery. The Foxp3 transcription factor is the master regulator for Tregs which can suppress a variety of immune cells including B cells, natural killer (NK) cells, NKT cells, CD4+, and CD8+ T cells, as well as monocytes and dendritic cells (DCs) (Schmidt, Oberle et al. 2012). Tregs can directly inhibit cells through the secretion of immunosuppressive cytokines, such as IL-10 and TGFβ. In addition, Tregs can cause antigen presenting cells (APCs) to have reduced expression of costimulatory molecules, indirectly influencing the activation of other immune cells (Cederbom, Hall et al. 2000). Loss of Foxp3 expressing Tregs can result in autoimmunity (Mouly, Chemin et al. 2010, Josefowicz, Lu et al. 2012). Similar to Tregs, Bregs can suppress the immune response though the secretion of regulatory cytokines such as IL-10 and TGFβ. In addition, Bregs express inhibitory molecules, such as FasL, that suppress pathogenic T cells and autoreactive B cells in a cell-to-cell contact-dependent manner (Lundy 2009, Yang, Rui

et al. 2013). MDSCs can cause cell contact-dependent antigen specific and antigen-independent immunosuppression. Other mechanisms of MDSC suppression include, but are not limited to mediators such as reactive oxygen species, nitric oxide, and suppressive cytokines (Movahedi, Guilliams et al. 2008, Youn, Nagaraj et al. 2008).

SYSTEMIC LUPUS ERYTHEMATOSUS

Characteristics

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease mediated by both innate and adaptive immune cells resulting in damage to multiple organs. SLE affects 1 in 2000 individuals with a female to male ratio of 9:1. Hallmark features of SLE include a loss of tolerance to nuclear antigens such as DNA, RNA, and histones resulting in the production anti-nuclear antibodies (ANAs) and immune complex (IC) deposition in multiple organs. For example, IC deposition in the joints or kidney results in arthritis and glomerulonephritis, respectively. SLE is also characterized by periods of disease quiescence and flares of disease activity (Tsokos 2011).

Diagnosis

Diagnosis of SLE can be difficult because manifestations of symptoms can vary widely among patients. Diagnosis of SLE requires 4 out of 11 American College of Rheumatology recognized symptoms, which are Serositis, Oral ulcers, Arthritis, Photosensitivity, Blood disorders, Renal involvement, ANAs, Immunologic phenomena (eg, dsDNA, anti-Smith antibodies, etc.), Neurologic disorder, Malar rash, and Discoid rash (Tsokos 2011). Patients often present with autoantibodies that can predict prognosis regarding specific disease manifestations (Table 1) (Rahman and Isenberg 2008).

Glomerulonephritis

Glomerulonephritis occurs when the glomeruli, which filter waste and excess fluid from the blood within the kidney, become inflamed and damaged. Kidney failure is one of the leading causes of mortality in SLE patients (Borchers, Leibushor et al. 2012). Antigens in the kidney can serve as targets for autoantibodies, or antibodies binding a soluble antigen in the circulation may form ICs and get deposited in the kidney. Tissue damage is caused by Fc receptor- (FcR)- and complement-mediated inflammation (Borchers, Leibushor et al. 2012). There are different classes of lupus nephritis describing the

different patterns of renal injury in SLE. Following a kidney biopsy, Class I is identified as normal glomeruli with minimal mesangial nephritis, Class II is purely mesangial lupus nephritis, Class III is focal proliferative glomerulonephritis, Class IV is diffuse proliferative glomerulonephritis, and Class V is membranous glomerulonephritis (Weening, D'Agati et al. 2004). Autoantibodies present in SLE patients that are associated with kidney disease involve anti-dsDNA, -nucleosome, -Ro, -Sm, -α-actinin, and –C1q.

Neuropsychiatric or Central Nervous System SLE

Central nervous system (CNS) or neuropsychiatric (NP) SLE occurs when there is a loss of tolerance to CNS related antigens resulting in damage to the CNS. 80% of SLE patients experience symptoms of NP SLE (Brey, Holliday et al. 2002). Symptoms of NP SLE include migraines, seizures, stroke, psychosis, cognitive dysfunction, and notably a very high incidence of mood disorders, particularly anxiety and depression (Popescu and Kao 2011). Autoantibodies against CNS antigens, such as the anti-N-methyl-D-aspartate receptor (anti-NMDAR), can be detected in the cerebrospinal fluid (CSF) and used in the diagnosis of NP SLE patients (Fragoso-Loyo, Cabiedes et al. 2008).

Autoantigen	Prevalence	Clinical Effect
dsDNA	70-80%	Kidney Disease, Skin Disease
Nucleosomes	60-90%	Kidney Disease, Skin Disease
Ro	30-40%	Kidney Disease, Skin Disease
Sm	10-30%	Kidney Disease
NMDAR	33-50%	Brain Disease
Phospholipid	20-30%	Brain Disease, Thrombosis
Actinin	20%	Kidney Disease, Brain Disease
C1q	20-50%	Kidney Disease

Table 1. Autoantibodies associated with disease manifestations. Listed are antigens targeted by autoantibodies that can be detected in subsets of SLE patients. These autoantibodies are useful in the diagnosis of SLE and in predicting specific disease manifestations. (Adapted from Rahman and Isenberg 2008).

SLE Disease Activity Index

SLE Disease Activity Index (SLEDAI) is a weighted and cumulative index of disease activity with scores ranging from 0 to 105, higher values indicating more disease activity. The SLEDAI assesses disease involvement in 12 organ systems, with each manifestation carrying a different weight. Leukopenia and thrombocytopenia each have a score of 1. Increased DNA binding, low complement, pericarditis pleurisy, mucosal ulcers, alopecia, and a new rash each carry a weight of 2. Pyuria, proteinuria, hematuria, urinary casts, myositis, and arthritis each have a score of 4. Vasculitis, cerebrovascular accidents, lupus headache, cranial nerve disorder, visual disturbance, organic brain syndrome, psychosis, and seizures each have a score of 8. The total sum of weights for each manifestation present in the patient represent the patient's SLEDAI score (Bombardier, Gladman et al. 1992).

Treatment

Current treatment options for SLE involve global suppression of the immune system leaving patients vulnerable to infections. Corticosteroids, Hydroxychloroquine, and Methotrexate, for patients with concurrent RA, are common immune suppressants given to SLE patients to manage their disease (Tsokos 2011). Benlysta, an anti-BAFF

monoclonal antibody, is the first new dug approved by the FDA in 50 years for the treatment of SLE but is not very effective and still leaves patients at risk of immune suppression (Tsokos 2011). A better understanding of the pathogenesis of SLE is necessary in order to develop more specific therapies to treat SLE.

Genetic Basis

Epidemiology studies have revealed a strong genetic component in the etiology of SLE. SLE develops with a high heritability rate and genetic factors have been identified as playing a role in the pathogenesis of SLE through association and family studies. Both naturally occurring polymorphisms and targeted mutations in numerous genes lead to lupus-like autoimmunity in mice.

GWAS Studies Identifying Polymorphisms in SLE Patients

Genome-wide Association Studies (GWAS) have rapidly advanced our understanding of the genetic basis of lupus. More than 40 susceptibility loci have been identified and confirmed to be associated with SLE (Cui, Sheng et al. 2013). Additionally, many genes with unknown functions have been identified as susceptibility loci for lupus, suggesting that additional molecular mechanisms contribute to the risk of developing SLE.

Polymorphisms in regulators of BCR signaling and PC accumulation, Lyn and Ets1, have been identified in SLE patients (Sullivan, Piliero et al. 2000, Brey, Holliday et al. 2002, Han, Zheng et al. 2009, Lu, Vidal et al. 2009, He, Liu et al. 2010, Yang, Shen et al. 2010, Zhong, Li et al. 2011, Wang, Ahlford et al. 2013).

Murine Models of Lupus

Murine models of lupus are an invaluable tool for researchers to gain a better understanding of the pathogenesis of SLE. Anti-Glomerular basement membrane disease (anti-GBM) is an inducible model in which transfer of rabbit serum and rabbit anti-basement membrane antibodies with Freund's adjuvant results in lupus-like glomerulonephritis, and is especially useful in the study of end organ damage in lupus (Lerner, Glassock et al. 1999). Spontaneous models of SLE develop without additional manipulation, due to their genetic background. MRL-lpr mice have a defect in the Fas receptor, which normally mediates negative selection and peripheral tolerance through programmed cell death, resulting in the onset of lupus-like autoimmunity by 3-4 months of age (Watson, Rao et al. 1992). In the NZM2410 murine model of lupus, susceptibility to lupus has been mapped to the Sle1, Sle2, and Sle3 loci (Morel, Mohan et al. 1997). Sle1 confers susceptibility to high titers of IgG ANAs in the absence of any severe nephritis, Sle2 is responsible for spontaneous development of elevated levels of IgM,

but not IgG Abs against several antigens, indicative of polyclonal activation or polyreactivity affecting the B cell lineage. Sle3 causes the production of IgM and IgG Abs against both nuclear and non-nuclear antigens and the development of severe lupus nephritis and is mediated by hyperstimulatory APC (Morel, Mohan et al. 1997, Zhu, Liu et al. 2005). Other spontaneous models of lupus include the F1 generation of the cross NZB x NZW (BWF1) and BXSB.Yaa mice, which develop antinuclear antibodies and glomerulonephritis (Helyer and Howie 1963, Hogarth, Slingsby et al. 1998, Henry and Mohan 2005). Inducible and spontaneous models of lupus are useful in analysis of the interplay between various susceptibility loci. Gene targeted mutations that result in a lupus-like syndrome without further intervention, are critical to in depth analysis of specific mechanisms that contribute to autoimmunity. One such model are Lyn-/- mice, in which loss of inhibition of BCR signaling results in a lupus-like phenotype by 6-8 months (Hibbs, Tarlinton et al. 1995, Nishizumi, Taniuchi et al. 1995, Chan, Meng et al. 1997). The appearance of autoantibodies in patients with SLE tends to follow a similar course seen in most murine models of lupus, with a progressive accumulation of specific autoantibodies before clinical onset of SLE, while patients are still asymptomatic (Arbuckle, McClain et al. 2003).

Pathogenesis Model of SLE

The adaptive immune cells maintain tolerance against self antigens through central and peripheral tolerance, but in autoimmune diseases such as lupus, this tolerance is lost. Innate immune cells recognize pathogen associated molecular patterns and are not educated against self antigens in the primary lymphoid tissue like lymphocytes. While innate cells do not contribute to autoimmunity through direct cognate recognition of self proteins they can be activated by endogenous ligands and are involved in the activation of other immune cells and secretion of inflammatory mediators that exacerbate disease. The model for SLE disease pathogenesis involves a positive feedback loop between the innate and adaptive immune system (Figure 1). A genetic predisposition and environmental triggers initiate the loss of tolerance to self. Activation of the adaptive immune system by the innate immune system begins a positive feedback loop resulting in a perpetuation of autoimmunity. The accumulation of autoreactive lymphocytes and autoantibodies can lead to IC deposition in various organs. Clinical disease onset is associated with systemic inflammation and tissue injury, which may result in irreversible tissue damage (Liu and Davidson 2012).

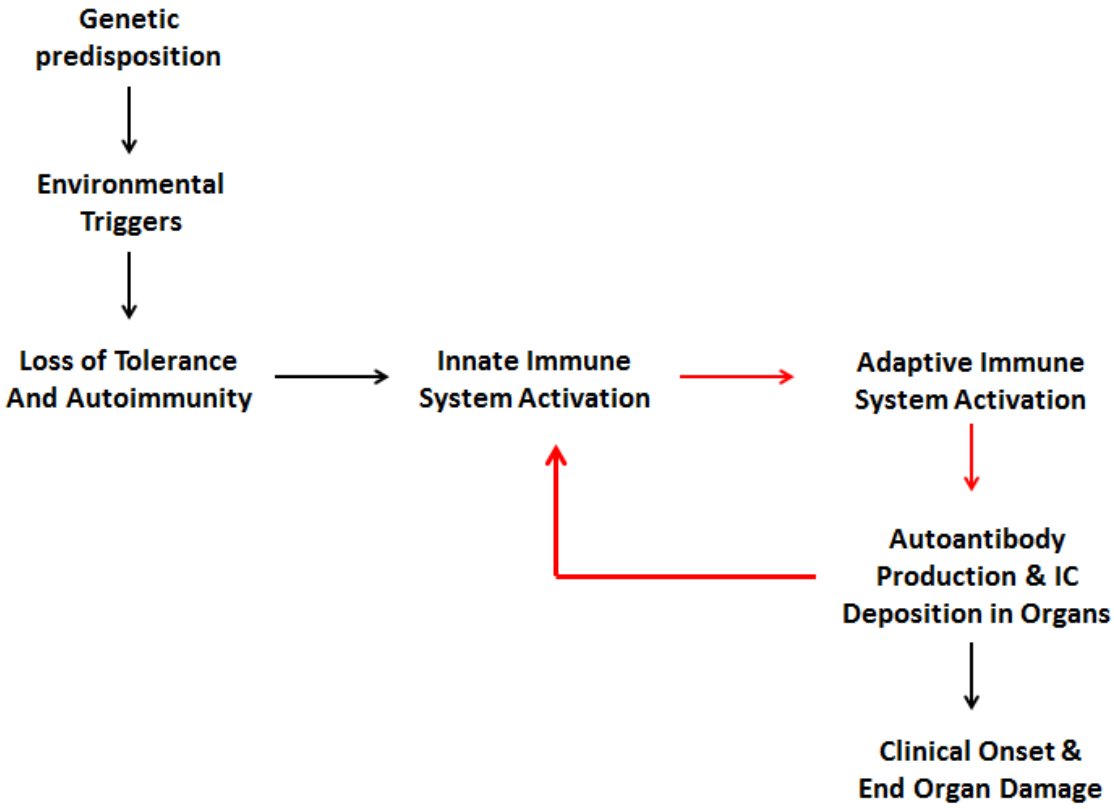


Figure 1. SLE disease model involving dysregulation of the innate and adaptive immune system. A genetic predisposition for SLE plus environmental triggers, contributes to the initiation of autoimmune disease. Innate immune cell activation results in increased antigen presentation, secretion of BAFF, IFNα, and other inflammatory cytokines. With a loss of adaptive immune tolerance, these events lead to activation of the adaptive immune system and autoantibody production and IC formation and deposition into tissue. IFN promotes B cell responses to TLR7, amplifying the production of antibodies against self antigens containing TLR7 ligands. Autoreactive B cells activate autoreactive T cells which secrete IFNɣ. A positive feedback loop between the adaptive and innate immune system occurs with further activation of the innate immune system by IFNα, IFNɣ, and ICs, amplifying autoimmunity. Clinical disease onset begins with systemic inflammation and tissue injury that can result in irreversible tissue damage (Adapted from Liu and Davidson 2012).

Neutrophils

Neutrophils are increased in SLE patients and their presence in the kidney is a sign of active nephritis. Neutrophils can be a major source of nuclear antigens following the secretion of neutrophil extracellular traps (NETs) and contribute to end organ damage through the production of proteases following activation by ICs. Immature neutrophils exposed to nucleic acid containing ICs are prone to death by NETosis, during which cells release DNA webs called NETs and release alarmins (Yu and Su 2013). Neutrophils activate plasmacytoid DCs (pDCs) through the secretion of these defensins as well as type I IFNs, amplifying the type I IFN response. Neutrophils secrete BAFF, maintaining autoreactive B cell survival (Liu and Davidson 2012).

Plasmacytoid Dendritic Cells

pDCs are activated by type I interferon (IFNα) which is secreted by neutrophils and by nucleic acid containing ICs through toll-like receptor 7 (TLR7). FcR-mediated recognition and internalization of nucleic acid containing ICs by pDCs contributes to disease manifestation. Within the endosome, DNA and RNA interact with TLR9 and TLR7, respectively, resulting in the secretion of type I IFN. Activated pDCs then secrete more IFNα which further activates pDCs and contributes to inflammation in SLE patients (Banchereau and Pascual 2006, Liu and Davidson 2012). IFNα also promotes B cell

responses to TLR7, amplifying the production of antibodies against self antigens containing TLR7 ligands (Lau, Broughton et al. 2005, Thibault, Graham et al. 2009).

Myeloid Dendritic Cells

Myeloid DCs (mDCs) also secrete IFNα, however pDCs are the main contributors to elevated type I IFNs. mDCs also take up apoptotic cells and process and present antigen to T cells and are the major stimulators of autoreactive T cells. Activation of cytotoxic CD8+ T cells may contribute to the availability of apoptotic cellular debris. mDCs and pDCs help expand autoreactive B cells. SLE serum induces differentiation to DCs and while there have been differences in reports regarding elevated or decreased levels of mDCs and pDCs in the blood, there is an increase of infiltrating DCs to inflammation sites (Banchereau and Pascual 2006).

Macrophages

Macrophages contribute to the pathogenesis of SLE in a variety of ways. Notably, macrophages have a reduced ability for phagocytosis and IC clearance (Kavai and Szegedi 2007, Chan, Nie et al. 2012). SLE macrophages have decreased expression of FcγRII and mannose binding receptors, which play a role in phagocytosis of apoptotic cells in SLE, and correlate with disease severity (Kavai and Szegedi 2007). Additionally,

macrophages in SLE patients have increased apoptosis and chemotaxis (Li, Lee et al. 2009). Increased apoptosis of macrophages and neutrophils, and the inability of macrophages to clear apoptotic debris through phagocytosis, increases the availability of self antigen to autoreactive cells in the periphery. Furthermore, enhanced chemotaxis in SLE macrophages contributes to the inflammatory immune cell recruitment and tissue injury (Li, Lee et al. 2009).

T cells

T effector cells, both CD4+ T helper (Th) cells and CD8+ cytotoxic T cells, contribute to autoimmunity. Abnormalities in SLE CD4+ T cells include increased expression of the co-stimulatory molecule, CD40 ligand, and the adhesion molecule, CD44. SLE Th cells secrete inflammatory cytokines and provide co-stimulation to autoreactive B cells. Increased secretion of Th1 cytokines TNFα, IFNϒ, and IL-2 contributes to the inflammatory mileu in SLE patients (Mak and Kow 2014). Th2 cells contribute to antibody mediated autoimmune disease through the secretion of cytokines, such as IL-4, which promote class switching in B cells. While both Th1 and Th2 cells play a role in the pathogenesis of SLE, Th1 cytokines are expressed at higher levels in lupus nephritis patients (Gomez, Correa et al. 2004). Th17 cells secrete IL-17, a pro-inflammatory cytokine, and have been associated with many autoimmune diseases

such as Multiple Sclerosis (MS) and Inflammatory Bowel Disease. Increased numbers of Th17 cells have been found in SLE patients, correlating with increased disease severity. Additionally, increased numbers of Th17 cells have been found in the kidney of lupus nephritis patients (Shin, Lee et al. 2011). Another subset of T cells, T follicular helper (Tfh) cells are expanded in murine models of SLE, sanroque, BWF1, BXSB, and Mrl-lpr. Tfh cells contribute to the activation and expansion of memory B cells and PCs within germinal centers (Luzina, Atamas et al. 2001, Vinuesa, Cook et al. 2005, Vinuesa, Sanz et al. 2009). The cytotoxic activity of CD8+ T cells is reduced in SLE patients (Stohl, Elliott et al. 1997). An inability to suppress infected cells may result in more polyclonal B cell activation and autoantibody production. Furthermore, T regulatory (Treg) cells maintain peripheral tolerance and are dependent on IL-2 for survival. Lower levels of both IL-2 and Tregs have been observed in SLE patients, resulting in decreased suppression of peripheral autoreactive and inflammatory T and B cells (La Cava 2008, Alexander, Sattler et al. 2013).

B CELLS IN AUTOIMMUNITY

B cells in Lupus

SLE is characterized by the presence of autoantibodies secreted by plasma cells (PCs), which are derived from autoreactive B cells. These autoantibodies mediate end organ damage through IC deposition in tissue. In SLE, the CD138+ expressing plasma cell subset is expanded. Additionally, monocytes, DCs, and neutrophils secrete BAFF, the target of Benlysta,and type I IFNs which contributes to the survival of autoreactive B cells(Scapini, Hu et al. 2010, Kiefer, Oropallo et al. 2012). The hyperactive phenotype of SLE B cells is a hallmark feature in SLE, and B cells have many functions that become dysregulated in lupus and that contribute to autoimmunity.

Antigen Presentation

B cells function as APCs to present antigen to CD4+ T cells in the context of MHC class II. Naïve B cells upregulate expression of CD80 and CD86 upon activation. CD80 and CD86 engage CD28, a costimulatory molecule expressed on T cells. Signal through the TCR and CD28 activates naïve T cells, while a signal through the TCR in the absence of costimulation leaves naïve T cells anergic. T cells activated by B cells in turn will

upregulate CD40L, which stimulates CD40 on B cells. In this way the B and T cells help each other. In many murine models of lupus, B cells are hyperactive and express increased levels of costimulatory molecules to activate T cells. Increased reciprocal B and T cell help expands the pool of activated autoreactive lymphocytes which can contribute to autoimmunity.

Cytokine Secretion

In addition to functioning as APCs, B cells can produce cytokines. B cells secrete cytokines including but not limited to IL-2, IL-4, IL-6, IL-10, IL-17, type I IFNs, TNFα, TGFβ, and LT, which can exert positive or negative effects on B cells themselves or other immune cells. Hyperactive B cells have an increased production of inflammatory cytokines which contribute to inflammation in autoimmunity. Studies have focused on defining B cell subsets with B effector 1 (Be1) cells secreting IFNϒ, TNFα, IL-12, and IL-10, and Be2 cells secreting IL-2, IL-4, IL-6, IL-13, IL10, and TNFα. In addition to T cell help, TLRs trigger robust cytokine production, including IL-1, IL-2, IL-6, IL-10, IL-12, IFNα, and IFNϒ. SLE B cells spontaneously secrete IL-1α, IL-4, and IL-6 which interact with their respective receptor in an autocrine manner stimulating the B cells to spontaneously proliferate and differentiate into antibody-secreting cells. Positive effects of B cell cytokine production include promoting macrophage, neutrophil, NK cell, and

DC activation, naïve T cell differentiation into Th1, Th2, and Th17 cells, as well as promoting PC development and antibody production. B cells can also exert negative, regulatory effects through cytokines. Mainly through IL-10 and TGFβ, B cells can inhibit Th1, Th2, and Th17 differentiation, antigen presentation by DCs and proinflammatory cytokine secretion by macrophages, induce Tregs, CD8+ anergy, and apoptosis of T effector cells (Bao and Cao 2014). In lupus, distinct subsets of B cells can have protective and pathogenic roles (Lemoine, Morva et al. 2009). Bregs have been found to be expanded in murine lupus models, such as BWF1 mice, and B cell derived IL-10 suppresses the inflammatory component of SLE by acting on myeloid and T cells in Lyn-/- mice (Haas, Watanabe et al. 2010, Scapini, Lamagna et al. 2011). Killer B cells expressing FasL in the MRL-lpr lupus model limit the suppressive effects of T cells, conferring protection of autoantibody-secreting FasL B cells, thus leading to poorer disease prognosis (Bonardelle, Benihoud et al. 2005)

Antibody Production

B cells produce and secrete antibodies which can enhance phagocytosis through opsonization, enhance complement activation, activate innate immune cells via FcRs, and neutralize pathogens or antigens, such as toxins. B cells which produce antibodies that recognize self antigen contribute to autoimmunity through the production of

autoantibodies. Autoantibodies can bind self antigens and the formation and deposition of IC can cause tissue damage. IC deposition mediates damage by binding to FcYRs and complement receptors on tissue resident cells, macrophages, and mast cells which secrete inflammatory cytokines and chemoattractants. Infiltrating inflammatory monocytes and neutrophils are recruited and become activated, contributing to the inflammatory environment and tissue damage (Mayadas, Tsokos et al. 2009).

Binding of an autoantibody to a self protein or receptor can also cause a loss or gain of function as discussed below with the anti-NMDAR antibodies. Other diseases with autoantibodies that can cause a loss or gain of function in a receptor include autoimmune hyperthyroidism, Graves' disease, and hypothyroidism, Hashimoto's thyroiditis. In Graves' disease and Hashimoto's thyroiditis, antibodies against the thyrotropin receptor, also known as thyroid stimulating hormone (TSH) receptor, serve as agonists and antagonists, respectively. In some cases, anti-TSH receptor autoantibodies have been detected in SLE patients (Pyne and Isenberg 2002).

Natural IgM autoantibodies (NAbs) play a role in regulating immune homeostasis and in protecting against autoimmune and inflammatory diseases. NAbs are responsible for the recognition and disposal of apoptotic cells and block TLR ligand and IC induced

inflammatory responses (Silverman, Vas et al. 2013). SLE patients have lower levels of NAbs and levels of NAbs negatively correlate with disease duration (Saiki, Saeki et al. 1987, Sivri and Hascelik 1995). Additionally, both IgM and IgG autoantibodies against β2 glycoprotein are protective against lupus nephritis and renal damage in SLE (Mehrani and Petri 2011). Anti-idiotype (anti-Id) antibodies recognize and bind to the antigen binding site of antibodies. In SLE and other autoimmune diseases, such as Type-I diabetes (T1D), anti-Id antibodies bind to and neutralize autoantibodies and inhibit further secretion of autoantibodies (Gronwall, Vas et al. 2012, Hampe 2012)

Plasma Cells

B cells differentiate into PCs or memory cells. PCs can be short or long-lived and secrete antibodies. Pax5 is responsible for maintaining B cell identity by functioning as a transcriptional activator of B-cell lineage and by limiting the expression of BLIMP1, a PC master regulator. Activating signals downregulate Pax5 expression, relieving Pax5 repression of BLIMP1, which in turn suppresses Pax5 and initiates PC differentiation (Yasuda, Hayakawa et al. 2012). In SLE, there is an accumulation of PCs resulting in increased autoantibody levels, contributing to disease pathogenesis. Studies using Rituximab, a monoclonal antibody targeting CD20, have shown a reduction in disease severity with the depletion of B cells SLE patients, however long-lived plasma cells

reside in the bone marrow and are not eliminated by this method (Cobo-Ibanez, Loza-Santamaria et al. 2014). In MS patients, antibody levels do not decrease with short-term treat of Rituximab, however in long-term treatment, antibody levels decrease (Naismith, Piccio et al. 2010).

Age Associated B cells

Age associated B cells (ABCs) are a subset of B cells that express the cell surface marker CD11c and accumulate in young lupus-prone mice at the onset of disease and secrete autoantibodies (Rubtsov, Rubtsova et al. 2011). ABCs secrete TNFα and contribute to the proinflammatory environment (Ratliff, Alter et al. 2013). ABCs also accumulate in aged female wild type C57BL/6 (B6) mice dependent on B cell TLR7 signaling (Rubtsov, Rubtsova et al. 2011, Rubtsov, Rubtsova et al. 2013). With age, decreased B lymphopoiesis results in a pool of B cells which have been chronically stimulated and expanded by antigen, including self antigen. The remaining B cells are more likely to be autoreactive and to secrete inflammatory cytokines, which can contribute to autoimmunity (Ratliff, Alter et al. 2013). In autoimmunity, the accelerated accumulation of ABCs, due to chronic stimulation by self antigen, could contribute to the pathogenesis of disease.

B cell Signaling Dysregulation in Lupus

In SLE, the failure of central tolerance and hyperactive B cell signaling leads to the accumulation of plasma cells and the production of autoantibodies. Activating and inhibitory signals maintain a balance in BCR signaling. Mutations in inhibitors of the BCR signaling pathway as well as regulators of PC differentiation and autoantibody production have resulted in autoimmunity.

Lyn Signaling in B Lymphocytes

Lyn is a src family tyrosine kinase expressed in B cells and myeloid cells. In B cell receptor signaling Lyn has both an inhibitory and activating role; however the absence of Lyn results in hyperactive B cells due to redundant positive regulators. Lyn phosphorylates several inhibitory receptors such as paired-immunoglobulin receptor (PIR-B) and CD22, triggering recruitment of the tyrosine phosphatase SHP-1 to the plasma membrane (Xu, Harder et al. 2005). This results in the dampening of BCR signaling. Lyn is also involved in the inhibitory pathway mediated by FcϒRIIb and SHIP-1 which counteracts PI3K signaling (Yu, Mamchak et al. 2003, Dorner and Lipsky 2006). Mutations in many components of this pathway, such as CD22, Siglec G, FcϒRIIb, SHIP-1, SHP-1, and Lyn lead to autoantibody production (Bolland and Ravetch 2000,

Pao, Lam et al. 2007, Croker, Lawson et al. 2008, Tsantikos, Maxwell et al. 2012, Muller and Nitschke 2014).

Polymorphisms in Lyn are associated with SLE and reduced expression of Lyn has been observed in B cells of SLE patients (Liossis, Solomou et al. 2001, Flores-Borja, Kabouridis et al. 2005, Harley, Alarcon-Riquelme et al. 2008, Lu, Vidal et al. 2009). The Src tyrosine kinase, CSK, phosphorylates C-terminal tyrosine residues in Lyn, leaving it in a closed and inactive conformation. A polymorphism in CSK is associated with SLE in which elevated levels of CSK result in increased inhibitory phosphorylation of Lyn (Manjarrez-Orduno, Marasco et al. 2012). Carriers of the CSK risk allele exhibit enhanced BCR mediated activation of mature B cells, transitional B cells, and IgM levels (Manjarrez-Orduno, Marasco et al. 2012).

Lyn-/- mice are a mouse model of lupus with the following phenotype: hyperactive B cells and loss of tolerance to self-antigens resulting in IgG autoantibodies, plasma cell accumulation, and Ig deposition in the kidney (Hibbs, Tarlinton et al. 1995, Chan, Meng et al. 1997, Satterthwaite, Lowell et al. 1998, Harder, Parsons et al. 2001, Chu and Lowell 2005, Lamagna, Hu et al. 2014). Additionally, increased expansion and activation of the myeloid compartment in Lyn-/- mice contributes to increased T cell

activation (Hibbs, Tarlinton et al. 1995, Harder, Parsons et al. 2001, Scapini, Hu et al. 2010, Scapini, Lamagna et al. 2011). There are two major checkpoints regulating autoantibody production in Lyn-/- mice. First, PCs accumulate in the periphery of Lyn-/- mice in a B cell intrinsic manner. This depends on Btk, a target of Lyn-dependent inhibitory signaling pathways, but is independent of the proinflammatory cytokine IL-6. Loss of tolerance at check point one results in a loss of tolerance to a wide range of self antigens recognized by IgM (Gutierrez, Halcomb et al. 2010). Then a subset of these cells, with specificities against lupus associated autoantigens, receive signals to class switch to pathogenic IgG autoantibodies, which is dependent on IL-6. (Gutierrez, Halcomb et al. 2010).

Ets1 Signaling in B and T Lymphocytes

Ets1, an Ets family transcription factor, is expressed in B and T cells. Ets1 is involved in the regulation of immune cells and is implicated in tumor aggressiveness, as it is expressed in high levels by these cells (Dittmer 2003). Ets1 regulates Th17 cell differentiation, Th1 inflammatory responses, as well as the development and function of Tregs (Grenningloh, Kang et al. 2005, Moisan, Grenningloh et al. 2007, Mouly, Chemin et al. 2010, Leng, Pan et al. 2011, Garrett-Sinha 2013). Ets1 upregulates the expression of the key B cell-restricted transcription factor Pax5 and inhibits the activity of the

plasma cell transcription factor Blimp1 to inhibit B cell differentiation to plasma cells (Lin, Angelin-Duclos et al. 2002, John, Clements et al. 2008). Polymorphisms in Ets1 have been associated with SLE and reduced expression of Ets1 has been observed in peripheral blood mononuclear cells (PBMCs) from SLE patients (Sullivan, Piliero et al. 2000, Han, Zheng et al. 2009, He, Liu et al. 2010, Li, Sun et al. 2010, Yang, Shen et al. 2010, Zhong, Li et al. 2011, Wang, Ahlford et al. 2013).

Ets1-/- mice exhibit a lupus like phenotype: hyperactive B cells, plasma cell accumulation, increased levels of IgM and IgG autoantibodies targeting lupus associated autoantigens, and Ig deposition in the kidney (Wang, John et al. 2005, John, Russell et al. 2014). Decreased IL-2 levels and Tregs have been observed in Ets1-/- mice and the Th17 cell compartment in these mice is expanded (Mouly, Chemin et al. 2010, Leng, Pan et al. 2011). The phenotype of Lyn-/- and Ets1-/- mice is strikingly similar, which led to the question of whether Lyn and Ets1 were functioning to limit autoimmunity on a shared pathway.

SUMMARY

SLE is difficult to diagnose. As previously discussed, there are some already known autoantibodies present in SLE patients which are used as a marker for disease and are associated with various disease manifestations. This allows physicians to predict certain disease complications such as glomerulonephritis, thrombosis, and NP SLE related events. However there are many autoantibodies present in SLE patients that have not been characterized for their association with disease prognosis and pathogenesis. This led me to attempt to identify novel autoantibodies and autoantigens in SLE patients and to identify the patient manifestations with which they are associated as well as their potential pathogenic role.

The presence of autoantibodies in SLE patients can contribute to disease through the deposition of ICs in the joints, glomeruli, and many other organs including the CNS. However, it is the steps leading to the production of these autoantibodies that are dysregulated in SLE patients and murine models of lupus. To determine which molecules are contributing to the accumulation of antibody secreting plasma cells I will focus on known regulators of BCR signaling and plasma cell accumulation, Lyn and Ets1, to determine their interaction and the downstream effects on disease.

The goals of my project:

- To identify novel autoantibodies in SLE patients as markers of disease and their targeted autoantigens to gain insight into disease pathogenesis.
- To identify autoantibodies specific to NP SLE patients which target CNS antigens that could be pathogenic.
    - To do this, I utilized a protein array to identify increased autoantibodies present in the serum from human SLE patients and focused on CNS autoantigens that had been previously implicated in neurological disease.

- To understand the mechanisms by which dysregulation of Lyn and Ets1 contributes to autoimmune disease.
- To determine whether there is an interaction between Lyn and Ets1 in a shared pathway to limit plasma cell accumulation.
- To determine whether partial loss of Lyn and Ets1 accelerates autoimmunity.
    - To do this, I utilized the mouse models of lupus, Lyn-/-- and Ets1-/-, to create compound heterozygotes of Lyn and Ets1. I observed their phenotype and assayed for accelerated disease.

CHAPTER TWO
METHODOLOGY

MATERIALS AND METHODS

Humans

Collection of peripheral blood from consented human subjects was overseen and approved by the University of Texas Southwestern Medical Center Institutional Review Board (IRB). Collection of blood and scoring of SLEDAI, Renal SLEDAI, and class of kidney disease was completed by doctors at The University of Texas Southwesten Medical Center's affiliated clinics and hospitals. Controls were matched where possible by age, gender, and ethnicity.

Mice

The Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center approved all experiments on mice. Mice were bred in a specific pathogen free (SPF) colony and housed in a barrier facility in a stress-free environment. Mice used for this study were 2 to 15 month old males and females as indicated in the text and figure legends. Daily health and husbandry needs for all mice

were overseen by the university's Animal Resource Center (ARC) veterinarians and staff. Congenic control (C57BL/6, wt) and MRL/lpr mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6.Lyn-/-, Ets1+/-, Lyn+/-, Lyn+/- Ets1+/-, CD19 cre IKK2 CA, B6.Sle1.Sle3, B6.Sle1.Sle2.Sle3 and BWF1 mice were bred in our mouse colony (Helyer and Howie 1963, Chan, Meng et al. 1997, Morel, Croker et al. 2000, Jimi, Strickland et al. 2008).Tail clips were obtained at weaning and used to prepare tail lysates for DNA extraction and PCR genotyping.

Human PBMC and Serum Isolation

Peripheral blood was collected in heparinized tubes and processed. Cells were centrifuged out and serum was extracted from the supernatant. PBMCs were isolated with density-gradient centrifugation over Ficoll. Cells were lysed for Western analysis, treated as described, or stained for flow cytometry assays.

Mouse Serum and Tissue Preparation

Mice were bled by either piercing the saphenous vein (for survival and microarray studies) or by cardiac puncture (at sacrifice). Serum was isolated by centrifugation and

stored at -20 °C. Upon collection, tissues were placed in complete RPMI medium (cRPMI) consisting of RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-β-mercaptoethanol. Single-cell suspensions were made from whole spleen by compressing the spleen between two frosted glass slides in a tissue culture dish, then washed and passed through a 70-micron nylon using a 23-gauge needle. Single-cell suspensions were depleted of red blood cells (RBCs) with RBC lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA). Cells were resuspended in cRPMI and a small aliquot was counted by Trypan Blue (Lonza/ Biowhittaker, Basel, Switzerland) exclusion using a microscope and hemacytometer.

Western Blotting

Human sera and PBMCs were isolated, lysed, and prepared for Western Blot analysis in sample buffer by boiling for 10 minutes. Western blot sample buffer (5x) contains 3.125ml Tris (pH=6.8), 5ml glycerol, 1g SDS, 1ml BME, 0.25g bromophenol blue, in 10ml ddH_2O. Samples were spun down and subjected to SDS-PAGE and transferred to nitrocellulose or polyvinyl fluoride (PVDF) according to standard procedures. In brief, blots were probed with the primary, mouse anti-human STIP1 (Abnova H00010963-

M35), and the secondary antibody, goat anti-mouse Ig (Biorad). Bands were visualized with ECL substrate and quantified using ImageJ®. For repeated probing with mouse anti-human Albumin (R&D Systems MAb 1455), blots were incubated on a shaker for 30 minutes at room temperature in stripping buffer containing 6.25ml 1M Tris (pH=6.8), 20ml 10% SDS, 0.714ml 10mM 2me, 73ml ddH_2O.

Flow Cytometry

RBC-depleted splenocyte suspensions were Fc-blocked with purified rat IgG2b anti-mouse CD16/CD32 (2.4G2) antibody for 5 minutes at 4°C prior to primary staining with monoclonal antibodies. The following fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP), Alexa-fluor or biotin-conjugated antibodies were used in various combinations to stain for distinct populations. Incubations for both primary and secondary antibodies were 15 minutes at 4°C. All antibodies used are listed below (Table 2).

Antigen	Fluorophore	Catalog Number	Company
B220	PE	553090	BD Biosciences
B220	PerCP	650452U100	Tonbo Biosciences
CD11b	FITC	553310	BD Biosciences
CD11b	PerCP	550993	BD Biosciences
CD11c	PE	557401	BD Biosciences
CD11c	Biotin	553800	BD Biosciences
CD138	PE	553714	BD Biosciences
CD138	Biotin	553713	BD Biosciences
CD19	FITC	553785	BD Biosciences
CD19	Biotin	553784	BD Biosciences
CD21	PE	123409	BioLegend
CD23	Biotin	553137	BD Biosciences
CD3	FITC	553062	BD Biosciences
CD4	PerCP	550954	BD Biosciences
CD44	PE	553134	BD Biosciences
CD62L	FITC	553150	BD Biosciences
CD69	Biotin	553235	BD Biosciences
CD8	PerCP	553036	BD Biosciences
CD8	FITC	11-0081-85	eBiosciences
CD86	PerCP	105026	BioLegend
CD86	Biotin	553690	BD Biosciences
Gr-1	PE	553128	BD Biosciences
IFNɣ	PE	562020	BD Biosciences
IgM	PE	553409	BD Biosciences
IgM	PerCP	550881	BD Biosciences
IL17a Alexa	APC	560224	BD Biosciences
Ly6c	FITC	553104	BD Biosciences
PDAC1	Biotin	130-091-962	Miltenyi Biotec

Table 2. Antibodies used in flow cytometry staining. Detailed above is the antigen targeted, the fluorophore, catalog number, and company the antibodies were acquired from.

When staining for intracellular antigens, cells were fixed and permeabilized according to the manufacturer's instructions for the Cytofix/Cytoperm™ kit (BD Biosciences). Briefly, cells were incubated overnight with media alone or PMA (50ng/ml) and ionomycin (250 ng/ml) in the presence of GolgiPlug™ (Brefeldin A) (BD Biosciences), which was used according to the manufacturer's instructions. To stain for cell surface markers, cells were incubated with antibodies targeting CD4 or CD8 for 15 minutes at 4°C. Cells were then treated with Cytofix/Cytoperm™ buffer for 20 minutes at 4°C. To stain for intracellular cytokines, cells were incubated with antibodies targeting IFNɣ and IL-17 for 30 minutes at 4°C. All samples were acquired on a FACSCalibur cytometer and analyzed using CellQuest software (both from BD Biosciences).

ELISA

For detection of total IgM, IgG, and IgA antibodies, flexible 96-well PVC plates (BD Biosciences) were coated with 2 µg/ml goat anti-mouse immunoglobulin (Ig) (Southern Biotech) and blocked with 1% bovine serum albumin (BSA) in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM, IgG, and IgA, Sigma-Aldrich) were diluted serially and added to wells in triplicate and incubated for 1 hour at room temperature.

For detection of anti-dsDNA autoantibodies, Immulon II plates (Dynatech Laboratories, Chantilly, VA) were pre-coated with 0.1 mg/ml methylated BSA (mBSA) and subsequently coated with 50 µg/ml calf thymus dsDNA (Sigma-Aldrich). For detection of anti-ssDNA antibodies, plates were coated as above except that the calf thymus DNA was boiled and quenched on ice immediately prior to coating. After plates were incubated overnight at 4°C with blocking buffer (PBS, 3% BSA, 0.1% gelatin, 3 mM EDTA), serial dilutions of serum were added in duplicate and incubated for 2 hours at room temperature.

For mice, both total Ig and anti-DNA ELISAs, bound IgM, IgG, and IgA was detected by an alkaline phosphatase-conjugated secondary antibody, goat anti-mouse IgM, IgG, and IgA (Southern Biotech for IgM and IgG, MABTECH for IgA) and developed with an alkaline phosphatase substrate kit (Bio-Rad Laboratories, Hercules, CA). For humans, both total Ig and anti-dsDNA ELISAs, bound IgM or IgG was detected by an HRP-conjugated secondary antibody, goat anti-human IgM or IgG (Southern Biotech), and developed with an TMB substrate (Bio-Rad Laboratories, Hercules, CA). The optical density was read at 405 nm and 450 nm on an absorbance microplate reader (Bio-Tek Instruments, Winooski, VT) for Alkaline phosphatase and HRP detection methods,

respectively. Total amounts of IgM and IgG were calculated based on the known concentrations of the Ig standards.

For detection of antibodies against STIP1, Immulon II plates (Dynatech Laboratories, Chantilly, VA) were coated with 2 µg/ml protein, STIP1 (ATGEN ATG0426) and incubated overnight at 4°C. Plates were subsequently incubated for 2 hours at room temperature in blocking buffer (PBS, 3% BSA, 0.1% gelatin, 3 mM EDTA). Serial dilutions of serum were added in duplicate and incubated overnight at 4°C. Bound IgM or IgG was detected by an HRP-conjugated secondary antibody, goat anti-human IgM (Bethyl E80-100) or IgG (Sigma A6029) and developed with TMB substrate (Bio-Rad Laboratories, Hercules, CA). The optical density was read at 450 nm on an absorbance microplate reader (Bio-Tek Instruments, Winooski, VT). All other protoarray validations were performed in the laboratory of Dr. Quan Li.

IL-6 was detected using an OptEIA™ anti-mouse IL-6 ELISA kit (BD Biosciences) according to the manufacturer's instructions. Briefly, Immulon II plates (Dynatech Laboratories) were coated with anti-IL-6 antibody overnight at 4°C. The plates were then blocked and serial dilutions of standards and serum samples or culture supernatants were plated in triplicate for 1-hour incubation at room temperature. IL-6

was detected with a biotin-conjugated anti-IL-6 antibody and strepavidin-conjugated horseradish peroxidase. Substrate buffer was then added to the plate, stopped after 30 minutes and the optical density was read at 450 nm using an absorbance microplate reader (Bio-Tek Instruments). Total amounts of IL-6 were calculated based on the known concentrations of the recombinant IL-6 standards (BD Biosciences).

ELISPOT

For enzyme-linked immunosorbent spot (ELISPOT) analysis, Millipore MultiScreen 96-well plates with Immobilon-P membranes were coated with 5 µg/ml of a polyclonal goat anti-mouse Ig (Southern Biotech Catalog # 1010-01). Splenocytes were depleted of red blood cells and plated in serial dilutions at 10,000, 25,000, 50,000, and 100,000 cells/well and incubated overnight in a tissue culture incubator. IgM-secreting ASCs were detected by using a biotin-conjugated rat anti-mouse IgM (KPL Fisher Catalog # 16-18-03) detection antibody, and IgG-secreting ASCs were detected by using a biotin-conjugated polyclonal anti-mouse IgG antibody (KPL Fisher Catalog # 16-18-18). ELISPOT plates were counted with an automated reader (Zellnet Consulting, Fort Lee, NJ, USA).

Serum Creatinine Colorimetric Assay

Serum creatinine was measured using a serum creatinine colorimetric assay kit (Cayman, Ann Arbor, MI) per the manufacturer's protocol. Serum was diluted 1 to 10.

Autoantigen Array

Serum was collected as described above from 4-7 month old mice. Blind samples were submitted to Dr. Quan-Zhen Li (Department of Immunology, The University of Texas Southwestern Medical Center). Autoantibodies were measured on an autoantigen proteomic array that has been described previously (Li et al., 2007). The array includes seventy autoantigens and four control proteins. 1 µl of each sample was diluted 1:100 and added to the arrays in duplicate. Detection was with Cy3- (indocarbocyanine) labeled anti-mouse IgM and Cy5- (indodicarbocyanine) labeled anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). A Genepix 4000B scanner (Molecular Devices, Inc., Sunnyvale, CA) with laser wavelengths 532 nm (for Cy3) and 635 nm (for Cy5) was used to generate images for analysis. Images were analyzed using Genepix Pro 6.0 software to generate a GPR file (Molecular Devices, Inc.). Net fluorescence intensities (nfi) were normalized using anti-mouse IgM or IgG spotted onto each array.

Values obtained from duplicate spots were averaged. These values were received from Dr. Li and hierarchical clustering analysis of autoantibodies was performed using Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm).

Protein Microarray Screen

Autoantibody specificities in HC and SLE serum samples were analyzed using ProtoArray® Human Protein Microarrays from Life Technologies through the laboratory of Dr. Quan Li. Per manufacturer's protocol: Over 9,000 non-redundant human proteins were printed on nitrocellulose coated glass slides. Arrays were blocked at 4°C for 1 hour, prior to probing. Serum samples (10ul) were diluted at 1:100 and incubated at 4°C for 90 minutes. Arrays were washed and subsequently probed with anti-human IgG conjugated to Alexa Fluor® 647 (Invitrogen) at 4°C for 90 minutes. Arrays were then washed, dried, and scanned with GenePIx® 4000B fluorescent scanner (Molecular Devices). Data was acquired with GenePix® Pro Software (Molecula Devices) and processed using ProtoArray® Prospector 4.0 (Invitrogen).

Statistical Analysis

P values where not otherwise noted were determined by a two-tailed, unpaired student's t test to determine significant differences between groups.

The protein microarrays are invaluable tools; however, current limitations on sample size (n=6 per group) due to cost impact what can be concluded from the array. A larger n would be necessary for sufficient power to definitively identify significant differences between HCs and SLE patients, especially when trying to identify a subset of patients. While multiple testing corrections were not performed on the array analysis, the majority of antigens tested were validated by ELISA to be differentially targeted in SLE vs. HC.

To determine whether there was a correlation between STIP1 antigen and STIP1 autoantibody levels with other patient parameters, a linear regression analysis was used.

Patients fell into two distinct groups, those with high and low anti-STIP1 antibody. Patients with STIP1 autoantibody levels 3 standard deviations (SD) or more above the HC mean were defined as having high anti-STIP1 autoantibody levels. Chi square

analysis was used to determine whether STIP1 antibody levels were independent of other patient parameters. P values from chi square analysis were determined by using a Fisher's Exact test.

Significance cutoffs for p values were set at 0.05. All analyses were performed using GraphPad® Prism Software.

CHAPTER THREE
IDENTIFICATION OF NOVEL AUTOANTIBODIES AND ANTIGENS IN SLE

INTRODUCTION

Autoantibodies are used in the diagnosis of SLE and predicting presentation of specific disease manifestations. Depending on the antigen targeted by the autoantibodies and the pathogenic potential of the autoantibodies, damage to specific organs and tissues may occur, which can be associated with disease manifestations. Although many autoantibodies have been identified in SLE, most are not specific, and many autoantibodies that can mediate damage have not been identified. Additional biomarkers of SLE would be helpful in the diagnosis and prognosis of patients, as well as understanding the mechanisms of disease pathogenesis for future therapeutic targets.

Neuropsychiatric SLE

Autoantibodies targeting CNS antigens are useful in the diagnosis of NP SLE and have been identified to play a role in the pathogenesis of NP SLE manifestations. The presence of antibodies against CNS antigens can be detected in the serum and cerebrospinal fluid (CSF) of many NP SLE patients. Autoantibodies against CNS

antigens can also be detected in the serum of non-NP SLE patients; however the presence of these autoantibodies in the CSF is specific for NP SLE patients. Through ubiquitous or ectopic expression of a CNS antigen by a tumor or other mechanisms such as molecular mimicry, B cells can lose tolerance to the self antigen through presentation by T cells and DCs. Activated T cells and memory B cells from the periphery can then cross the blood brain barrier (BBB) normally. In the CNS, memory B cells undergo re-stimulation, antigen-driven affinity maturation, clonal expansion and differentiation into antibody-secreting PCs. In addition, autoantibodies systemically produced in the periphery by antibody secreting plasma cells can cross a disrupted or leaky BBB. Antibodies produced within the CNS by plasma cells or reaching the CNS by BBB disruption bind CNS antigens. Autoantibodies binding a receptor in the CNS can cause internalization of the receptor and subsequent dysfunction of the receptor. Additionally, autoantibodies binding a soluble CNS antigen can attenuate the protein's function by limiting available protein and be pathogenic or protective.

Many autoantibodies have been proposed to be involved in the mechanism of NP SLE, including anti-neuronal antibodies (Zandman-Goddard, Chapman et al. 2007). Many studies have identified an association between lupus psychosis and anti-ribosomal P protein (anti-P) antibodies (Bonfa, Golombek et al. 1987, Stein, Conrad et al. 2013). In

addition to CNS involvement, kidney disease in SLE patients with anti-P antibodies has been reported (Toubi and Shoenfeld 2007). Anti-P antibodies are able to enhance the production of pro-inflammatory cytokines in both the CNS and kidney. Similar to the ability of anti-dsDNA antibodies, anti-P antibodies are able to penetrate into living cells, leading to cell dysfunctions such as cell apoptosis (Toubi and Shoenfeld 2007). Anti-P antibodies bind to neuronal antigens and cause increased calcium influx in neurons, resulting in apoptotic cell death (Matus, Burgos et al. 2007).

NMDAR is a glutamate receptor critical in synaptic plasticity, a cellular mechanism for learning and memory function. NMDA binds specifically to the NMDAR, opening the ion channel controlling calcium flux through NMDARs, which is thought to be critical to synaptic plasticity. Antibodies against the NR2A and NR2B subunits of the NMDAR act as agonists of the NMDAR resulting in NP SLE symptoms including cognitive deficits, psychosis, memory deficits, mood disorders, and seizures.(Moscato, Jain et al. 2010). A subset of anti-DNA antibodies crossreact with the NR2A subunit of the NMDAR in SLE patients, which mediates apoptotic death of neurons in vivo and in vitro (DeGiorgio, Konstantinov et al. 2001, Kowal, Degiorgio et al. 2006). Anti-NMDAR, as well as anti-DNA antibodies that cross react with the NR2 subunit of NMDAR have been associated

with cognitive dysfunction and depression in SLE (DeGiorgio, Konstantinov et al. 2001, Kowal, Degiorgio et al. 2006, Lapteva, Nowak et al. 2006).

Antibodies against the NR1 subunit of the NMDA and GluR1／2 subunit of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, another glutamate receptor, cause other immune-mediated disorders of the CNS. In Anti-NMDARencephalitis and Anti-AMPA receptor encephalitis, autoantibodies cause decreased synaptic NMDA or AMPA receptor levels, reducing their functional capacity (DeGiorgio, Konstantinov et al. 2001, Kowal, Degiorgio et al. 2006, Dalmau, Gleichman et al. 2008, Lai, Hughes et al. 2009, Hughes, Peng et al. 2010, Kivity, Katzav et al. 2013). In mice anti-N2 antibodies cause hippocampus-related memory impairment. Additionally, an anti-Id targeting anti-dsDNA, anti-16/16 Id, can cross react with brain tissue and cause NP SLE symptoms in SLE (Kivity, Katzav et al. 2013). Other antibodies identified in NP SLE patients or associated with NP SLE manifestations include, but are not limited to, anti-phospholipid antibodies, anti-tubulin antibodies, and anti-histone antibodies (Mak, Ho et al. 2009).

Proteomic Array as a Tool for Identifying Novel Biomarkers and Autoantibodies in SLE Patients

Previous autoantibody arrays produced by the UTSW Microarray core and used in our lab contained 70 autoantigens to screen for autoantibodies. The Protoarray (LifeTechnologies) is a commercially available proteomic array that includes over 9,000 non-redundant human recombinant proteins. Using the Protoarray, my aim was to identify a candidate biomarker or panel of potential biomarkers for applications in disease diagnosis, stage, progression, or response to therapy. The protein array identified several novel autoantibodies elevated in SLE patient serum, including autoantibodies against the autoantigen, Stress Induced Phosphoprotein 1(STIP1), which is known to have neuroprotective functions in the CNS.

Stress Induced Phosphoprotein 1

STIP1, also referred to as STI1 or heat shock protein (HSP) 70/90 organizing protein (HOP), is a 62.6 kDa protein involved in holding HSP70 and HSP90 together in the HSP90 chaperone machinery (Odunuga, Longshaw et al. 2004). STIP1 is a ubiquitously expressed cytoplasmic protein that can be detected in many tissues. STIP1 is phosphorylated by the cell cycle kinases, casein kinase II (CKII) at S189 and cdc2-

kinase at T198 (Odunuga, Longshaw et al. 2004). Additionally, calcium/calmodulin-dependent protein kinase II (CaMKII) delta phosphorylates STIP1 on Ser189 (Masaoka, Nishi et al. 2008). Phosphorylation of STIP1 by cdc2-kinase could inhibit Hop-Hsp90 interactions (Odunuga, Longshaw et al. 2004) and phosphorylation of STIP1 promotes nuclear localization of STIP1, suggesting that calcium signals via CaMKII delta may regulate subcellular localization of STIP1 (Masaoka, Nishi et al. 2008). These results suggest that the subcellular localization of STIP1 is dependent on stress levels of the cell, particularly heat stress, and controlled by cell cycle arrest. They also suggest that calcium signals via CaMKII delta may regulate subcellular localization of STIP1 (Odunuga, Longshaw et al. 2004, Masaoka, Nishi et al. 2008). Nuclear translocation of STIP1 occurs under heat shock, and its proposed nuclear localization signal is involved in Hsp90 binding (Odunuga, Longshaw et al. 2004)

Autoantibodies against HSP90 have been identified in SLE patients and murine models of lupus, suggesting a potential link between autoantibodies produced against HSPs and STIP1 in SLE patients (Minota, Koyasu et al. 1988, Panchapakesan, Daglis et al. 1992, Stephanou, Latchman et al. 1998). In addition, high-titer autoantibodies against STIP1 have been described in patients with Neuro-Behcet's Disease (Vural, Ugurel et al. 2011). The pathogenic potential for anti-STIP1 autoantibodies in Neuro-Behcet's

Disease has not been investigated, however it is intriguing that anti-STIP1 antibodies are present in these patients and could be contributing to their neurological complications. STIP1 can be detected in the serum of healthy individuals and has been found to be secreted by astrocytes, microglial cells, and by various cancer cells resulting in elevated levels of serum STIP1 protein in patients with ovarian cancer (Lima, Arantes et al. 2007, Wang, Chao et al. 2010, Fonseca, Romao et al. 2012). This suggests that STIP1 could be secreted under certain conditions and serve as an autoantigen for autoreactive cells. Finally, STIP1 can function as a ligand for cellular prion protein (PrPc), and the interaction of STIP1 and PrPc induced neuroprotective signals that rescue cells from apoptosis (Zanata, Lopes et al. 2002).

The interaction of STIP1 with PrPc promotes neuritogenesis and neuroprotection in wildtype neurons but not in PrPc-null cells. This effect was abolished by antibodies against either PrPc or STIP1 and was dependent on the STIP1 domain that binds PrPc (Lopes, Hajj et al. 2005). Additionally, the PrPc -STIP1 interaction enhances astrocyte development by promoting survival, regulating proliferation, and enhancing differentiation of astrocytes (Hartmann, Martins et al. 2013). The PrPc-STIP1 interaction was also found to enhance memory consolidation and inhibition of this interaction impaired both short- and long-term memory formation (Coitinho, Lopes et al. 2007).

Furthermore, STIP1 and PrPc are upregulated in the brain in models of ischemic stroke and PrPc-STIP1 signaling facilitated stroke recovery in part by recruiting bone marrow derived cells to the ischemic brain and promoting their proliferation (Lee, Lai et al. 2013). Finally, a fundamental feature of Alzheimer's disease is the overproduction and accumulation of Amyloid β (Aβ) in the brain. Aβ Oligomer induced cell death was found to be limited by the Prpc-STIP1 interaction (Ostapchenko, Beraldo et al. 2013). Prpc inhibits NMDAR, and Aβ toxicity depends on the interactions between Prpc and NMDAR (Khosravani, Zhang et al. 2008, You, Tsutsui et al. 2012). Collectively, these studies revealed the neuroprotective effect of the Prpc-STIP1 interaction.

PrPc has functions outside of the CNS, and has also been implicated in regulation of immune responses (Hu, Kieseier et al. 2008). PrPc is constitutively expressed on T cells and APCs (Durig, Giese et al. 2000). PrPc is responsible for macrophage phagocytosis, and in its absence, macrophage phagocytosis was decreased (de Almeida, Chiarini et al. 2005). PrPc is a negative regulator of T cell activation (Hu, Nessler et al. 2010). It was found to interact with ZAP-70, and has been identified as a component of the multimolecular signaling complex involved in T cell activation and is upregulated on the surface of activated T cells (Mabbott, Brown et al. 1997, Mattei, Garofalo et al. 2004). PrPc has also been found to play a role in experimental autoimmune encephalitis

(EAE). Clinical and histological manifestations of EAE worsen with loss of PrPc or with decreased PrPc (Tsutsui, Hahn et al. 2008, Ingram, Isaacs et al. 2009, Hu, Nessler et al. 2010). Overexpression of PrPc reduced EAE severity, while siRNA of PrPc increased T cell activation, Th1 and Th17 differentiation, and EAE severity (Hu, Nessler et al. 2010). These studies clearly define a role for PrPc not only the CNS, but in immune responses, and particularly autoimmunity.

Summary

With the use of a protein array, I will identify multiple autoantibodies and autoantigens that are elevated in SLE patients compared to HCs. In addition to the identification of specific autoantibodies, further analysis of the targeted antigens can reveal pathways that may be involved in SLE pathogenesis. Many antibodies against CNS antigens were observed to be elevated in the serum of SLE patients. Therefore I followed up with validating the elevation of these autoantibodies in the serum of SLE patients as well as NP SLE patients. Due to the previous implications of STIP1 in Neuro Behçet's disease, its interaction with the HSPs which serve as an autoantigen in SLE, and its interaction with the PrPc and possible role in the immune system, I focused my studies on determining what might cause the increase in STIP1 autoantibodies in SLE patients and whether they had a potential pathogenic role in SLE.

RESULTS

Protein array identified multiple autoantibodies elevated in SLE patient serum

The Protoarray (LifeTechnologies) is a proteomic array that includes over 9,000 non-redundant human recombinant proteins. The protein array identified 1218 IgM autoantibodies to be significantly elevated in SLE patients over HCs (Figure 2). Out of 449 IgG autoantibodies identified to be significantly different between SLE and HC, 446 were elevated and 3 were decreased in SLE patients compared to HCs (Figure 3.A). STIP1 was one autoantigen that was targeted by autoantibodies in a subset of SLE patients. While the Protoarray did not identify anti-STIP1 autoantibodies to be significantly different between HC and SLE serum, the fold increase between the mean of SLE patients to HC was 49, and the elevation of anti-STIP1 autoantibodies in a subset of patients suggested that it could be associated with specific disease manifestations (Figure 3.B).

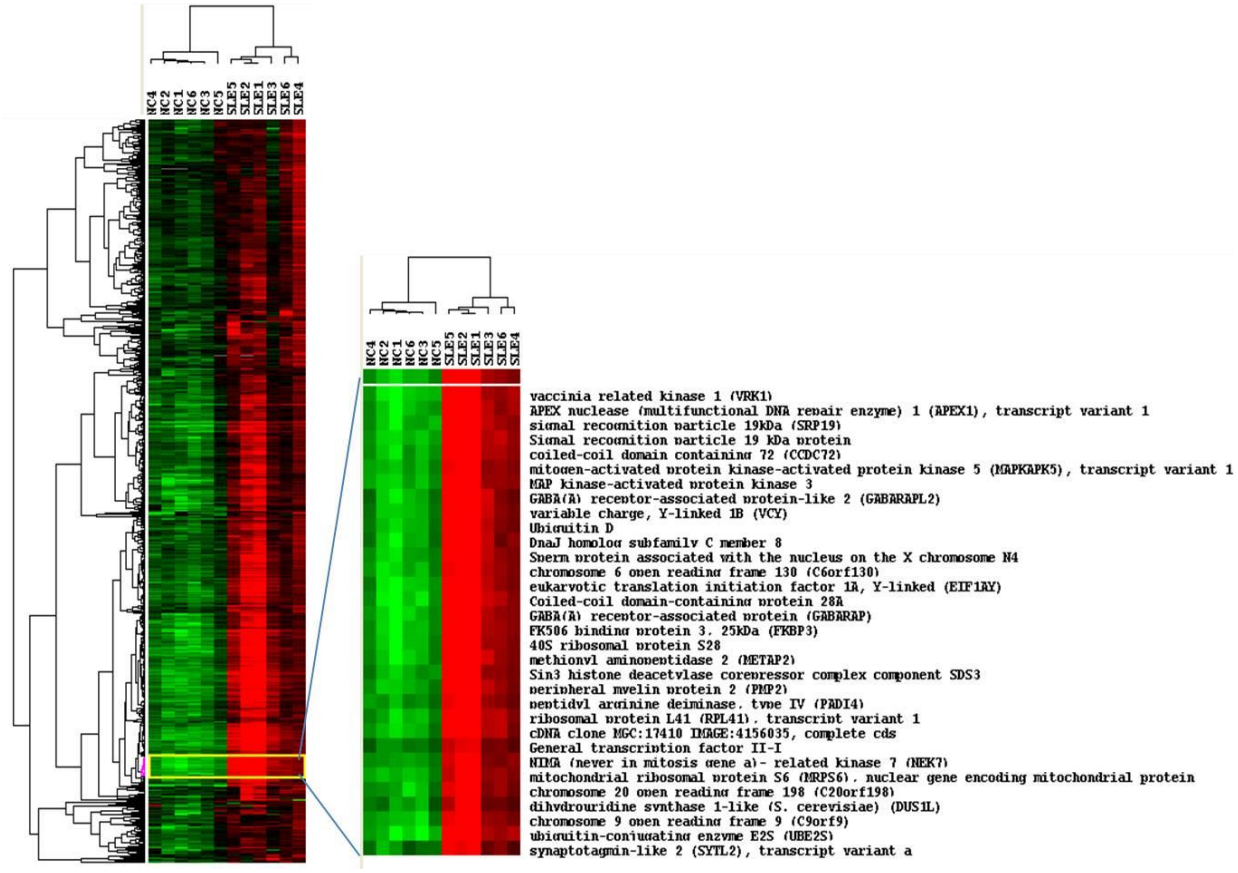
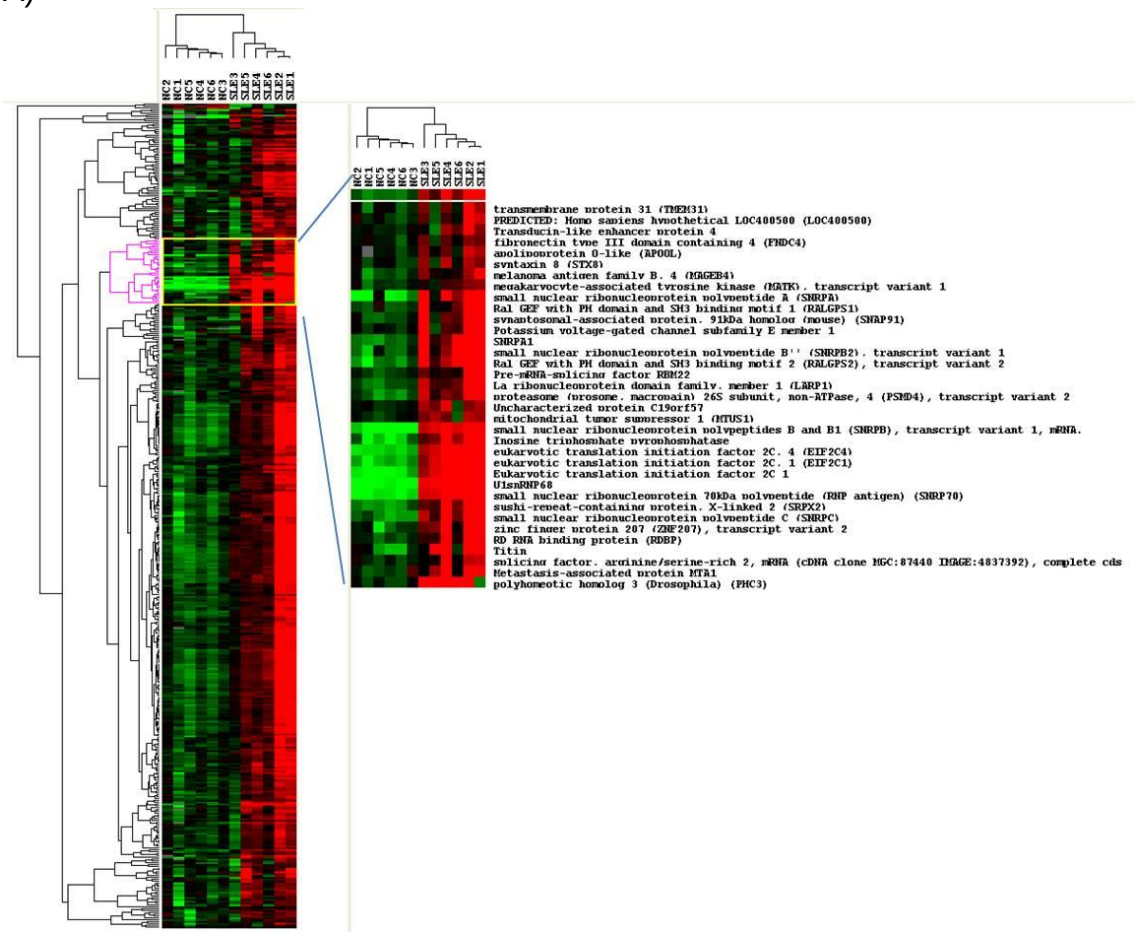


Figure 2. Protein array revealed IgM autoantibodies that were elevated in human SLE. The Protoarray identified 1218 IgM autoantibodies to be significantly different between SLE (n=6) and Healthy Control (NC, n=6) serum, 1218 up in SLE. Green, black, and red represent net fluorescent intensities below, close to, and above the mean, respectively. Significance was determined by a student's t-test.

A)


HC2
HC1
HC5
HC4
HC6
HC3
SLE3
SLE5
SLE4
SLE6
SLE2
SLE1
transmembrane protein 31 (TMEM31)
PREDICTED: Homo sapiens hypothetical LOC400500 (LOC400500)
Transducin-like enhancer protein 4
fibronectin type III domain containing 4 (FNDC4)
apolipoprotein O-like (APOOL)
syntaxin 8 (STX8)
melanoma antigen family B, 4 (MAGEB4)
megakaryocyte-associated tyrosine kinase (MATK), transcript variant 1
small nuclear ribonucleoprotein polypeptide A (SNRPA)
Ral GEF with PH domain and SH3 binding motif 1 (RALGPS1)
synaptosomal-associated protein, 91kDa homolog (mouse) (SNAP91)
Potassium voltage-gated channel subfamily E member 1
SNRPA1
small nuclear ribonucleoprotein polypeptide B'' (SNRPB2), transcript variant 1
Ral GEF with PH domain and SH3 binding motif 2 (RALGPS2), transcript variant 2
Pre-mRNA-splicing factor RBM22
La ribonucleoprotein domain family, member 1 (LARP1)
proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (PSMD4), transcript variant 2
Uncharacterized protein C19orf57
mitochondrial tumor suppressor 1 (MTUS1)
small nuclear ribonucleoprotein polypeptides B and B1 (SNRPB), transcript variant 1, mRNA.
Inosine triphosphate pyrophosphatase
eukaryotic translation initiation factor 2C, 4 (EIF2C4)
eukaryotic translation initiation factor 2C, 1 (EIF2C1)
Eukaryotic translation initiation factor 2C 1
U1snRNP68
small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen) (SNRP70)
sushi-repeat-containing protein, X-linked 2 (SRPX2)
small nuclear ribonucleoprotein polypeptide C (SNRPC)
zinc finger protein 207 (ZNF207), transcript variant 2
RD RNA binding protein (RDBP)
Titin
splicing factor, arginine/serine-rich 2, mRNA (cDNA clone MGC:87440 IMAGE:4837392), complete cds
Metastasis-associated protein MTA1
polyhomeotic homolog 3 (Drosophila) (PHC3)

B)

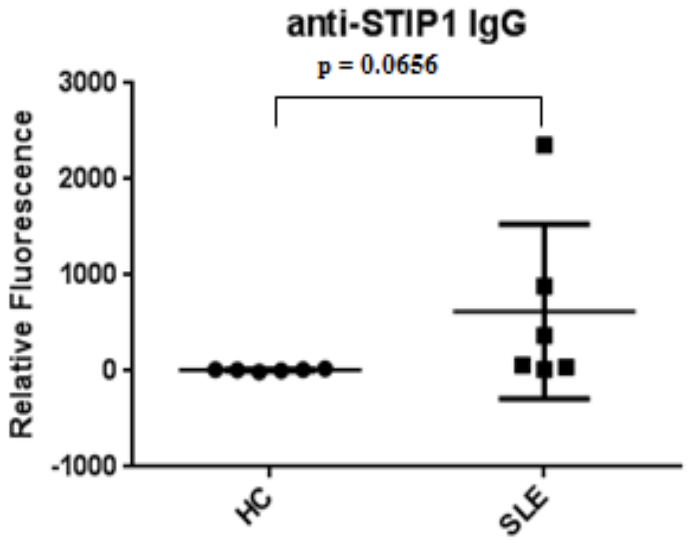


Figure 3. Protein array revealed IgG autoantibodies that were elevated in human SLE. A) The Protoarray identified 449 IgG autoantibodies were significantly different between SLE (n=6) and Healthy Control (NC, n=6) serum. Green, black, and red represent net fluorescent intensities below, close to, and above the mean, respectively. B) Quantification of protoarray data for autoantibodies against STIP1. Significance was determined by a student's t-test

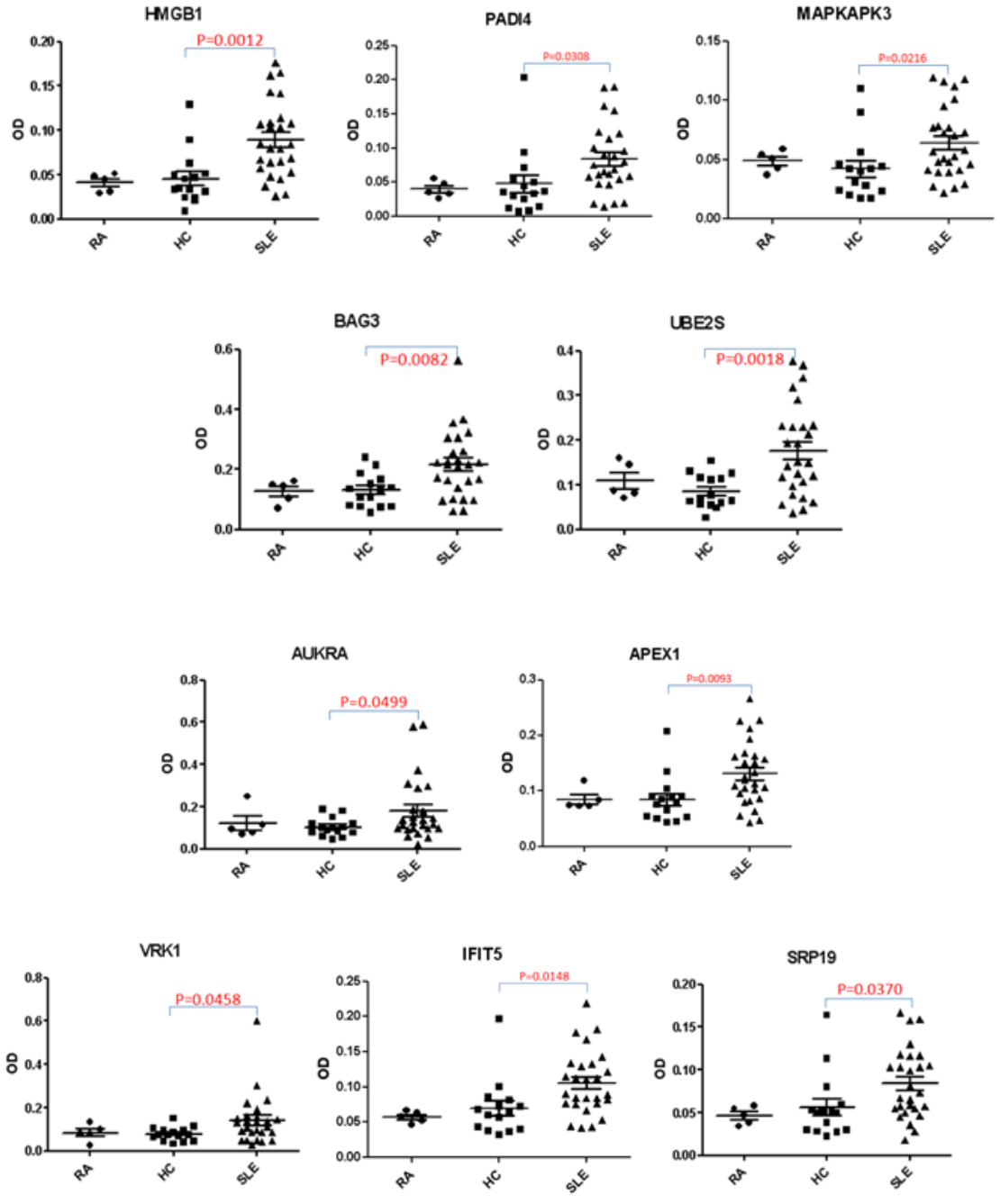
Elevated anti-STIP1 autoantibodies in SLE patients

Validation of autoantibodies found to be elevated by the protein array

To validate the findings from the protein array, several autoantibodies that were elevated in the SLE patients serum were tested in a larger cohort by ELISA. Of 16 autoantigens tested, 10 were validated to be significantly elevated in SLE serum compared to HC (Figure 4). Additionally, serum samples from Rheumatoid arthritis (RA) patients were tested to confirm specificity in SLE. Serum autoantibody levels in RA serum were comparable to HCs confirming specificty of elevated autoantibodies in SLE patient serum (Figure 4).

Elevated anti-STIP1 IgM and IgG in a larger cohort of SLE patients

To determine whether anti-STIP1IgM and IgG autoantibodies are significantly elevated in the serum of SLE patients, a larger cohort of HC and SLE serum were analyzed by ELISA. Anti-STIP1 IgM and IgG were significantly elevated in the serum of SLE patients compared to HC (Figure 5). To determine whether anti-STIP1 autoantibodies were specific for NP SLE patients, anti-STIP1 IgM and anti-STIP1 IgG was measured by ELISA in samples from patients with a known diagnosis of NP-SLE (red dots) (Figure 5). Anti-STIP1 IgM and IgG autoantibodies are not specific for NP SLE serum, and there are some NP SLE patients without elevated anti-STIP1 antibodies (Figure 5).


HMGB1
P=0.0012
PADI4
P=0.0308
MAPKAPK3
P=0.0216
BAG3
P=0.0082
UBE2S
P=0.0018
AUKRA
P=0.0499
APEX1
P=0.0093
VRK1
P=0.0458
IFIT5
P=0.0148
SRP19
P=0.0370
OD
RA
HC
SLE

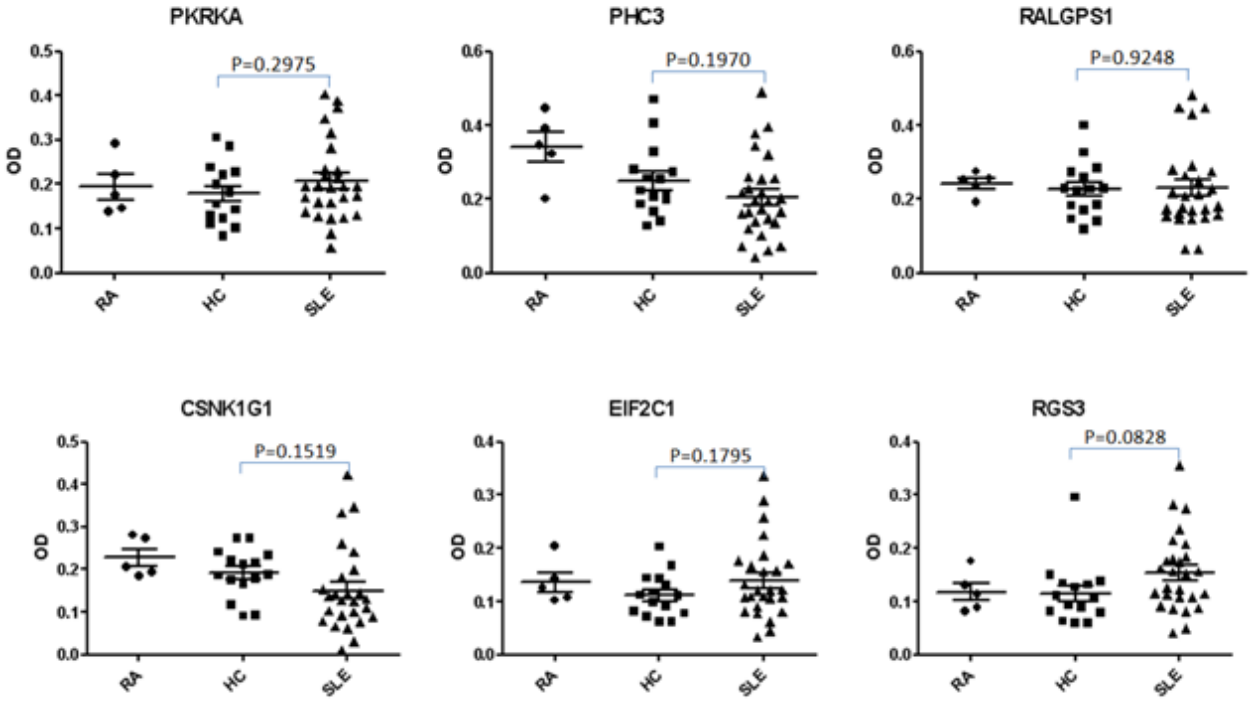
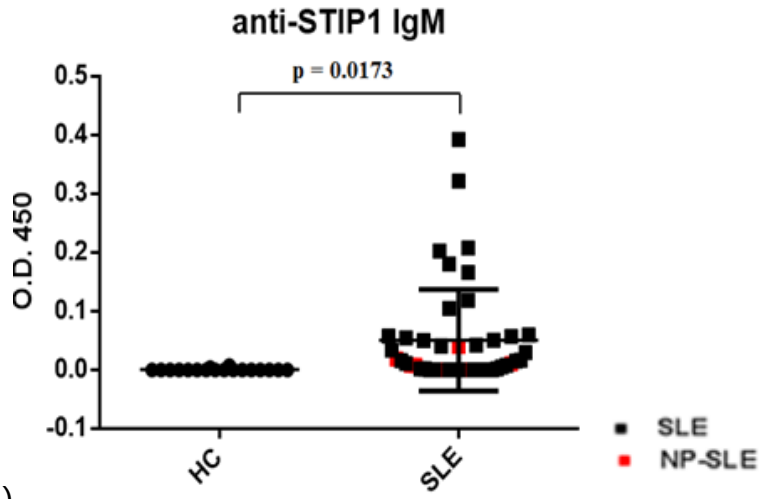


Figure 4. ELISA validation of autoantibodies identified by protein array to be elevated in SLE serum. Serum (1:100) from Rheumatoid Arthritis (RA, n=5), Healthy Control (HC, n=15), and Systemic Lupus Erythematosus (SLE, n=26) was analyzed by ELISA for IgG autoantibodies against the following indicated autoantigens.

A)



B)

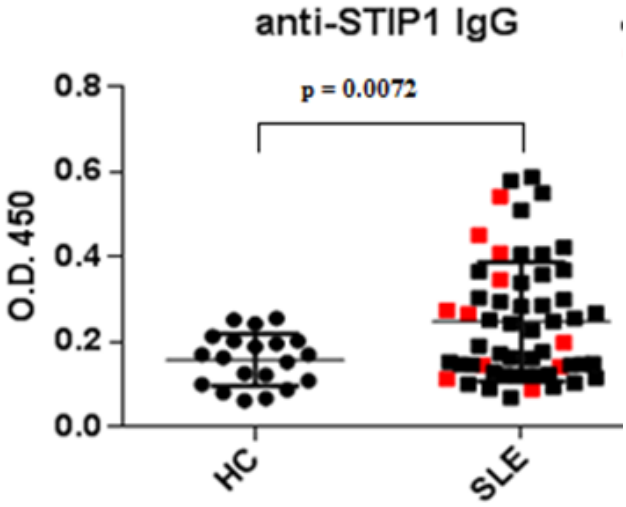
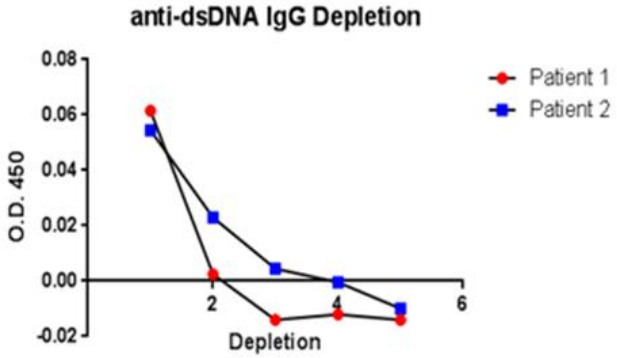


Figure 5. Anti-STIP1 IgM and IgG autoantibodies are elevated in SLE serum. Serum (1:100) was analyzed by ELISA for anti-STIP1 A) IgM in Healthy Control (HC, n=18), Systemic Lupus Erythematosus (SLE, n=36), and red dots indicate Neuropsychiatric SLE (NP SLE, n=10) and B) IgG in Healthy Control (HC, n=20), Systemic Lupus Erythematosus (SLE, n=45), and red dots indicate Neuropsychiatric SLE (NP SLE, n=10).

Anti-dsDNA antibodies do not react with STIP1

One hallmark feature of SLE patients is elevated anti-dsDNA antibodies. Anti-dsDNA antibodies crossreact with many other self antigens and can cause tissue damage by binding to the crossreacting, non-DNA target antigen. For example, a subset of anti-DNA autoantibodies that crossreact with the NR2 subunit of NMDAR in SLE. (DeGiorgio, Konstantinov et al. 2001). To confirm that the anti-STIP1 autoantibodies were indeed anti-STIP1 IgG autoantibodies and not anti-dsDNA antibodies crossreacting with STIP1, I designed an anti-dsDNA depletion ELISA assay. After measuring the levels of anti-STIP1 IgG from HC and SLE samples, the serum was serially depleted of anti-dsDNA IgG antibodies by incubating the serum in wells coated with dsDNA until there was no measurable anti-dDNA IgG. Anti-STIP1 IgG levels were then measured again to confirm that anti-STIP1 IgG reactivity was still present. Anti-STIP1 IgG was still present after the anti-dsDNA antibodies had been depleted, proving that the validated levels of anti-STIP1 IgG were not anti-dsDNA antibodies crosssreacting with the STIP1 protein (Figure 6).

A)



B)

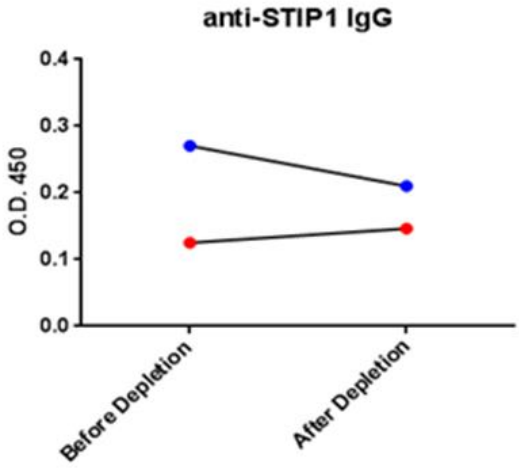


Figure 6. Anti-dsDNA autoantibodies do not crossreact with STIP1. An anti-dsDNA depletion assay was performed on SLE (n=2) sera (1:100) by ELISA. A) Sera was serially incubated for 1 hour at room temperature in wells coated with dsDNA until no more anti-dsDNA autoantibodies could be detected, for a total of 5 incubations. B) Anti-STIP1 IgG reactivity was measured before and after patient serum was depleted of anti-dsDNA antibodies.

STIP1 antigen levels in the serum and PBMCs are normal in SLE patients

STIP1 mRNA levels are not elevated in SLE PBMCs

To determine whether the increase in autoantibody production against STIP1 was due to an overexpression of STIP1 by PBMCs, STIP1 mRNA levels were measured from the PBMCs of HC and SLE patients. No significant difference in PBMC STIP1 mRNA levels was observed between HCs and SLE patients (Figure 7), although this does not rule out a change in STIP1 expression in other tissues.

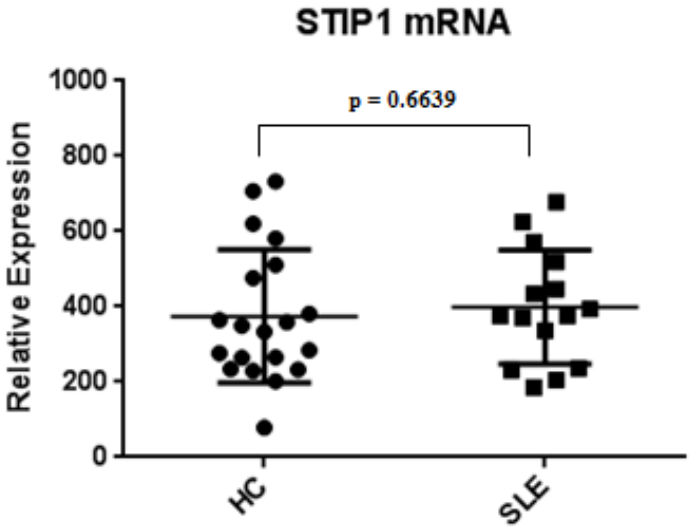


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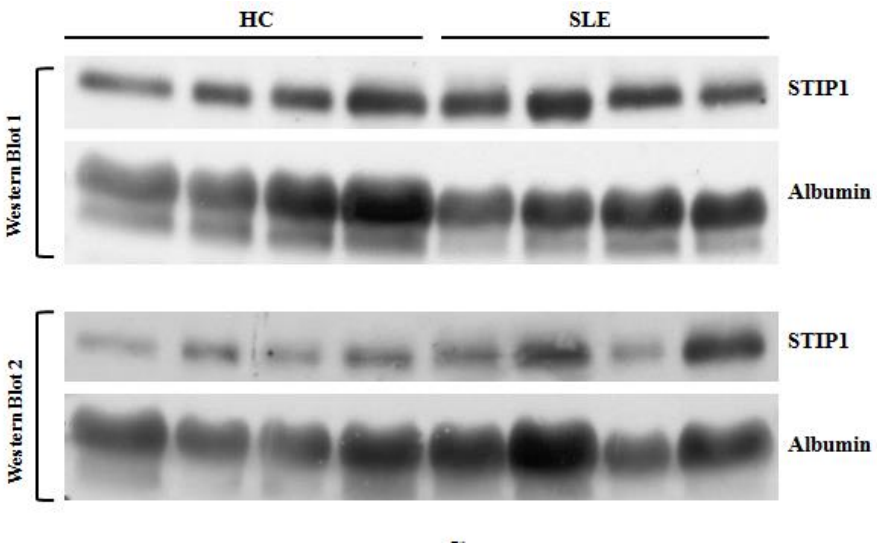
Figure 7. STIP1 mRNA levels are not elevated in SLE PBMCs. Expression of STIP1 in healthy controls (n = 20) and SLE (n = 15) PBMCs was determined by microarray analysis (Affymetrix array HG-U133A as described in Becker, Dao et al. 2013). Healthy Controls (HC) and Systemic Lupus Erythematosus (SLE). Student's t-test was two sided.

STIP1 protein levels are not elevated in SLE serum

Another possible mechanism by which B cells could be overexposed to STIP1, resulting in the overproduction of anti-STIP1 antibodies, would be if STIP1 levels were elevated in the serum. This could occur by increased secretion of STIP1 into the serum as is seen in the case of ovarian cancer, or if the intracellular protein is overabundant in the serum due to the loss of clearance of apoptotic debris by macrophages.

STIP1 serum protein levels from SLE patients and HCs were measured by Western blot and normalized to albumin levels. There was no significant difference in serum protein level of STIP1 between HCs and SLE patients (Figure 8.A & B). Decreased serum albumin levels have been reported in SLE patients and are a marker for disease severity (Yip, Aghdassi et al. 2010). However no significant difference in serum STIP1 protein levels was observed between HCs and SLE without normalization to albumin levels (Figure 8.C). Furthermore, serum STIP1 protein levels did not correlate with anti-STIP1 IgM or IgG levels (Figure 9.A & B).

A)



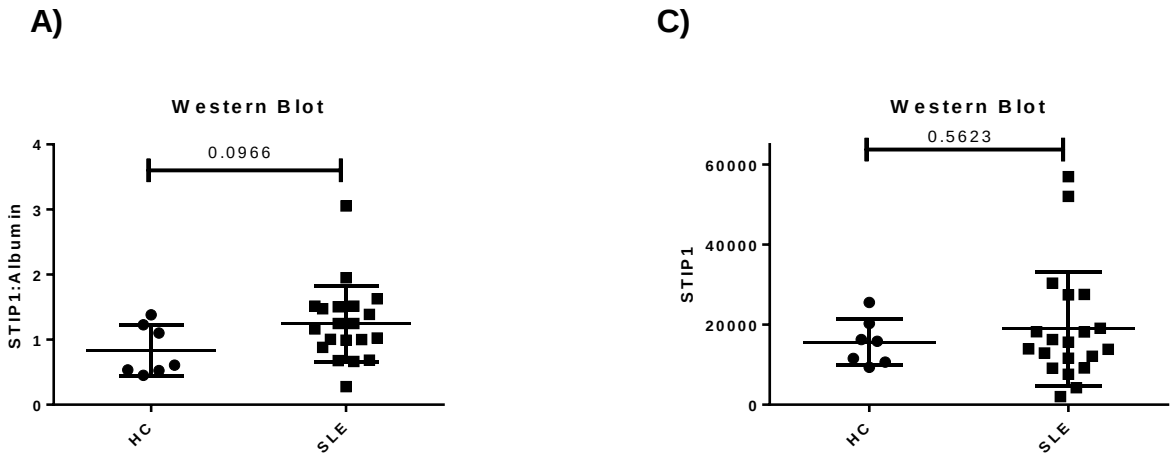
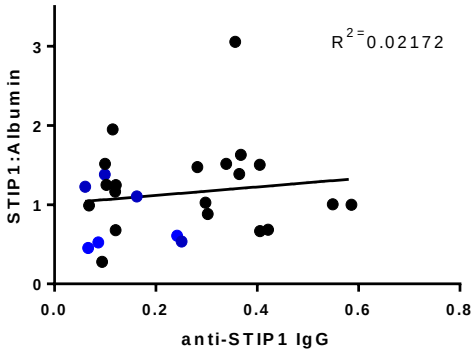


Figure 8. Serum STIP1 levels are not elevated in SLE patient serum. A) STIP1 levels were determined in serum from HC (n = 8) and SLE patients (n = 8) for two separate Western blots using the same conditions. B) Western blot data were quantified using ImageJ software and STIP1 levels were normalized to the loading control (albumin) levels. C) Quantification of Western blot data for STIP1 protein levels (not normalized to albumin). Student's t-test was two tailed.

A)



B)

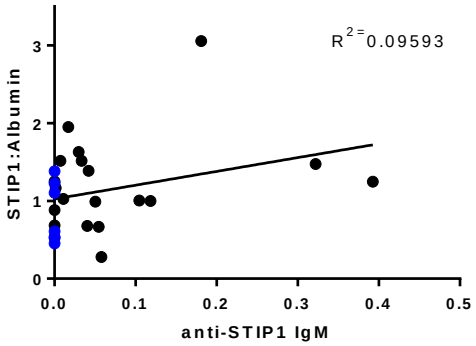


Figure 9. Serum STIP1 levels do not correlate with anti-STIP1 IgG autoantibodies. A) Linear regression of STIP1 serum levels to anti-STIP1 IgG levels. B) Linear regression of STIP1 serum levels to anti-STIP1 IgM levels. Blue dots represent HCs. R^2 was determined by linear regression.

Patients with high anti-STIP1 IgG levels are less likely to have high parameters of disease activity

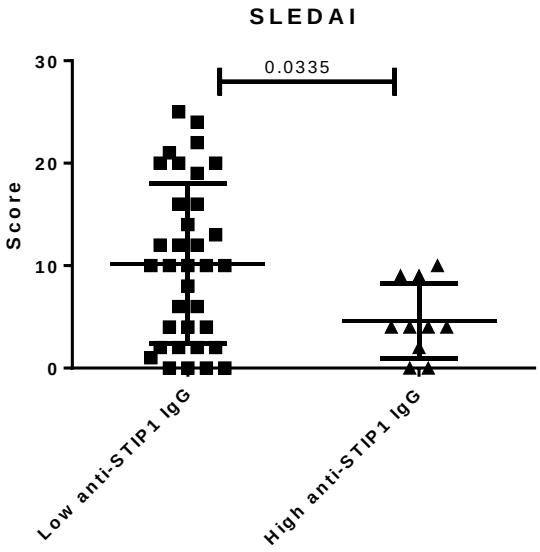
Interestingly, the frequency of patients with elevated serum anti-STIP1 (defined as >= mean of HC + 3 SD) for the IgM isotype (23/36 = 64%) (Low anti-STIP1 IgM n=13, High anti-STIP1 IgM n=23) was much higher than for the IgG isotype (10/45 = 22%) (Low anti-STIP1 IgG n=35, High anti-STIP1 IgG n=10). A relatively high frequency of patients with anti-STIP1 IgM may indicate that loss of tolerance to STIP1 is a common occurrence in SLE, and a low frequency of patients with high anti-STIP1 IgG suggests that class switching to IgG occurs only in a subset of patients, which may be associated with disease severity or a specific disease manifestation.

SLE patients with higher levels of serum anti-STIP1 IgG, but not IgM, had significantly lower SLEDAI (Figure 10.A & B) and Renal SLEDAI (Figure 10.E & F) scores, decreased anti-dsDNA IgG levels (Figure 10.K & L), and higher age (Figure 10.Q & R) compared to SLE patients with lower serum anti-STIP1 IgG levels. Additionally, chi square analysis revealed that SLE patients with elevated anti-STIP1 IgG, but not IgM, were less likely to have high SLEDAI scores (Figure 10.C & D), high Renal SLEDAI scores (Figure 10.G & H), and proliferative kidney disease (Figure 10.I & J) compared to

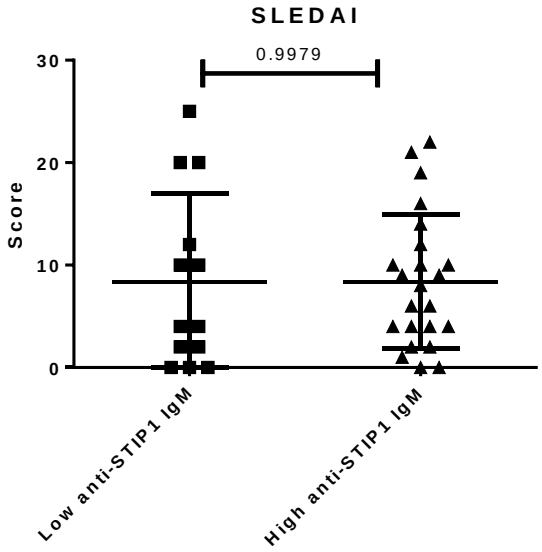
SLE patients with lower levels of anti-STIP1 IgG. Increased SLEDAI, Renal SLEDAI, proliferative kidney disease, and anti-dsDNA IgG are markers for increased disease severity; therefore SLE patients with increased levels of anti-STIP1 IgG are less likely to have high disease activity.

SLE patients with higher levels of serum anti-STIP1 IgM, but not IgG, had significantly lower levels of serum C4 (Figure 10.M & N). Chi square analysis revealed that SLE patients with high anti-STIP1 IgM are less likely to have high levels of serum C4 (Figure 10.O & P). Low levels of C4 correlate with increased disease severity, therefore high anti-STIP1 IgM may correlate with flares in patients with active and ongoing inflammation. No difference was observed between patients with low and high anti-STIP1 IgM and IgG for the following parameters: Serum creatinine, BUN, HCO_3, WBC, Platelets, C3, erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), Urine protein (UProtein), UProtein/UCr, gender, and ethnicity (Appendix A).

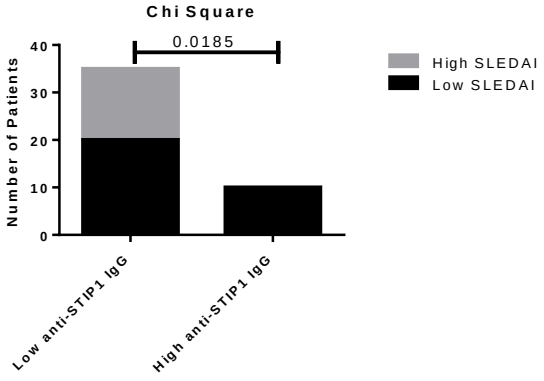
A)


SLEDAI
0.0335
Score
30
20
10
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

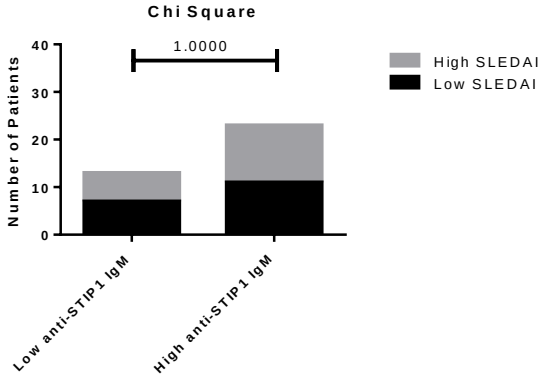
B)


SLEDAI
0.9979
Score
30
20
10
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

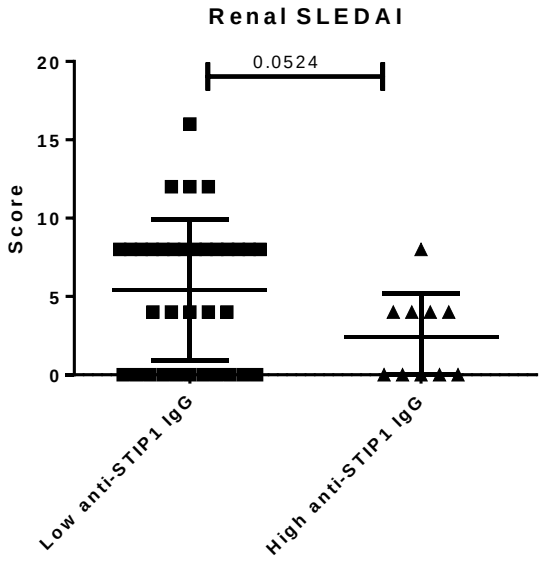
B)


Chi Square
0.0185
Number of Patients
40
30
20
10
0
High SLEDAI
Low SLEDAI
Low anti-STIP1 IgG
High anti-STIP1 IgG

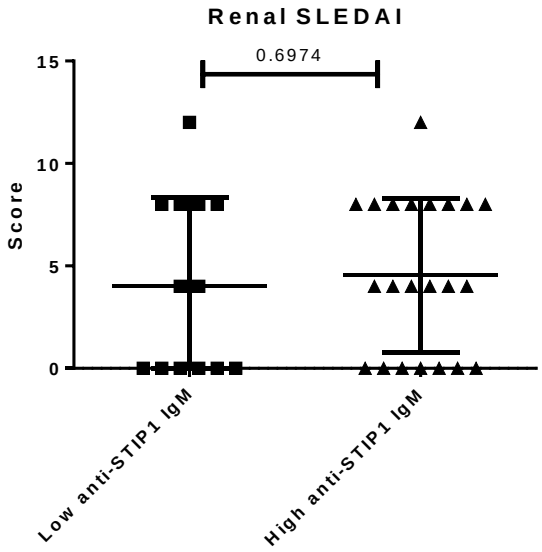
D)


Chi Square
1.0000
Number of Patients
40
30
20
10
0
High SLEDAI
Low SLEDAI
Low anti-STIP1 IgM
High anti-STIP1 IgM

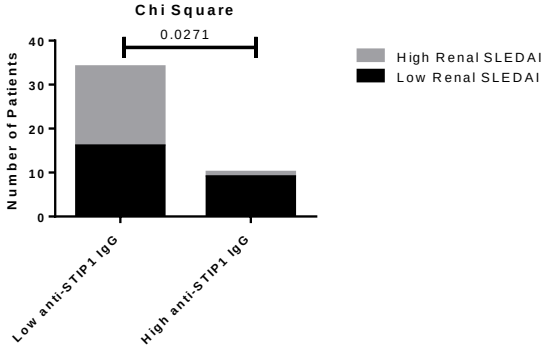
E)


Renal SLEDAI
0.0524
Score
20
15
10
5
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

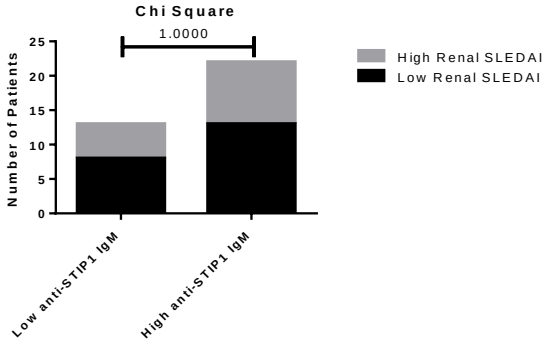
F)


Renal SLEDAI
0.6974
Score
15
10
5
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

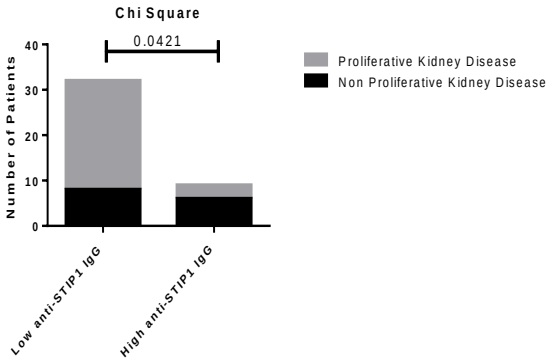
G)


Chi Square
0.0271
Number of Patients
40
30
20
10
0
High Renal SLEDAI
Low Renal SLEDAI
Low anti-STIP1 IgG
High anti-STIP1 IgG

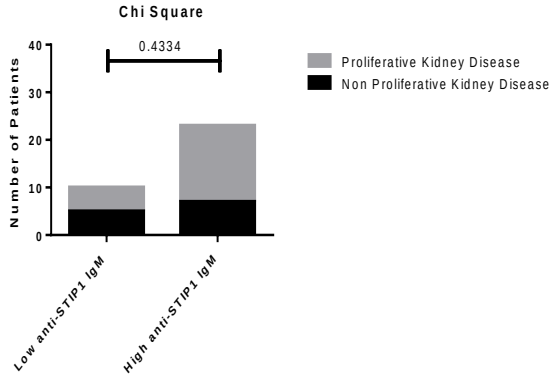
H)


Chi Square
1.0000
Number of Patients
25
20
15
10
5
0
High Renal SLEDAI
Low Renal SLEDAI
Low anti-STIP1 IgM
High anti-STIP1 IgM

I)


Chi Square
0.0421
Number of Patients
40
30
20
10
0
Proliferative Kidney Disease
Non Proliferative Kidney Disease
Low anti-STIP1 IgG
High anti-STIP1 IgG

J)


Chi Square
0.4334
Number of Patients
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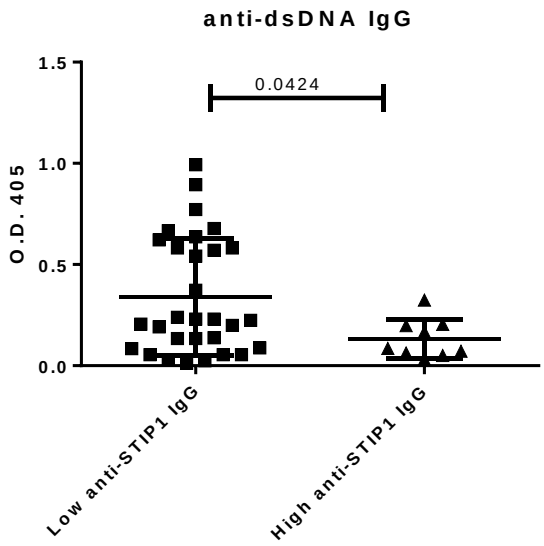
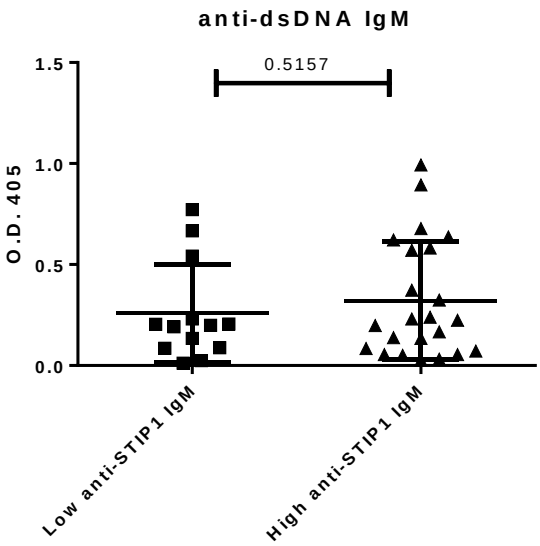
30
20
10
0
Proliferative Kidney Disease
Non Proliferative Kidney Disease
Low anti-STIP1 IgM
High anti-STIP1 IgM

K)


anti-dsDNA IgG
0.0424
O.D. 405
1.5
1.0
0.5
0.0
Low anti-STIP1 IgG
High anti-STIP1 IgG

L)


anti-dsDNA IgM
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O.D. 405
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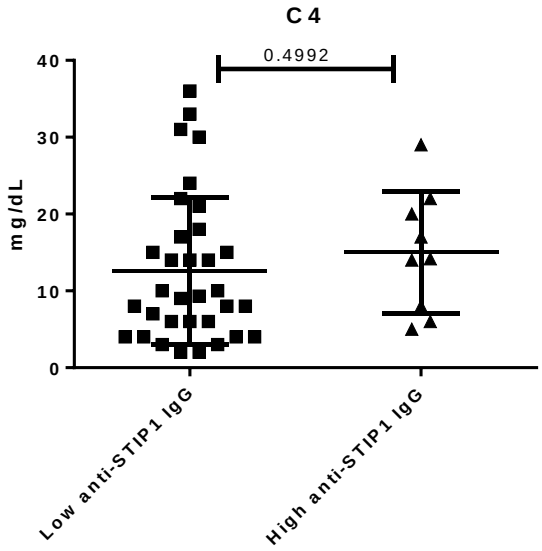
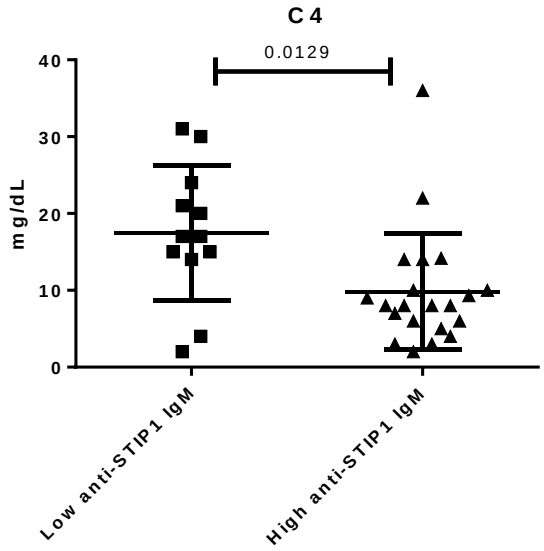
1.0
0.5
0.0
Low anti-STIP1 IgM
High anti-STIP1 IgM

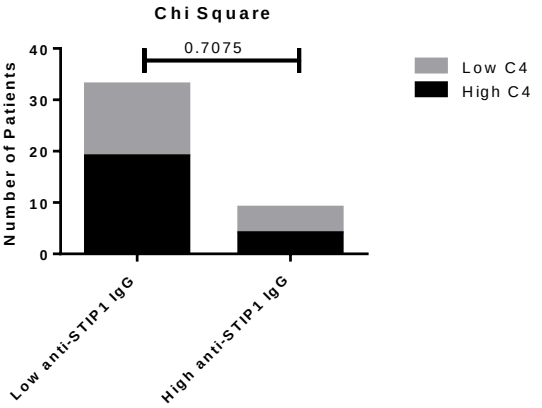
M)


C4
0.4992
mg/dL
40
30
20
10
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

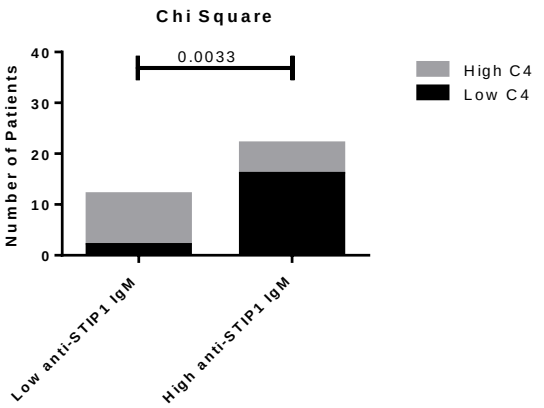
N)


C4
0.0129
mg/dL
40
30
20
10
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

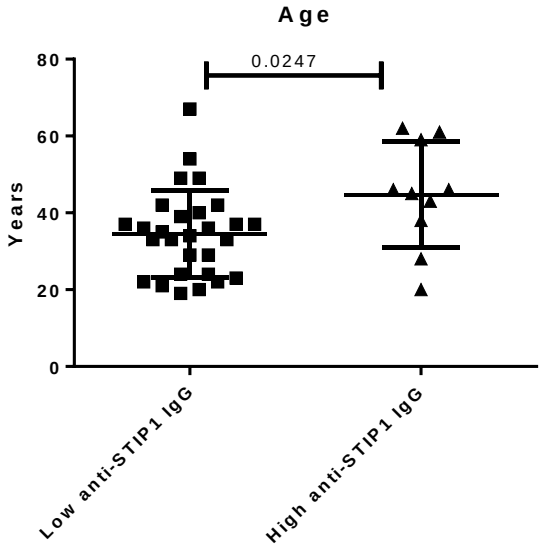
O)


Chi Square
0.7075
Number of Patients
40
30
20
10
0
Low C4
High C4
Low anti-STIP1 IgG
High anti-STIP1 IgG

P)


Chi Square
0.0033
Number of Patients
40
30
20
10
0
High C4
Low C4
Low anti-STIP1 IgM
High anti-STIP1 IgM

Q)



R)

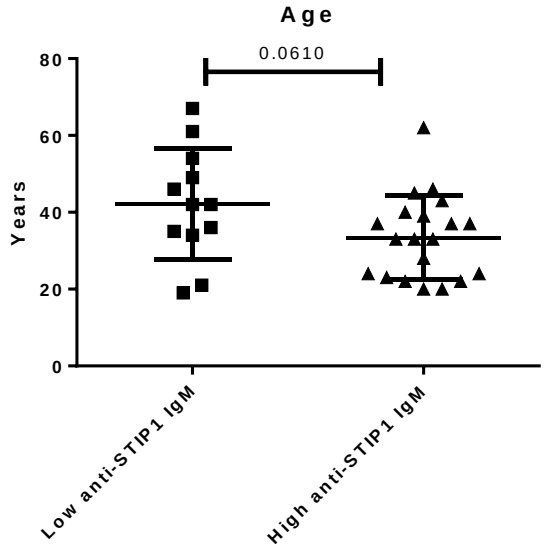


Figure 10. SLE patients with elevated anti-STIP1 IgG levels are less likely to have increased disease activity. Low (less than the mean of HC + 3 SD) and high (greater than mean of HC + 3SD) serum anti-STIP1 IgG and IgM SLE patients were grouped and compared for A), B), C), & D) SLEDAI, E), F), G), & H) Renal SLEDAI, I) & J) Kidney Disease Class (NonProliferative = I,II, & V and Proliferative = III & IV) K) & L) serum anti-dsDNA IgG levels, M), N), O), & P) serum C4 levels, and Q) & R) Age.

Anti-STIP1 IgG autoantibodies detected in the MRL-lpr murine model of lupus

To determine whether murine models of lupus can be used to study the importance of anti-STIP1 autoantibodies in SLE, anti-STIP1 IgG levels were measured in several strains of lupus-prone mice including BWF1, SLE123, SLE13, Lyn-/-, and MRL-lpr and were compared to the wild-type mouse, B6. Since many of the murine models of lupus have different disease mechanisms as described above, several strains were analyzed to determine if anti-STIP1 autoantibodies are general to autoimmunity and SLE or would be observed in a strain specific manner. Anti-STIP1 IgG autoantibodies were detected in the murine lupus strain MRL-lpr, but not in the other lupus mouse models (Figure 11).

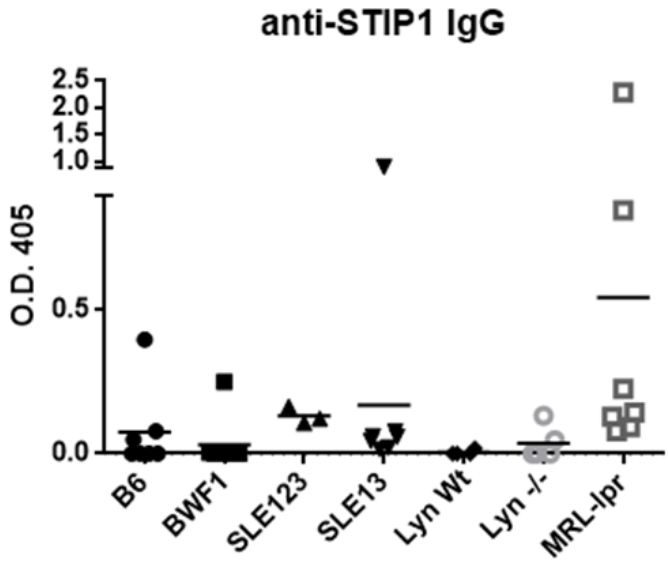


Figure 11. Anti-STIP1 IgG autoantibodies are detected in a subset of murine models of lupus. Levels of anti-STIP1 autoantibodies were detected by ELISA using serum (1:100) from B6 (n = 7), BWF1 (n = 8), SLE123 (n = 3), SLE13 (n = 7), Lyn+/+ (n = 4), Lyn-/- (n = 5), and MRL-lpr (n = 7) mice.

DISCUSSION

The protein array was able to identify many novel IgM and IgG autoantibodies elevated in SLE patients compared to HCs. This technology will be useful in identifying autoantibody profiles in SLE patients and novel autoantigens which could reveal pathways involved in the pathogenesis of SLE. Additionally, novel autoantibodies detected in SLE patients can be used as biomarkers for diagnosis and response to treatment, as well as for identifying potential therapeutic targets.

Anti-STIP1 IgM and IgG autoantibodies were elevated in the serum of SLE patients and are a novel marker for a subset of SLE patients. Given the neurological implications of STIP1, I explored its role as a possible autoantigen in NP SLE, however they were not specific for NP SLE patients as the increase was seen in the serum of SLE patients with and without a diagnosis of NP SLE. SLE patients with high levels of IgG autoantibodies had significantly lower SLEDAI and Renal SLEDAI score, lower levels of anti-dsDNA IgG, higher age, and were less likely to have a high SLEDAI and Renal SLEDAI score. Patients with high levels of IgM autoantibodies had significantly lower serum C4 levels, and were less likely to have a high serum C4 levels. This suggests that anti-STIP1 IgG may be protective. However, further studies into role of STIP1 in the immune system

and more specifically, in NP SLE, through its interactions with PrPc is necessary to determine whether anti-STIP1 autoantibodies are playing a protective or pathogenic role.

As previously mentioned, 80% of SLE patients will have a NP SLE manifestation along their disease course and it is unknown whether the patients with elevated STIP1 antibodies represents that subset of patients or if the patients that will later be diagnosed with NP SLE (Brey, Holliday et al. 2002). Alternatively, the patients with high anti-STIP1 IgG could represent the patients that do not present with NP SLE manifestations, since anti-STIP1 IgG could be protective. Intriguingly, the percent of patients with high anti-STIP1 IgG is the same frequency of patients that do not display NP SLE manifestations; however some NP SLE patients had high anti-STIP1. A longitudinal study of patients with elevated STIP1 antibodies following their disease progression and manifestations could answer this question.

Finally, the elevated STIP1 antibodies present in SLE patients was measured in the serum and not the CSF. The anti-NMDAR antibody specific for NP SLE, is present in both SLE and NP SLE patient serum. However, it is only present in the CSF of NP SLE patients and not SLE patients without NP manifestations (Fragoso-Loyo, Cabiedes et al.

2008). Only once the antibodies are able to pass the BBB and get into the CSF will there be the opportunity for them to gain access to the target antigen and become pathogenic, causing NP SLE manifestations. SLE patients could have a loss of tolerance to the STIP1 in the periphery; however without the breach to their BBB, they would not exhibit NP SLE manifestations. Therefore, STIP1 antibodies could still have a specific pathogenic effect in NP SLE if found to be elevated in the CSF of NP SLE patients but not SLE patients. It is also possible that anti-STIP antibodies could be protective in the CNS.

STIP1 mRNA levels in SLE PBMCs were not elevated compared to healthy controls. This suggests that the mechanism by which there are elevated autoantibodies against STIP1 in SLE patients does not result from increased exposure to STIP1 through an increased expression of STIP1 by PBMCs, however this does not rule out overexposure to the antigen due to hyperexpression of STIP1 in the CNS or another tissue.

STIP1 protein levels were not elevated in the serum of SLE patients compared to healthy controls and do not correlate with anti-STIP1 IgG. This suggests that while there is some STIP1 in the serum of SLE patients and HCs, the increased anti-STIP1 antibodies present in the serum of SLE patients were not due to heightened active

secretion of STIP1 into the serum as occurs during ovarian cancer. Additionally, since there is not increased STIP1 protein in the serum, the autoantibodies are not likely being formed due to exposure to intracellular STIP1 protein that has accumulated due to failed apoptotic debris clearance.

Furthermore, anti-STIP1 IgM and IgG autoantibody levels do not correlate with STIP1 protein serum levels. This is further evidence that the accumulation of STIP1 autoantibodies in the serum is not due to exposure to an overabundance of antigen in the periphery, but occurs through another mechanism. A possible mechanism by which there could be a loss of tolerance to STIP1 is through molecular mimicry. Molecular mimicry occurs when a foreign antigen shares sequence or structure similarity with self antigens. It is suggested that pathogens mimic host proteins to avoid detection; however in some cases antibodies are formed against a pathogen that later react with self antigens resulting in autoimmunity. For example, *C. jejuni* infections can lead to the production of cross-reactive antibodies able to recognize gangliosides, which induce Guillan-Barre′ syndrome (Shahrizaila and Yuki 2011, Cusick, Libbey et al. 2012).

Additionally, it is possible that the interaction of STIP1 with the HSPs, which serve as autoantigens in SLE, could explain the mechanism by which STIP1 autoantibodies are

increased in SLE patients (Minota, Koyasu et al. 1988, Panchapakesan, Daglis et al. 1992, Stephanou, Latchman et al. 1998, Odunuga, Longshaw et al. 2004). HSPs are recognized by PRRs as endogenous danger signals and elicit a TLR-mediated inflammatory response (Asea, Rehli et al. 2002, Warger, Hilf et al. 2006). Furthermore, HSP specific autoreactive BCRs could internalize the entire HSP-STIP1 complex bound to the BCR. Upon reaching the endosomes, this HSP-associated STIP1 would get degraded, and presented by MHCs, which in turn can activate autoreactive STIP1 specific T cells. These T cells could then in turn activate B cells autoreactive for STIP1. This could be explored by seeing whether anti-HSP antibody levels correlate with anti-STIP1 antibody levels in SLE patients.

Another possibility is differential modification of STIP1 such as phosphorylation. STIP1 is phosphorylated by upstream kinases upon cellular stress, heat stress or changes in the cell cycle (Odunuga, Longshaw et al. 2004, Masaoka, Nishi et al. 2008). As previously mentioned, STIP1 phosphorylation and subcellular localization could be regulated by calcium flux, and elevated calcium responses are seen in Lyn -/- mice (Chan, Lowell et al. 1998, Masaoka, Nishi et al. 2008). Phosphorylation of STIP1 at residues S189, T198, and Ser189 could be measured by Western Blot within SLE and HC PBMCs, to determine whether STIP1 phosphorylation levels are different in SLE

patients. Differences in subcellular localization could also be analyzed by comparing nuclear and cytoplasmic extracts for levels of phosphorylated STIP1. Other post-translational modifications are implicated in autoimmune diseases, such as citrullination which results in the inability of immune cells to recognize the citrullinated peptide as self, resulting in autoimmunity. Citrullinated peptides and anti-citrullinated peptide antibodies play a critical role in initiating an inflammatory response in RA (Luban and Li 2010).

Elevated anti-STIP IgM occurs much more frequently in SLE patients than does increased anti-STIP IgG. This could suggest that loss of tolerance to the STIP1 autoantigen occurs in most SLE patients, but that class switching to the IgG isotype only occurs in a subset of patients and could be associated with a particular disease manifestation or phenotype. Few of the NP SLE have anti-STIP1 IgM levels above healthy controls. This could suggest that there is more class switching occurring in NP-SLE patients.

SLE patients with high anti-STIP1 IgG, but not IgM, are less likely to have a high SLEDAI or Renal SLEDAI score and elevated anti-dsDNA IgG, suggesting a protective role for anti-STIP1 IgG. This does not rule out the role of anti-STIP1 IgG in NPSLE

because many NP SLE events do not always correlate with other disease parameters such as increased SLEDAI, nephritis, or ANA levels (Demirkaya, Bilginer et al. 2008).

Another possibility for why high anti-STIP1 IgG levels might be associated with lower disease severity is because as patients disease progresses they start to accumulate anti-STIP1. With increased disease severity, they might be more heavily medicated. Therefore, anti-STIP1 could still be associated with increased disease activity; however the immune suppressants could be causing the drop in SLEDAI and other markers for disease. This is not likely however, because based on medical charts, patients with high anti-STIP1 IgG were taking little to no immune suppressants. Also, there was a very low frequency of patients with the more severe proliferative form of kidney disease, class III and IV, that had high anti-STIP1 IgG. STIP1, PrPc, and HSPs are expressed in the kidney, and STIP1 and HSPs are renal cell carcinoma antigens (Dinda, Mathur et al. 1998, Scanlan, Gordan et al. 1999, Bosque, Ryou et al. 2002, Somji, Ann Sens et al. 2002, Atkins, Lichtenfels et al. 2005, Ramljak, Asif et al. 2008). Therefore, anti-STIP1 autoantibodies could be playing a protective role locally in the kidney, as well as systemically. It would be interesting to determine whether STIP1 expression is altered in the kidneys of SLE patients, and whether anti-STIP1 antibodies bind to kidney tissue.

There was an observed strain specific increase in anti-STIP1 IgG autoantibodies in the murine lupus model, MRL-lpr mouse. Notably, the MRL-lpr mouse is not only a murine model of lupus, but is a mouse model for NP SLE. Many models of SLE such as the NZB and BXSB strains mice have a high incidence of inherited brain abnormalities that are unrelated to SLE, making it difficult to distinguish pre-existing CNS conditions with autoimmunity-related brain conditions. However, the MRL-lpr mouse displays many hallmark features of SLE including the female to male sex bias, making it a good model of SLE. In addition to the typical peripheral SLE symptoms of autoantibodies, arthritis, and nephritis, the MRL-lpr mouse develop anti-cardiolipin antibodies, which are implicated in NP SLE events, and NP symptoms collectively referred to as autoimmunity-associated behavioral syndrome (Gulinello and Putterman 2011). The MRL-lpr mice reproducibly display depression, decreased activity, and fatigue. These feelings of helplessness and despair are measured by failing a swim test and anhedonia, or loss of the ability to receive pleasure or reward, is measured by a failure to prefer a sweet solution (Gulinello and Putterman 2011).

The presence of anti-STIP1 autoantibodies in the NP SLE model, but not other models of SLE may suggest an association between anti-STIP1 autoantibodies and NP SLE pathogenesis. These antibodies could also be induced in response to neurological

damage and be protective. Furthermore, the presence of anti-STIP1 autoantibodies in the MRL-lpr strain, a NP SLE model, offers the possibility to study the potential pathogenic or protective roles of anti-STIP1 autoantibodies in NP SLE.

Future Directions

PrPc is known to modulate T cell activation and limit T cell mediated inflammation in EAE (Tsutsui, Hahn et al. 2008, Hu, Nessler et al. 2010). It is also known that STIP1 and PrPc interact and have protective effects in the CNS (Zanata, Lopes et al. 2002, Lopes, Hajj et al. 2005, Lima, Arantes et al. 2007, Hartmann, Martins et al. 2013). However, it is not known whether STIP1 affects T cell activation via PrPc. Blockade or deficiency of PrPc exacerbated EAE, therefore it could also be possible that the interaction between STIP1 and PrPc may play a role in regulating autoimmunity (Tsutsui, Hahn et al. 2008, Hu, Nessler et al. 2010). Future studies would be necessary to determine a role for STIP1 in T cell activation and inflammation as well as autoimmunity. Furthermore, if the STIP1-PrPc interaction has a regulatory effect on T cells, it would be interesting to determine whether anti-STIP1 autoantibodies could modulate this effect. Alternatively, the interaction between STIP1-PrPc could be functioning as co-stimulation for T cells. Therefore STIP1, through PrPc, could be

increasing T cell activation and causing inflammation. An increase in anti-STIP1 could then reduce this effect explaining the trends observed with increased anti-STIP1 and lower measures of disease activity and severity. These T cell effects would likely occur regardless of NP status and regardless of whether an individual has anti-STIP1 Abs in CSF.

Additional studies are necessary to determine whether anti-STIP1 antibodies are enriched in the CNS of NP-SLE patients. Ongoing experiments using CSF from HCs, NP SLE patients, and MS patients, as well as MRL-lpr and control mice are aimed at determining whether this is the case. If anti-STIP1 autoantibodies are detected in the CSF of NP SLE patients, that would suggest a possible pathogenic or protective potential for anti-STIP1. However it would not determine whether they are NP SLE specific, since the ongoing experiments do not include non-NP SLE CSF. This control would be necessary to determine whether anti-STIP1 autoantibodies in the CSF are specific to NP SLE or rather present in the CSF of all lupus patients.

If anti-STIP1 autoantibodies were detected in the CSF of NP SLE patients, I would then ask whether anti-STIP1 antibodies can bind neural tissue. I would also be interested in determining the effect of anti-STIP1 autoantibodies from SLE patients on neural tissue,

whether they are neuroprotective or neurotoxic. Finally, it would be useful to know whether anti-STIP1 autoantibodies present in other neuroimmunological diseases. As previously mentioned, CSF from MS patients are being analyzed for the presence of anti-STIP1 autoantibodies. If anti-STIP1 is present in MS CSF that would confirm that anti-STIP1 autoantibodies are not SLE or NP SLE specific, but a consequence of autoimmunity or autoimmunity involving the CNS. However, if anti-STIP1 autoantibodies are not detected in the CSF of MS patients, this may suggest a specific role for anti-STIP1 autoantibodies in SLE. Possible models described above for the interaction between anti-STIP1 Abs, STIP1, and PrPc in the CNS and the immune system are depicted in Figure 12.

In addition to its interaction with PrPc, STIP1 is a chaperone to the HSPs, HSP70 and HSP90. HSPs induce TLR-mediated inflammatory cytokine secretion (Asea, Rehli et al. 2002, Warger, Hilf et al. 2006). SLE patients have anti-HSP autoantibodies, and anti-HSP amplify HSP-mediated inflammation (Minota, Koyasu et al. 1988, Yokota, Minota et al. 2006). STIP1 could be required for or amplify the HSP-mediated inflammatory response. Anti-STIP1 autoantibodies might sequester STIP1 or the STIP1-HSP complex, reducing HSP-mediated inflammation (Figure 13). Furthermore, one explanation for why anti-STIP1 IgG, but not IgM, was associated with less severe

disease parameters could be explained by the interaction of anti-STIP1 IgG and FcϒIIb. Anti-STIP1 IgG bound to the HSP-STIP1 complexes could dampen BCR signaling in HSP-specific B cells through its interaction with FcϒIIb and thus exert protective effects.

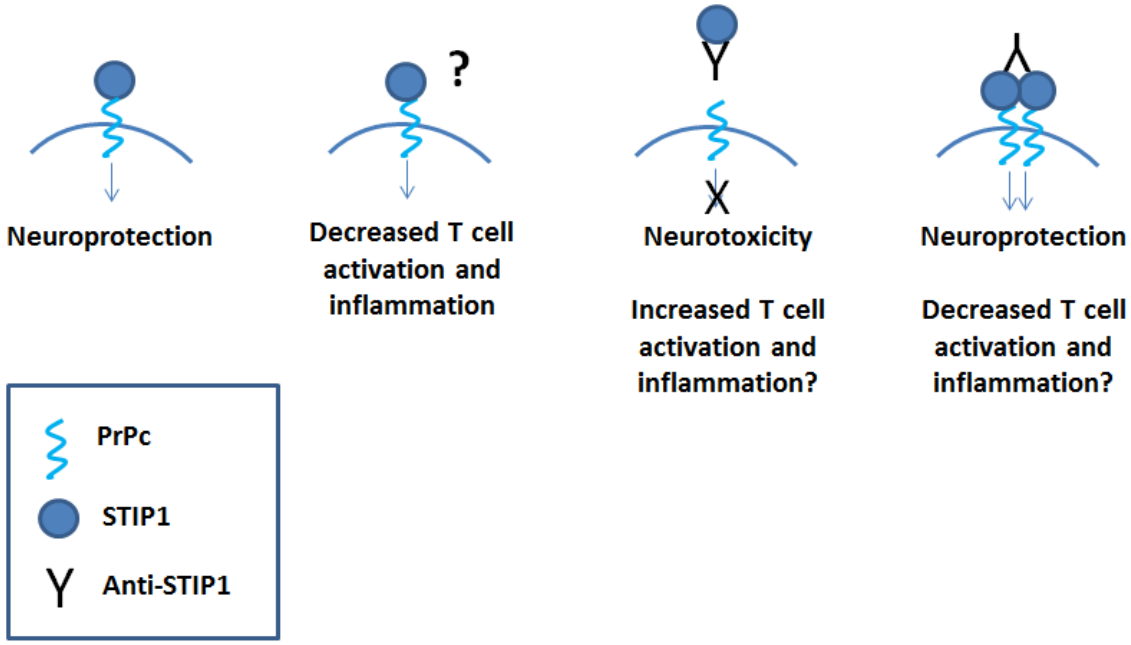


Figure 12. Model of anti-STIP1 autoantibodies and STIP1 antigen in the immune system and the CNS. Cellular prion protein (PrPc) is known to decrease activation of T cells and T cell-mediated inflammation in EAE. In the CNS, the interaction between PrPc and STIP1 promotes memory, survival, and neuritogenesis. This model proposes that the interaction of PrPc and STIP1 might also have a function in the immune system, in which the interaction of Prpc and STIP1 decreases T cell activation and inflammation. Depending on the epitope recognized by autoantibodies against STIP1, anti-STIP1 could either block the proposed effect of PrPc-STIP1 on T cells or could amplify the inhibitory signal.

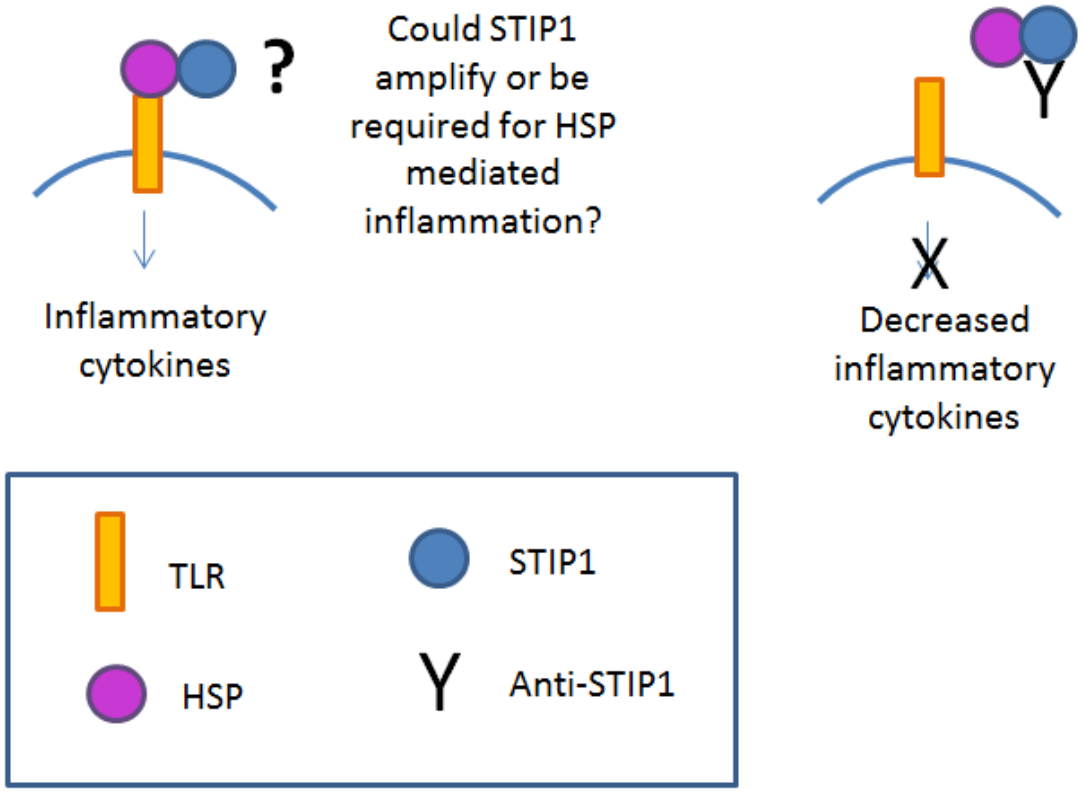


Figure 13. Model of anti-STIP1 and the STIP1-HSP complex in inflammation and autoimmunity. HSPs induce TLR-mediated inflammatory cytokine secretion which is amplified by anti-HSPs. STIP1 may be required for or amplify the HSP induced inflammatory response. Anti-STIP1 could bind to and sequester STIP1 or the STIP1-HSP complex decreasing the HSP induced inflammatory response.

CHAPTER FOUR
GENETIC INTERACTION BETWEEN LYN AND ETS1 IN AUTOIMMUNITY

INTRODUCTION

SLE is an autoimmune disease characterized by autoantibodies. Hallmark autoantibodies in SLE such as anti-dsDNA and anti-Ro or anti-cardiolipin are useful in the diagnosis of SLE and can contribute to the pathogenesis of SLE. While specific autoantibodies are useful as biomarkers for disease, the protein array done using human SLE serum revealed a widespread loss of tolerance to self antigens beyond the usual ANAs, such as anti-STIP1 autoantibodies. Understanding the regulation of B cells differentiating into PCs and the production of autoantibodies is critical to identifying the mechanisms of SLE pathogenesis.

The tyrosine kinase, Lyn, is expressed in B cells and myeloid cells. It plays both an activating and inhibitory role in BCR signaling, however loss of Lyn leads to hyperactive B cells (Chan, Meng et al. 1997, Nishizumi, Horikawa et al. 1998, Xu, Harder et al. 2005, Tsantikos, Maxwell et al. 2012, Lamagna, Hu et al. 2014). Lyn deficient mice develop increased autoantibody production and deposition of IC into the kidney by 6 to 8 months and serve as a mouse model of lupus (Hibbs, Tarlinton et al. 1995).

Polymorphisms in Lyn have been associated with SLE and reduced expression of Lyn is observed in SLE B cells (Liossis, Solomou et al. 2001, Flores-Borja, Kabouridis et al. 2005, Harley, Alarcon-Riquelme et al. 2008, Lu, Vidal et al. 2009). Polymorphisms in CSK, which has been shown to reduce Lyn activity, have also been identified in SLE patients (Manjarrez-Orduno, Marasco et al. 2012). Ets1, a transcription factor expressed in B and T cells which limits PC and Th17 differentiation, respectively, also has SLE associated polymorphisms (Sullivan, Piliero et al. 2000, Moisan, Grenningloh et al. 2007, Han, Zheng et al. 2009, He, Liu et al. 2010, Zhong, Li et al. 2011, Yasuda, Hayakawa et al. 2012, Wang, Ahlford et al. 2013, John, Russell et al. 2014). Decreased levels of Ets1 have been observed in SLE patients PBMCs (Li, Sun et al. 2010).

In addition, the phenotypes of Lyn-/- and Ets1-/- mice resemble SLE and are strikingly similar (Luo, Mayeux et al. 2014). In these mouse models, B cells are hyperactive and there is an observed accumulation of PCs and autoantibodies, resulting in IC deposition and organ damage. Lyn negatively regulates B cell activation and Ets1 negatively regulates PC accumulation. This, taken with the shared lupus-like phenotype in Lyn or Ets1 deficient mice lead to the hypothesis that Lyn and Ets1 are in a common pathway which limits PC cell differentiation.

Lyn maintains Ets1 levels, thus limiting plasma cell accumulation

In collaboration with Wei Luo and Lee Ann Garrett-Sinha the following biochemical pathway by which Lyn maintains Ets1 expression, thus limiting PC differentiation, was defined (Figure 14). Ets1 was found to be downregulated in B cells, through BCR or TLR stimulation in a pathway dependent on PI3K, Btk, IKK2, and JNK (Luo, Mayeux et al. 2014). Loss of Lyn, the tyrosine phosphatase SHP-1, or the inhibitory receptors CD22 and Siglec G, resulted in decreased Ets1 expression (Luo, Mayeux et al. 2014). Of note, decreased Ets1 levels in Lyn-/- B cells occur prior to the development of disease. Restoration of Ets1 expression limits PC differentiation of Lyn or SHP1 deficient B cells *in vitro* (Luo, Mayeux et al. 2014). Therefore, Ets1 levels are downregulated in response to activating signals, allowing for PC differentiation, and inhibitory signals maintain high Ets1 levels, limiting PC accumulation.

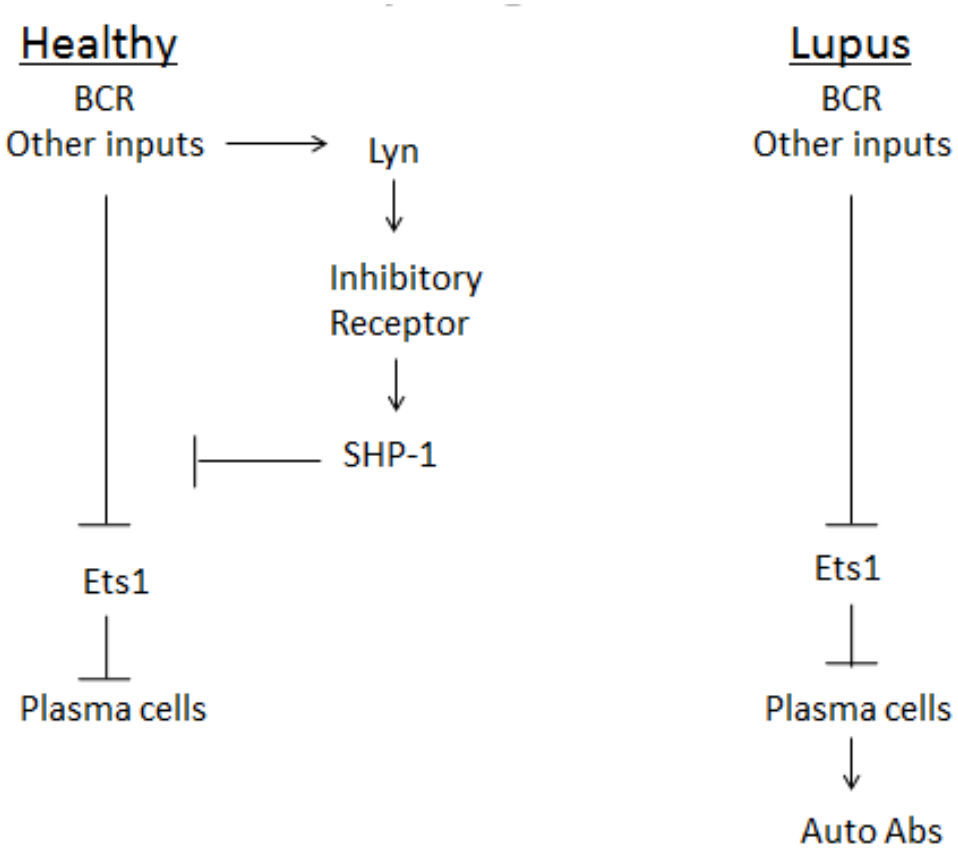


Figure 14. Activating signals downregulate Ets1 levels and inhibitory signals maintain Ets1 levels. Below is a pathway identified by which Lyn maintains Ets1 levels, limiting plasma cell accumulation. Loss of Lyn and other inhibitory signaling components, such as SHP1, results in the downregulation of Ets1, PC accumulation, autoantibody production, and autoimmunity.

Compound Heterozygotes

Compound heterozygotes can be used to study a genetic and functional interaction between signaling molecules in a common pathway. We found that Lyn, SHP-1, and Ets1 were linked in a pathway. Lyn is known to activate the inhibitory phosphatase, SHP-1. Reducing levels of both Lyn and SHP-1 would be expected to significantly impair SHP-1 dependent inhibitory receptor signaling, thus leading to disease. Indeed, Tarlington and Hibbs, through the use of compound heterozygotes of Lyn and SHP-1, showed that partial inactivation of SHP-1 amplified the consequences of Lyn haploinsufficiency, leading to an accelerated development of autoantibodies and disease (Tsantikos, Maxwell et al. 2012). Partial loss of Lyn and SHP-1 together resulted in kidney pathology and the development of ANAs at a significantly faster rate than in either single heterozygote. Autoantibody levels in Lyn SHP-1 compound heterozygotes were comparable to those in Lyn-/- mice. These results demonstrate a clear genetic interaction between Lyn and SHP-1 in autoimmunity (Tsantikos, Maxwell et al. 2012).

Summary

Lyn and Ets1 are in a common pathway in which Lyn maintains Ets1 levels, and therefore inhibits PC accumulation. Since deficiency of either Lyn or Ets1 alone leads to a full blown lupus-like phenotype with PC accumulation and autoantibody production, I hypothesized that the partial disruption of both Lyn and Ets1 would accelerate plasma cell accumulation and autoimmunity. To test this, the disease severity of compound heterozygotes of Lyn and Ets1 were compared to Lyn and Ets1 single heterozygotes.

RESULTS

To determine whether partial loss of Lyn and Ets1 together causes increased autoimmunity, compound heterozygotes of Lyn and Ets1 were created and assayed for disease. Lyn+/- Ets1+/- mice were compared to wt, Ets1+/-, and Lyn+/- mice to determine whether the compound heterozygotes developed accelerated disease compared to the single heterozygotes, Lyn+/- and Ets1+/-. Parameters that were altered in either the Lyn-/- or Ets1-/- mice were analyzed in Lyn Ets1 compound heterozygotes, Lyn+/- Ets1+/-. These include splenomegaly, T cell activation, cytokine production, myeloid defects, autoantibody production, and kidney function. Additional parameters common to SLE patients, murine models of lupus, or the Lyn SHP1 compound heterozygotes, but not known to be present in either Lyn or Ets1 deficient mice were also analyzed. To determine whether autoimmunity was accelerated in the compound heterozygotes, young and old mice were used to measure these parameters prior to and following disease development.

Accelerated Splenomegaly in Lyn Ets1 compound heterozygotes

Splenomegaly is often seen in autoimmune mice as a result of increased infiltrating inflammatory immune cells accumulating in the spleen and extramedullary hematopoiesis. Thus, the spleen weight of wt, Ets1+/-, Lyn+/-, and Lyn+/- Ets1+/- mice was measured. Spleen weights were significantly elevated in young Lyn+/- Ets1+/-, but not Ets1+/- or Lyn+/- , mice compared to wt mice (Figure 15.A). Spleen weights were significantly higher in older Lyn+/- Ets1+/- and Lyn+/-, but not Ets1+/-, mice compared to wt mice (Figure 15.B). However the total number of splenocytes was not different between genotypes (Figure 15.C). Splenomegaly was accelerated in the compound heterozygotes; therefore Lyn and Ets1 synergize to control the rate of splenomegaly.

Lyn and Ets1 cooperate to limit T cell activation

Increased CD4+ T cell activation in Lyn Ets1 compound heterozygotes

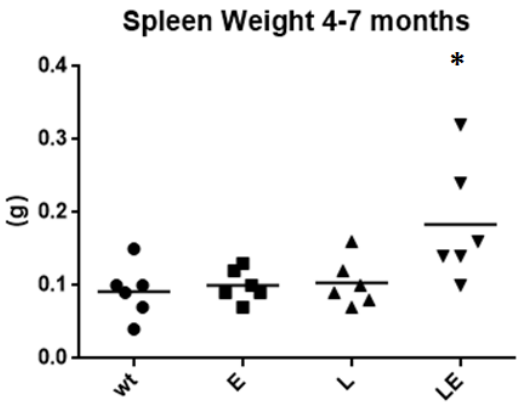
To determine whether there is increased T cell activation in Lyn+/- Ets1+/- mice compared to Lyn+/- or Ets1+/- mice, I stained splenocytes for the cell surface markers, CD4 and CD69, a marker of activation in T lymphocytes. Lyn+/- Ets1+/- mice, but not Lyn+/- or Ets1+/- mice, had significantly increased percentages of activated

CD4+CD69+ T cells compared to wt mice in both young and old mice (Figure 16.A & B). While the difference was not significant, Lyn+/- and Ets1+/- mice, had intermediate percentages of activated T cells compared to wt and the Lyn+/- Ets1+/- mice (Figure 16.A, B, & C).

Increased memory and reduced naïve T cells in Lyn Ets1 compound heterozygotes

To determine whether Lyn+/- Ets1+/- mice had any differences in their naïve and memory T cell pools, I stained splenocytes and gated on CD4+ T cells. CD62L is expressed on naïve T cells and is reduced in memory cells, while expression of the surface marker CD44 is low in naïve cells and increases in memory cells. Therefore naïve cells were identified as being CD4+CD62L+CD44lo/-, and memory cells were identified as being CD4+CD62L-CD44hi. In younger mice there is a trend towards decreased naïve T cells in Lyn+/- Ets1+/- mice which becomes more pronounced with age (Figure 17.A). In older mice, the percentage of naïve T cells was significantly lower in Lyn Ets1 compound heterozygotes, but not the single heterozygotes, compared to wt mice (Figure 17.A). Additionally, in younger mice there was a marked increase in the ratio of memory to naïve cells in Lyn+/- Ets1+/- mice compared to Lyn+/-, Ets1+/-, and wt mice (Figure 17.B).

A)



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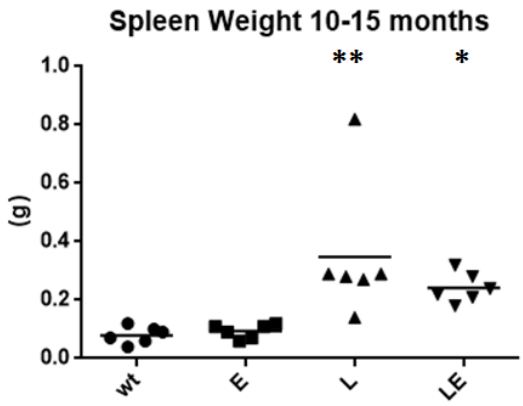


C)

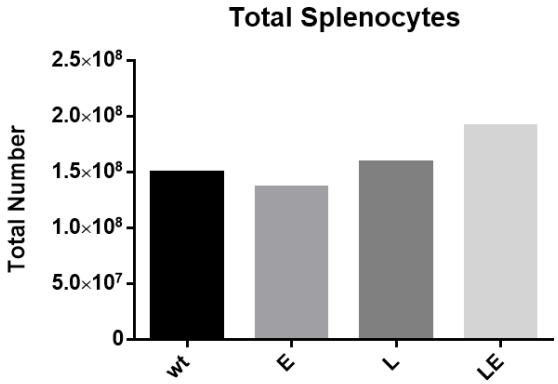


Figure 15. Accelerated Splenomegaly in Lyn Ets1 compound heterozygotes. Spleen weight was measured to assess splenomegaly in A) Young (4-7 months, n=6) and B) Old (10-15 months, n=6 wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice. C) Total number of splenocytes was determined using a hemocytometer. *p<0.05 vs wt and **p,0.0001 vs wt.

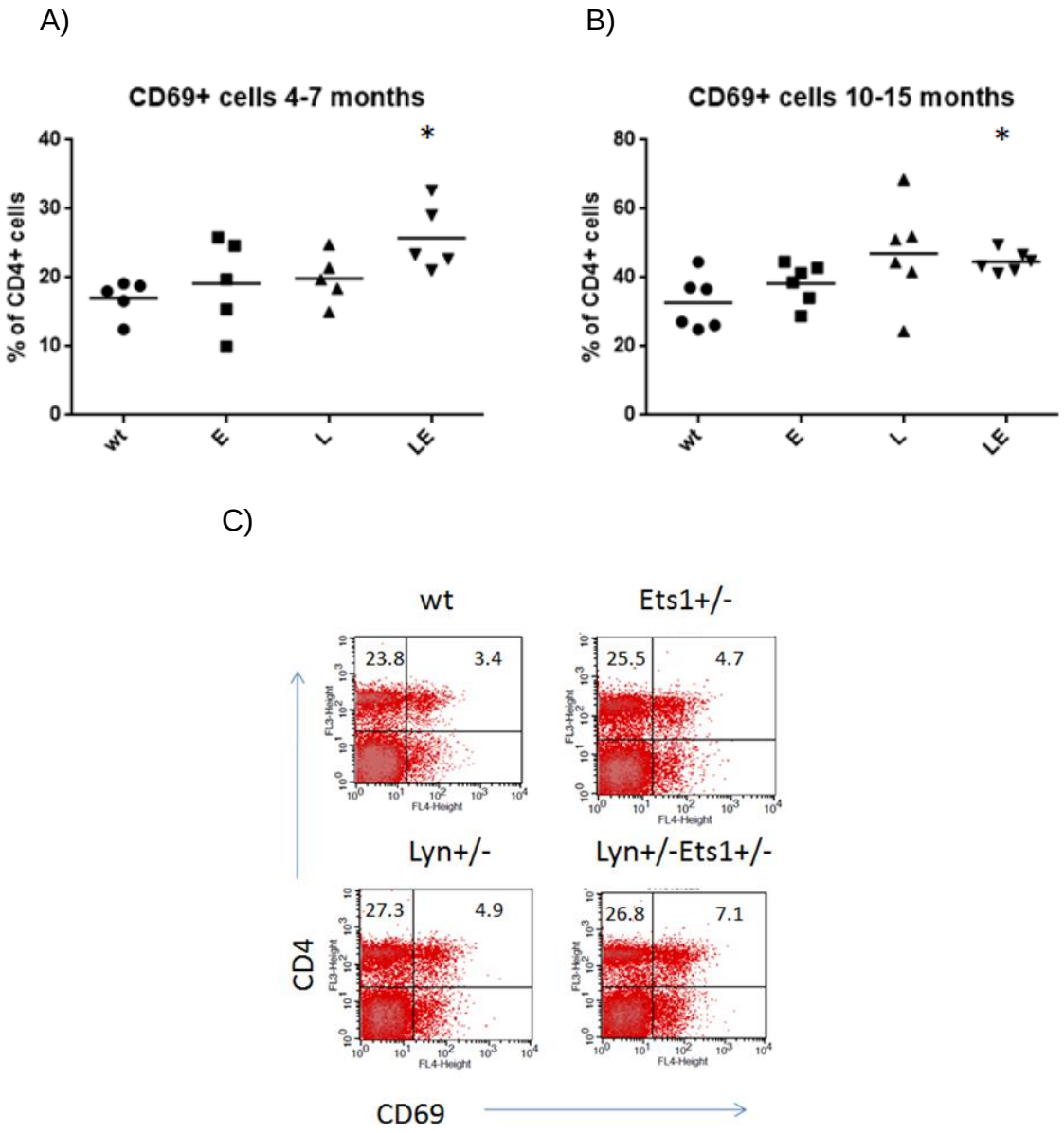
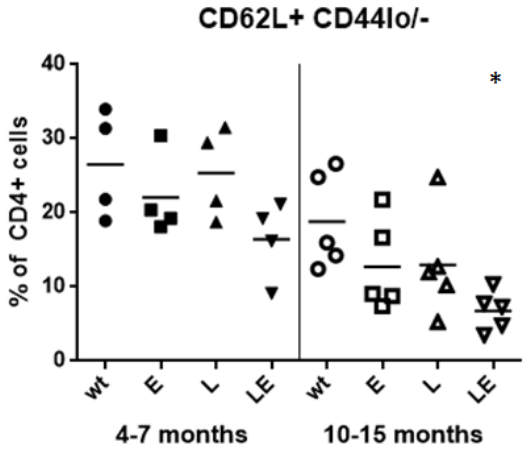


Figure 16. Increased CD4+ T cell activation in Lyn Ets1 compound heterozygotes. Total splenocytes were stained and gated on CD4+ cells. CD69+ cells were identified in A) young (4-7 months, n=5) and B) old (10-15 months, n=6) wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice. C) Representative dot plot CD4+ CD69+ stain. *$p < 0.01$ vs wt.

A)



B)

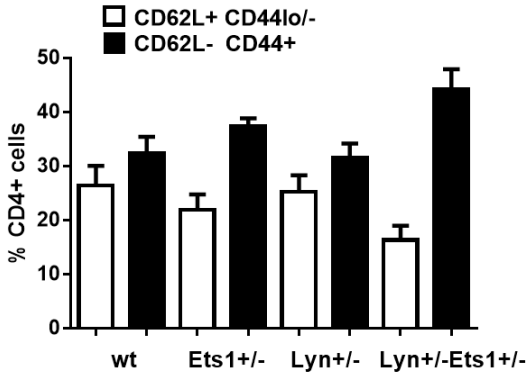
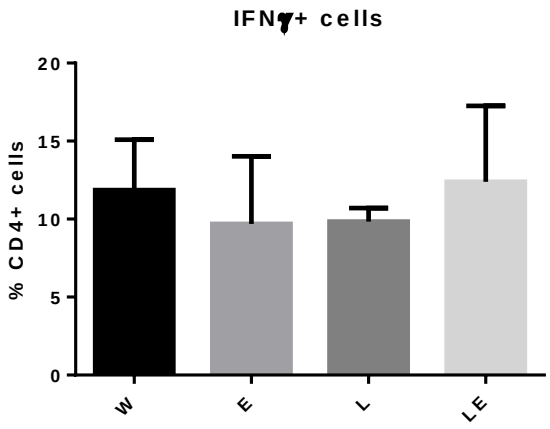


Figure 17. Increased memory and reduced naïve CD4+ T cells in compound heterozygotes. Total splenocytes were stained and gated on CD4+ cells. A) Percentages of CD4+ cells that are CD62L+ CD44lo/- naïve T cells in young (4-7 months, n=4) and old (10-15 months, n=5) wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice. B) Percentages of CD4+ cells that are CD62L+ CD44lo/- naïve T cells and CD62L- CD44+ memory T cells in young (4-7 months, n=4) mice. *$p < 0.005$ vs wt.

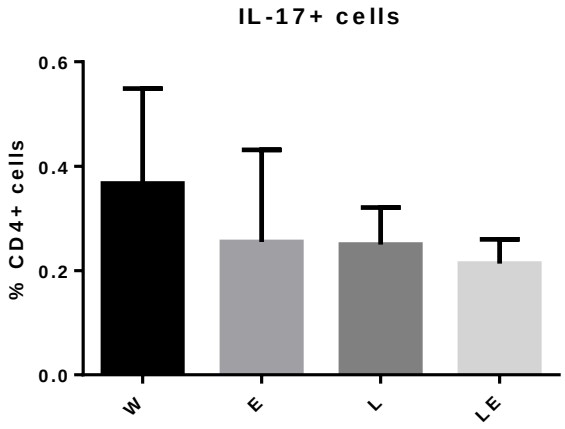
No shift in T cell cytokine profile

Ets1 is a negative regulator of Th17 differentiation. In Ets1-/- mice, T cells are skewed towards a Th17 phenotype and secrete increased levels of IL-17 (Moisan, Grenningloh et al. 2007, Garrett-Sinha 2013). T cells from Lyn-/- mice produce elevated IFNϒ (Chu and Lowell 2005, Scapini, Hu et al. 2010). To determine whether Lyn+/- Ets1+/- mice would have a greater frequency of cells producing IFNϒ, IL-17 or both, an intracellular cytokine assay was done to measure IFNϒ and IL-17. There was no increase in the frequency of CD4+ cells that expressed IFNϒ (Figure 18.A), IL-17 (Figure 18.B), or both (Figure 16.C) in either Lyn+/-, Ets1+/-, or Lyn+/-Ets1+/- mice. This suggests that complete loss of Ets1 is necessary to skew to IL-17 and complete loss of Lyn is necessary to result in increased IFNϒ.

A)



B)



C)

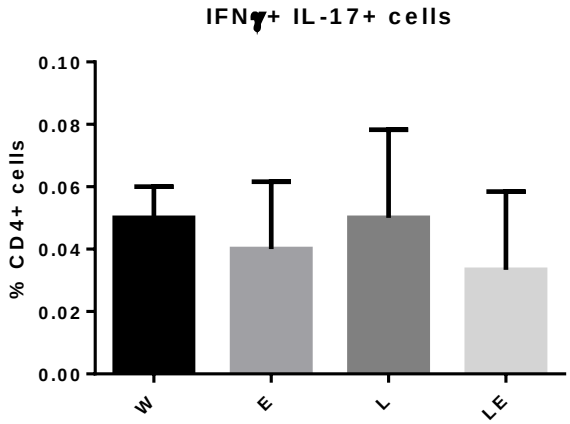


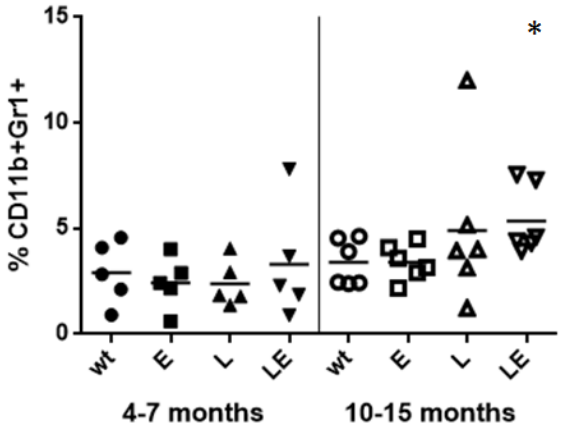
Figure 18. No shift in cytokine profiles in Lyn Ets1 compound heterozygotes. Total splenocytes from young (4-7 months, n=3) wildtype (W), Ets1+/- (E), Lyn+/- (L), and Lyn+/- Ets1+/- (LE) mice were stimulated with PMA and ionomycin for 12 hours in the presence of brefeldin A, gated on CD4+ cells and assayed for cytokine production by Intracellular cytokine staining for A) IFNϒ + cells, B) IL-17+ cells, and C) IFNϒ + IL-17+ cells

Lyn and Ets1 also cooperate to limit Myeloid Dendritic cells and Age Associated B cells

Increased Neutrophils and CD11c+ cells in aged Lyn Ets1 compound heterozygotes

Neutrophils and DCs, such as pDCs and mDCs, contribute to SLE pathogenesis as previously described. Therefore, total splenocytes were stained to determine the percentages of neutrophils and dendritic cells in Lyn Ets1 compound heterozygotes. In old mice, but not young mice, I observed an increase in percentages of neutrophils, identified as being CD11b+Gr1+, in Lyn+/- Ets1+/- but not Lyn+/- or Ets1+/-, mice compared to wt (Figure 19.A). Similarly, there was a significant increase in CD11c+ cells in old Lyn+/- Ets1+/- mice compared to wt, which was not seen in either Lyn+/- or Ets1+/- mice (Figure 19.B). To determine which subset of CD11c+ cells were contributing to the increased percentages of CD11c+ cells in the Lyn+/- Ets1+/- mice, I stained splenocytes for various markers to determine the percentages of pDCs, mDCs, and ABCs.

A)



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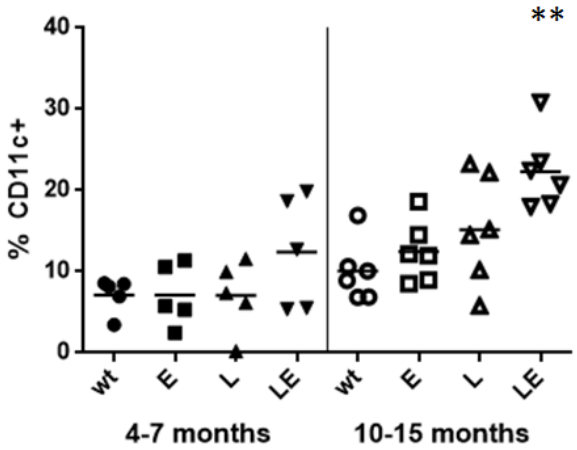


Figure 19. Increased Neutrophils and CD11c+ cells in Lyn Ets1 compound heterozygotes. Total splenocytes from young (4-7 months, n=5) and old (10 - 15 months, n=6) wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice were stained for A) Neutrophils and B) CD11c+ cells *$p<0.05$ and **$p < 0.005$ vs wt..

No increase in Plasmacytoid Dendritic cells in Lyn Ets1 compound heterozygotes

CD11c+Gr1+ cells were identified to determine whether pDCs were contributing to the increased percentage of CD11c+ cells in Lyn+/- Ets1+/- mice. There was no difference in percentages of pDCs in Lyn+/- Ets1+/- mice compared to Lyn+/-, Ets1+/-, and wt mice (Figure 20.A & B). Similar results were observed with Ly6C, also a marker of pDCs (Data not shown). Instead, the increase was in CD11c+Gr1- cells (Figure 20.B).

Increased Myeloid Dendritic cells and Age Associated B cells in Lyn Ets1 compound heterozygotes

Gating on CD11c+ cells, mDCs (CD11b+CD19-) and ABCs (CD11b+CD19+) were identified to determine whether these cell subsets contributed to the increase in CD11c+ cells observed in Lyn+/- Ets1+/- mice. Both percentages of mDCs and ABCs were significantly elevated in Lyn+/- Ets1+/- mice compared to wt and Ets1+/- mice (Figure 21.A & B).

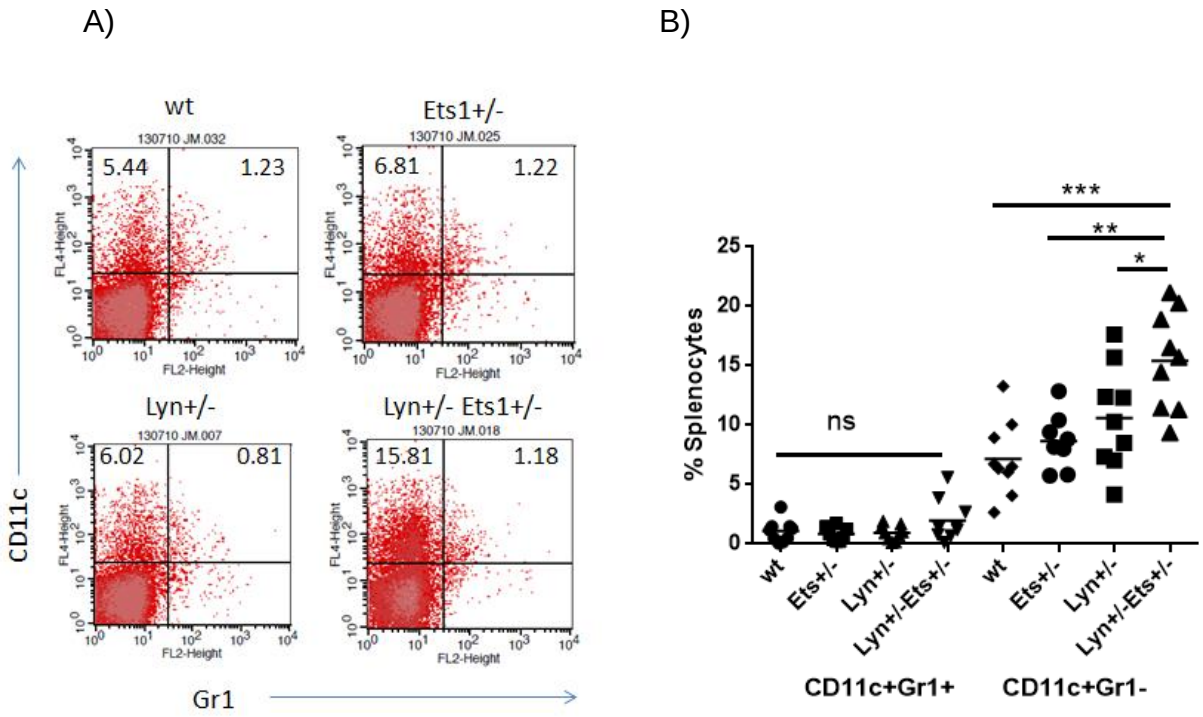


Figure 20. pDCs are not increased in Lyn Ets1 compound heterozygotes. Total splenocytes from 10-15 month old wildtype (wt, n=9), Ets1+/- (n=8), Lyn+/- (n=9), and Lyn+/- Ets1+/- (n=9) mice were stained for A) CD11c and Gr1. B) Percentage of CD11c+Gr1+ and CD11c+Gr1- cells. *p < 0.03, **p < 0.002, and ***p < 0.0003 vs wt.

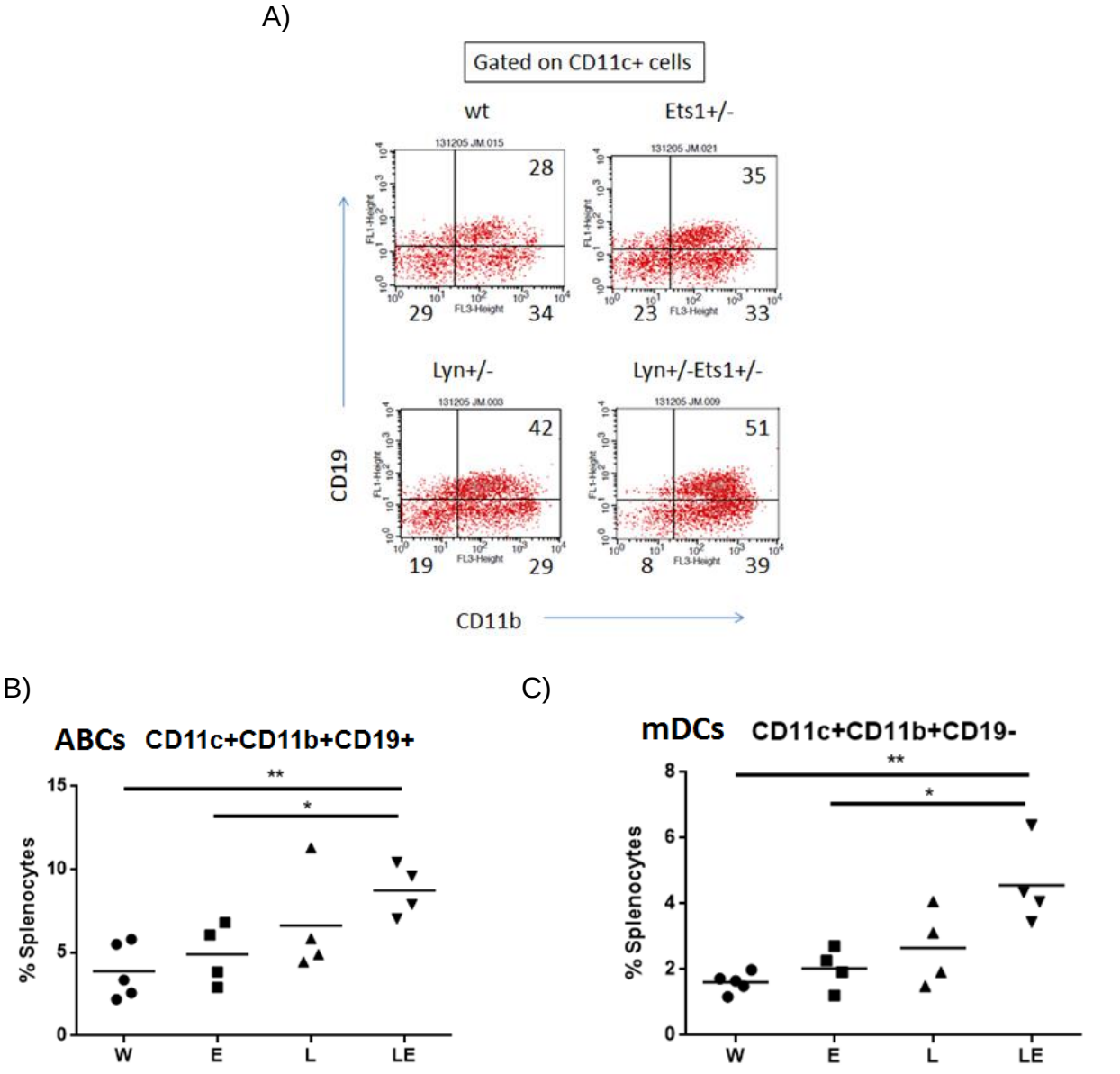


Figure 21. mDCs and ABCs are elevated in Lyn Ets1 compound heterozygotes. Total splenocytes from 10-15 month old mice were gated on CD11c and stained for A) CD19 and CD11b. B) Percentages of CD11c+CD19+CD11b+ and CD11c+CD19-CD11b+ cells in wildtype (W, n=5), Ets1+/- (E, n=4), Lyn+/- (L, n=4), and Lyn+/- Ets1+/- (LE, n=4) mice.

Partial loss of Lyn and Ets1 together accelerates the production of IgM autoantibodies but not IgG autoantibodies

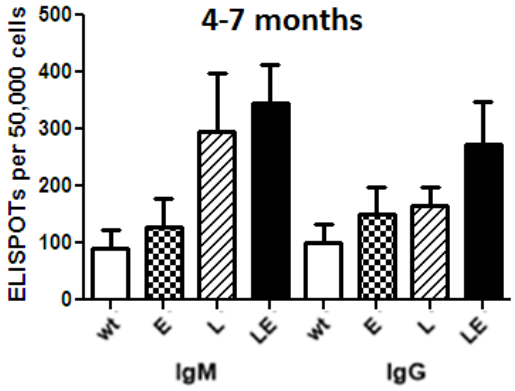
Trend towards increased antibody secreting cells and plasma cells in Lyn Ets1 compound heterozygotes

Because Lyn and Ets1 limit PC accumulation and Lyn and Ets1 knockouts accumulate autoantibodies, I enumerated antibody secreting cells and plasma cells. As determined by ELISPOT, there is a trend toward increased IgM and IgG antibody secreting cells in young Lyn+/- Ets1+/- mice, but only IgM antibody secreting cells in old Lyn+/- Ets1+/- mice compared to wt, Ets1+/-, and Lyn+/- mice (Figure 22.A & B). Additionally, I observed a trend for increased percentages of CD138, a marker for PCs, expressing cells in young but not old Lyn+/- Ets1+/- mice compared to wt, Ets1+/-, and Lyn+/- mice (Figure 22.C).

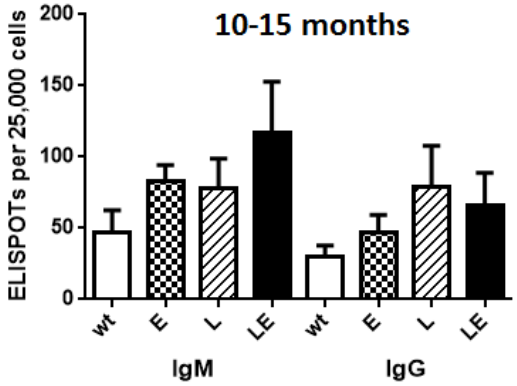
Normal levels of total IgM, IgG, and IgA in Lyn Ets1 compound heterozygotes

To determine whether partial loss of Lyn and Ets1 was contributing to increased levels of serum Ig, ELISAs were performed on young and old mice for total IgM, IgG, and IgA. There was no observed difference in the amount of serum IgM, IgG, and IgA among the genotypes (Figure 23).

A)



B)



C)

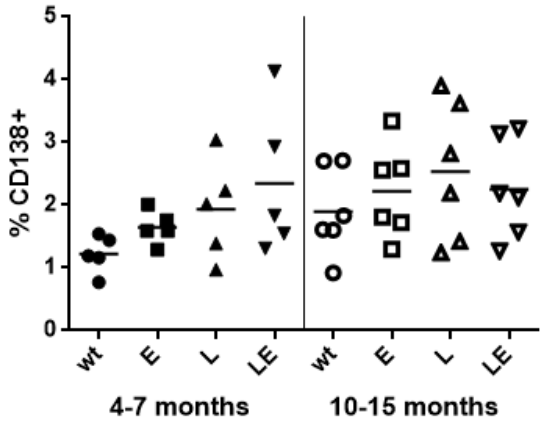


Figure 22. Trend towards increased antibody-secreting cells and plasma cells in Lyn Ets1 compound heterozygotes. Total splenocytes were analyzed by ELISPOT for IgM and IgG antibody-secreting cells in A) young (4-7 months, n=3 IgM and n=4 IgG) and B) old (10-15 months) wildtype (wt, n=4), Ets1+/- (E, n=5), Lyn+/- (L, n=3), and Lyn+/- Ets1+/- (LE, n=4) mice. C) Percentage of CD138+ cells in young (4-7 months, n=5) and old (10-15 months, n=6) mice.

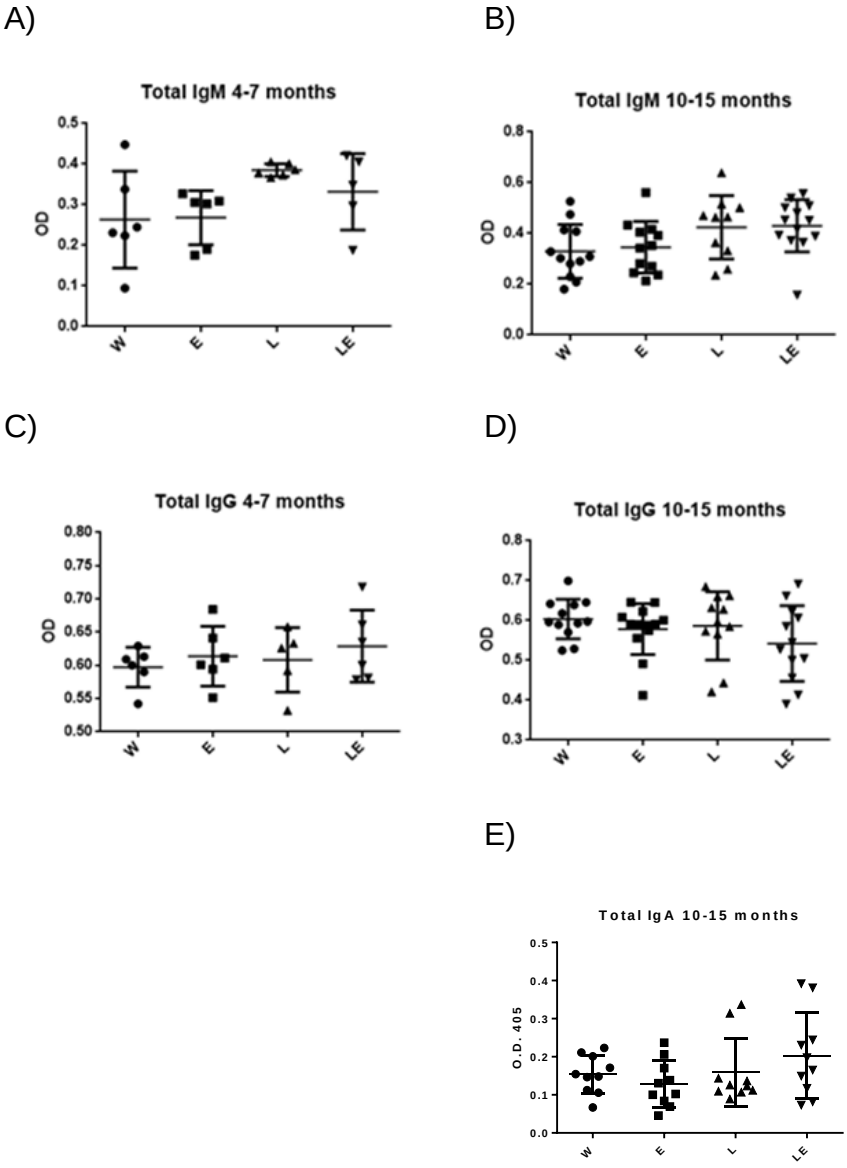


Figure 23. Normal levels of total IgM, IgG, and IgA in Lyn Ets1 compound heterozygotes. ELISAs using sera from A & C) young (4-7 months, n=6) and B, D, & E) old (10-15 months) wildtype (W, n=12), Ets1+/- (E, n=12), Lyn+/- (L, n=10), and Lyn+/- Ets1+/- (LE, n=12) mice was analyzed for A) & B) Total IgM (serum 1:1000), C) & D) Total IgG (serum 1:1250), & E) Total IgA (serum 1:250).

Accelerated IgM, but not IgG, or IgA, autoantibodies in Lyn Ets1 compound heterozygotes

To determine whether Lyn+/- Ets1+/- mice demonstrate increased autoantibodies, anti-ssDNA and anti-dsDNA ELISAs were performed. Both young and old Lyn+/- and Lyn+/- Ets1+/- mice had significantly increased anti-ssDNA IgM autoantibodies compared to all other genotypes (Figure24.A & B). While the autoantibody levels of Lyn+/- mice catch up to those of Lyn+/- Ets1+/- mice by the age of 10-15 months, partial loss of both Lyn and Ets1 accelerated the accumulation of anti-ssDNA IgM (Figure 24.A). Similarly, with the young mice, only Lyn+/- Ets1+/- mice have significantly elevated anti-dsDNA IgM compared to wt. In addition, young Lyn+/- Ets1+/- mice are significantly different than either Ets1+/- or Lyn+/- mice. However, by age 10-15 months Ets1+/-, Lyn+/-, and Lyn+/- Ets1+/- mice all have significantly higher levels of anti-dsDNA IgM than wt, but Lyn+/- Ets1+/- is still significantly different than Ets1+/- (Figure 24.B).

The acceleration in IgM autoantibody production observed in Lyn+/- Ets1+/- mice was not observed with IgG autoantibodies. Aged Lyn+/- and Lyn+/- Ets1+/- mice both had significantly higher levels of anti-ssDNA IgG compared with wt and there was no significant difference observed between genotypes for anti-dsDNA IgG (Figure 24.C).

There was little to no anti-DNA IgG detected in young mice (Figure 24.D). Finally, there was little to no anti-ssDNA IgA or anti-dsDNA IgA (Figure 24.E).

Autoantigen array and autoantibody profiles in Lyn Ets1 compound heterozygotes

Autoantigen arrays allow for the detection autoantibody profiles and patterns in antigen recognition by autoantibodies, similar to the SLE IFN signature in cytokine arrays. Serum samples from wt, Ets1+/-, Lyn+/- and Lyn+/- Ets1+/- mice were analyzed for reactivity against self antigens using an autoantigen array bearing roughly 80 antigens commonly associated with various autoimmune diseases. An increase in IgM, but not IgG, autoantibodies was observed in young Lyn+/- Ets1+/- serum compared to wt, Ets1+/-, and Lyn +/- (Figure 25).

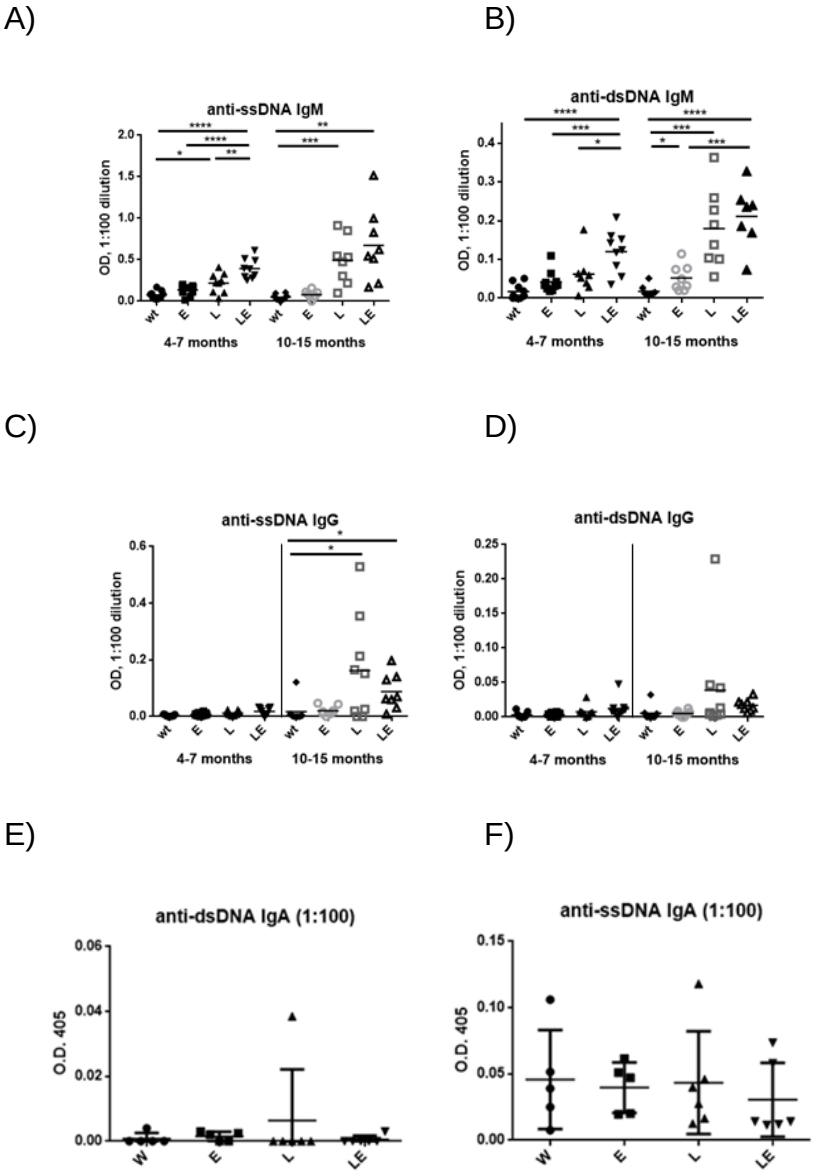


Figure 24. Accelerated IgM, but not IgG or IgA, autoantibodies in Lyn Ets1 compound heterozygotes. Serum (1:100) from young (4-7 months) and old (10-15 months) wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice (n=5-10)was analyzed by ELISA for A) anti-ssDNA IgM, B) anti-dsDNA IgM, C) anti-ssDNA IgG, D) anti-dsDNA IgG, D) anti-ssDNA IgA, and E) anti-dsDNA IgA.

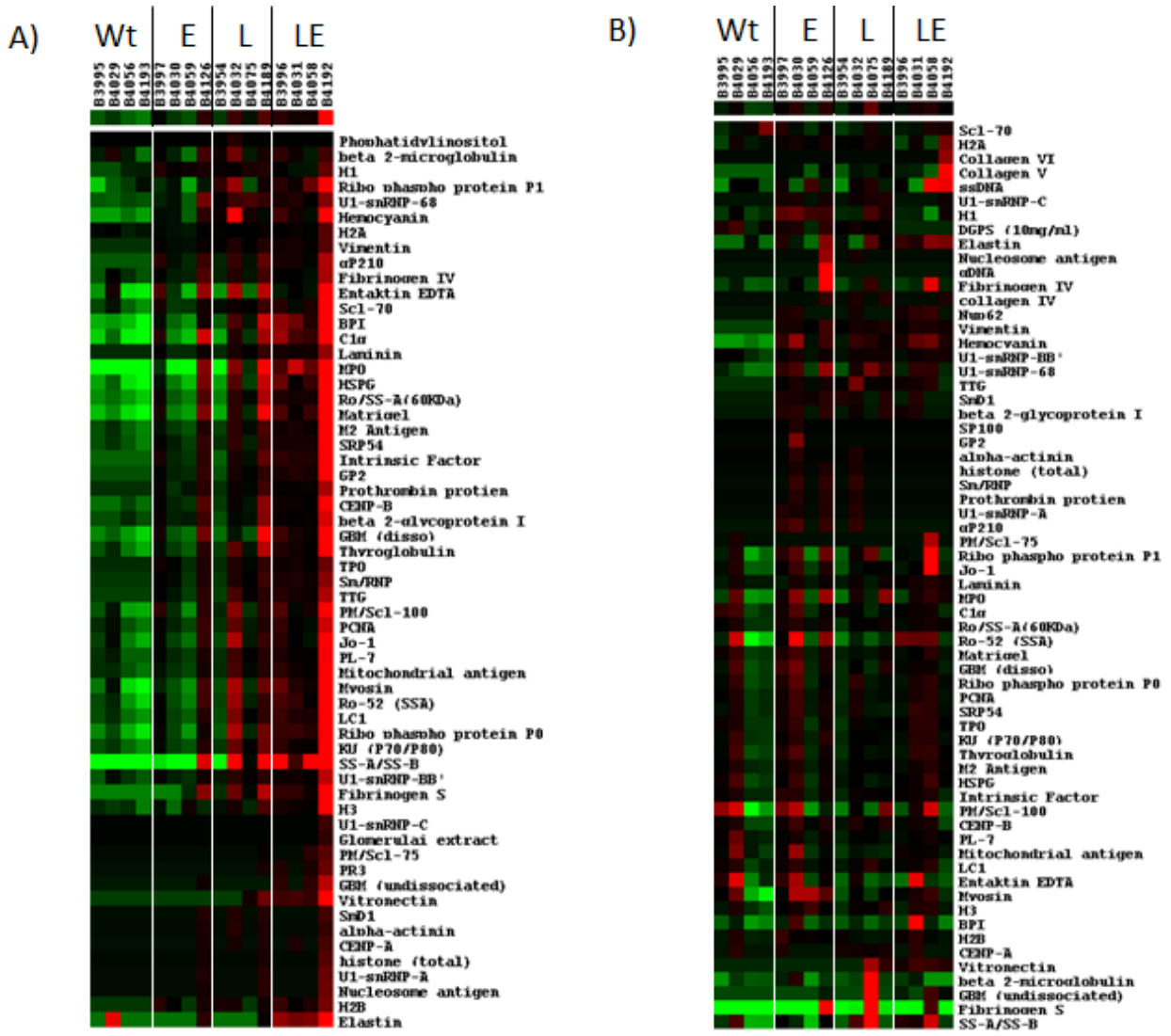


Figure 25. An autoantigen array reveals increased IgM, but not IgG, autoantibodies in Lyn Ets1 compound heterozygotes. Serum from young (4-7 months) wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice was analyzed by an autoantigen array (n=4).for A) IgM autoantibodies and B) IgG autoantibodies. Green, black, and red represent net fluorescent intensities below, close to, and above the mean for each antigen, respectively.

Overall signs of disease in Lyn Ets1 compound heterozygotes

IL-6 levels are not elevated in Lyn Ets1 compound heterozygote

IL-6 can induce class switching to pathogenic IgG and can contribute to inflammation (Suematsu, Matsuda et al. 1989, Minges Wols, Underhill et al. 2002, Cassese, Arce et al. 2003, Gutierrez, Halcomb et al. 2010, Tsantikos, Oracki et al. 2010). To determine whether the compound heterozygotes had increased serum IL-6 levels, an ELISA was performed. Serum IL-6 levels were not significantly elevated in Lyn+/- Ets1+/- mice compared to Ets1+/- and Lyn+/- mice (Figure 26).

Normal body weight and serum creatinine levels in Lyn Ets1 compound heterozygote

To determine whether Lyn and Ets1 increased autoimmune disease severity, body weight was measured. Body weight was not significantly decreased in Lyn Ets1 compound heterozygotes compared to the other genotypes (Figure 27.A). To determine whether Lyn Ets1 compound heterozygotes had increased kidney disease, serum creatinine levels were measured in old mice. There was no significant increase in serum creatinine levels observed in Lyn +/- Ets1+/- compared to the single heterozygotes (Figure 27.B).

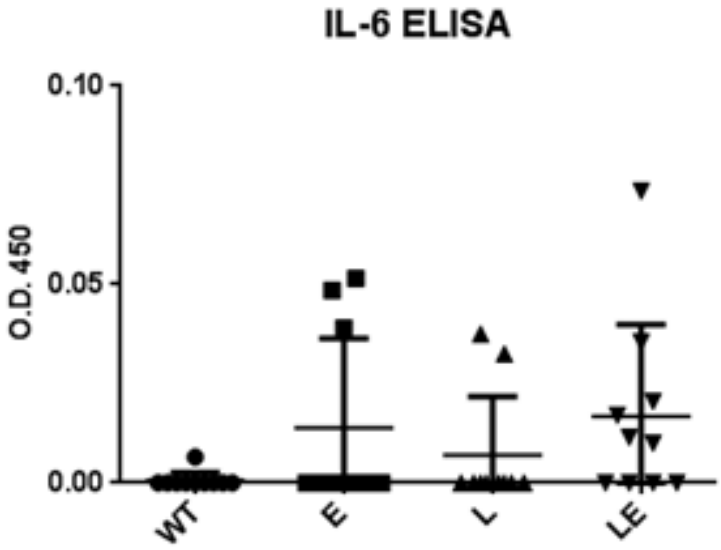
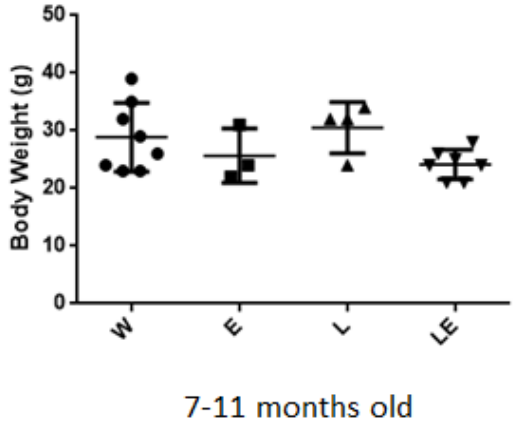


Figure 26. No increase in serum IL-6 levels in Lyn Ets1 compound heterozygotes. Serum (1:10) from old (10-15 months) wildtype (WT), Ets1+/-), Lyn+/- (L), and Lyn+/- Ets1 (LE) mice (n=10) were analyzed for IL-6 levels by ELISA.

A)



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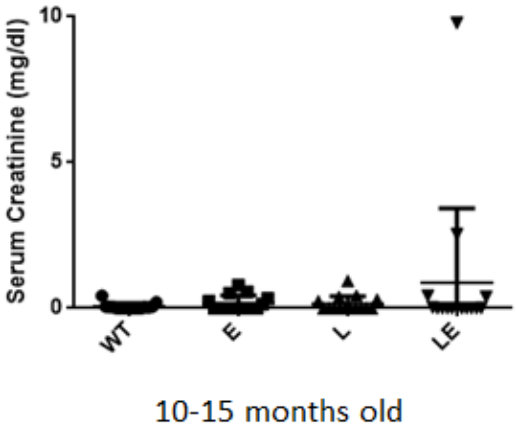


Figure 27. Normal body weight and serum creatinine levels in Lyn Ets1 compound heterozygotes. A) Body weight was measured in old (7-11 months) mice. B) Serum (undiluted) from old (10-15 months) wildtype (wt), Ets1+/- (E), Lyn+/- (L), and Lyn +/- Ets1+/- (LE) mice (n=5) was analyzed by a serum creatinine colorimetric assay.

DISCUSSION

Compound heterozygotes were created to determine whether partial loss of both Lyn and Ets1, two components in a common pathway limiting PC accumulation, would accelerate autoimmunity compared to single heterozygotes of Lyn or Ets1. Synergy between Lyn and Ets1 was observed in the activation of CD4+ T cells, the accumulation of neutrophils, mDCs, and ABCs, and the acceleration of splenomegaly and IgM autoantibodies. There were no differences were detected in percentage of pDCs, body weight, serum creatinine and IL-6 levels, or IgG autoantibody production in the Lyn Ets1 compound heterozygotes compared to the single heterozygotes. Thus, partial loss of both Lyn and Ets1 does make mice more prone to become autoreactive, however it does not result in overt autoimmune disease.

Partial loss of Lyn, Ets1, or both was not sufficient to alter Th subset distribution (all mice had predominantly Th1 cells). However partial loss of Lyn and Ets1 together was sufficient to cause an increase in the percentage of activated CD4+ T cells. Activated T cells contribute to autoimmunity by secreting inflammatory cytokines and chemoattractants and serving as costimulators for autoreactive B cells.

There was an observed increase in the ratio of memory to naïve T cells in Lyn+/- Ets1+/- mice compared to Lyn+/-, Ets1+/-, and wt mice. In aged mice, the frequency of naive T cells was significantly lower than wt, while loss of either Lyn or Ets1 alone was not sufficient. This supports the observation of increased T cell activation. Memory T cells have encountered their antigen and subsequently respond in a more rapid and robust manner because a secondary signal is no longer required for activation. In SLE, there is increased apoptosis of cells and a loss of antigen clearance by macrophages resulting in more available antigen for autoreactive T cells to encounter. Memory T cells can then contribute to autoimmunity through their effector mechanisms and by activating B cells. In SLE patients, there is increased stimulation of T cells resulting in the accumulation of terminally differentiated memory T cells (Fritsch, Shen et al. 2006).

Synergy between Lyn and Ets1 in multiple cell types, rather than just within B cells, may be contributing to increased T cell activation. Lyn is not expressed in T cells and Ets1 is primarily expressed in lymphoid rather than myeloid cells. However, the loss of Lyn in B cells and myeloid cells and the loss of Ets1 in B and T cells could collectively work together to increase T cell activation. Increased activation of T cells could result from hyperactive B and myeloid signaling and a change in the cytokine mileu produced by these cells. Additionally, presentation of antigen by APCs and subsequent T cell

activation could be enhanced through increased costimulatory marker expression (Chan, Nie et al. 2012). These effects in APCs could combine with T cell intrinsic activities of Ets1 to promote T cell activation.

In T cells, Ets1 is a negative regulator for the differentiation to Th17 cells. Th17 cells are known to contribute to the inflammatory phenotype of autoimmune disease such as Crohns and MS (Lovett-Racke, Yang et al. 2011, Waite and Skokos 2012). In MS patients, myelin oligodendrocyte glycoprotein (MOG) specific Th17 cells preferentially traffic to the brain and are enriched in the CNS and increased levels of IL-17 mRNA was detected in the CSF of MS patients (Venken, Hellings et al. 2010). IL-17 producing cells are increased in SLE patients and are present in the inflamed kidney tissue of patients with lupus nephritis and mouse models of lupus (Crispin, Oukka et al. 2008, Zhang, Kyttaris et al. 2009, Wang, Ito et al. 2010). Partial loss of Ets1 in T cells might thus be expected to skew the T cell subset toward a more inflammatory phenotype. Partial loss of Lyn and Ets1 however did not result in increased IL17 producing cells. One possible explanation for this is that partial loss of Ets1 is not sufficient to promote Th17 differentiation, and that a complete loss of Ets1 protein would be necessary to prevent inhibition of Th17 differentiation. Another possibility is that the effect of the loss of Lyn on IFN□ production is dominant and prevents skewing towards a Th17 phenotype.

Furthermore, Ets1 binds to Foxp3 transcriptional regulatory sequences and controls their methylation status, suggesting that Ets1 can contribute to epigenetic changes required for stable Foxp3 expression in Tregs (Mouly, Chemin et al. 2010). Partial loss of Ets1 therefore could result in reduced Treg numbers or reduced suppressive potential of autoreactive cells.

While B cell specific deletion of Lyn results in autoimmunity, myeloproliferation and increased T cell activation (Lamagna, Hu et al. 2014), myeloid intrinsic roles of Lyn also contribute to disease. As in B cells, Lyn is a negative regulator of myeloid signaling, mainly through the phosphorylation of ITM-containing inhibitory receptors. In Lyn-/- mice overproduction of BAFF by myeloid cells induces IFNϒ secretion by T cells, which in turn stimulates more BAFF release by myeloid cells (Scapini, Hu et al. 2010). Lyn deficiency in myeloid cells resulted in myeloid expansion and an autoimmune phenotype, similar but less severe than Lyn-/- mice (Scapini, Hu et al. 2010). Deletion of Lyn in DCs resulted in spontaneous activation of B and T cells, autoantibody production, and nephritis (Chu and Lowell 2005). Partial loss of Lyn in myeloid cells could result in increased myeloproliferation, while partial loss of Lyn in DCs could result in the accumulation of activated B cells, T cells, and autoantibodies. Therefore, partial loss of

Lyn could initiate the inflammatory loop between myeloid cells and T cells to promote activation of T cells with reduced Ets1 levels.

Lyn and Ets1 also cooperate to limit the frequency of neutrophils and CD11c+ cells. Neutrophils have a known contribution to the pathogenesis of SLE through massive infiltration to site of tissue damage increased NETosis, and secretion of type I IFN and BAFF. Lyn plays a negative role in the regulation of adhesion-mediated signaling in neutrophils. Neutrophils from Lyn-/- mice have hyperresponsive integrin-mediated effector functions, including adhesion, respiratory burst and secondary granule release (Pereira and Lowell 2003). MDSCs are potent T cell suppressors and also express CD11b and Gr1. Analysis of additional markers on the increased CD11b+Gr1+ cells observed in Lyn+/- Ets1+/- mice would be necessary to determine whether they are neutrophils or MDSCs. Neutrophils expressing low levels of CD11b have been suggested to represent MDSCs due to their suppressive abilities (Pillay, Tak et al. 2013). Additionally, CD11b+Gr1+CD244+ cells were also found to be suppressive (Pillay, Tak et al. 2013). MDSCs can therefore be distinguished from neutrophils by their level of CD11b expression, CD244 expression, or by directly analyzing the CD11b+Gr1+ population’s suppressive potential. If MDSCs were elevated in Lyn Ets1

heterozygotes, the anti-inflammatory nature of MDSCs could be limiting disease and explain why Lyn Ets1 heterozygotes did not develop full blown autoimmunity.

In further analysis to determine which CD11c expressing cells were contributing to the increased percentages of CD11c+ cells in Lyn+/- Ets1+/- mice , mDCs and ABCs were found to be significantly elevated to both wt and Ets1+/- mice. mDCs activate and induce class switching in autoreactive B cells either directly though the secretion of BAFF, or indirectly through autoreactive Th cells (Castigli, Wilson et al. 2005). Myeloproliferation, resulting in increased activation of B and T cells by mDCs, begins a positive feedback loop of hyperactive immune cells. In addition to other IFN producing cells, such as pDCs, mDCs are one important contributor to the IFN signature observed in SLE. mDCs are the main activators of T cells, and contribute to the activation of autoreactive T cells and B cells.

ABCs contribute to the secretion of autoantibodies in autoimmune prone models and in aged females (Rubtsov, Rubtsova et al. 2011). Expansion of this population could be contributing to SLE pathogenesis. In these experiments, males were used to ensure that the increase in ABCs was due to genotype and not a sex difference. Furthermore, there is no gender bias in the development of autoimmune disease in Lyn-/- mice (data

not shown). ABC expansion occurs via TLR7 signaling in B cells (Rubtsov, Rubtsova et al. 2011, Rubtsov, Rubtsova et al. 2013). Anti-RNA autoantibodies were not evaluated by ELISA in the Lyn Ets1 heterozygotes; however, the autoantigen array indicates that these mice have IgM against several RNA containing self antigens. Independent of ABCs, BCR plus TLR9 stimulation of B cells is much more effective at downregulating Ets1 in wt cells. This suggests that nucleic acid specific cells reactive with RNA containing antigens (BCR plus TLR7) or DNA containing antigens (BCR plus TLR9) may be particularly sensitive to partial loss of Lyn and Ets1. Interestingly, IgM memory cells with a similar phenotype, that also express CD11c, develop in response to ehrlichial (*Ehrlichial muris*) infection (Jones, DeIulio et al. 2012). Antibodies against *E. muris* and *E. chaffeensis*, in human ehrlichiosis patients, are self reactive against nuclear antigens, as seen in SLE (Jones, DeIulio et al. 2012). These B cells may contribute to autoimmunity, but it is unclear whether they are ABCs.

Partial loss of Lyn and Ets1 together accelerates the production IgM autoantibodies but not pathogenic IgG autoantibodies. While the compound heterozygotes have a partial loss of Lyn and Ets1, residual expression of Lyn and Ets1 in B cells may be sufficient to limit the accumulation of PCs and IgG autoantibodies. As previously discussed, there are two checkpoints present in the loss of tolerance that occurs in SLE. The first is B

cell intrinsic and involves hyperactive B cells and IgM autoantibody accumulation. The second checkpoint involves class switching of B cells with lupus associated autoantigen specificities and pathogenic autoantibodies, however only a subset of B cells receive signals for the production of these focused IgG autoantibodies. The second checkpoint depends on IL-6 and requires complete deficiency of Lyn, as anti-dsDNA IgG autoantibodies were not observed in Lyn+/- mice (Figure 24) (Gutierrez, Halcomb et al. 2010). Serum IL-6 and creatinine levels were not significantly elevated in Lyn Ets1 heterozygotes compared to the Ets1 and Lyn heterozygotes. The fact that complete loss of Lyn and IL-6 is necessary to develop pathogenic IgG autoantibodies could explain why acceleration in IgM, but not IgG, autoantibodies was observed in Lyn Ets1 heterozygotes. This suggests that partial loss of Ets1 is not enough to break the second checkpoint in Lyn+/- mice and cause class switching to pathogenic IgG autoantibodies. However, the breach in checkpoint 1 is accelerated in mice heterozygous for both Lyn and Ets1.

Lyn Ets1 compound heterozygotes did not develop autoimmunity as severe as the Lyn or Ets1 knockouts or the Lyn SHP-1 compound heterozygotes. This could be because SHP-1 is upstream of Ets1 and could be affecting multiple processes in B cells that the deficiency of Ets1 would not (Figure 28). Additionally SHP-1 is expressed in T, B, and

myeloid cells, while Ets1 is expressed in T and B cells. Both Lyn SHP-1 and Lyn Ets1 compound heterozygotes lose tolerance at checkpoint one, resulting in accumulation of PCs and IgM autoantibodies. However, additional hits may be necessary in the myeloid compartment to break tolerance at checkpoint two, resulting in a subset of autoreactive B cells class switching to produce IgG autoantibodies (Figure 29). These additional myeloid defects would occur in Lyn SHP-1, but not Lyn Ets1, compound heterozygotes.

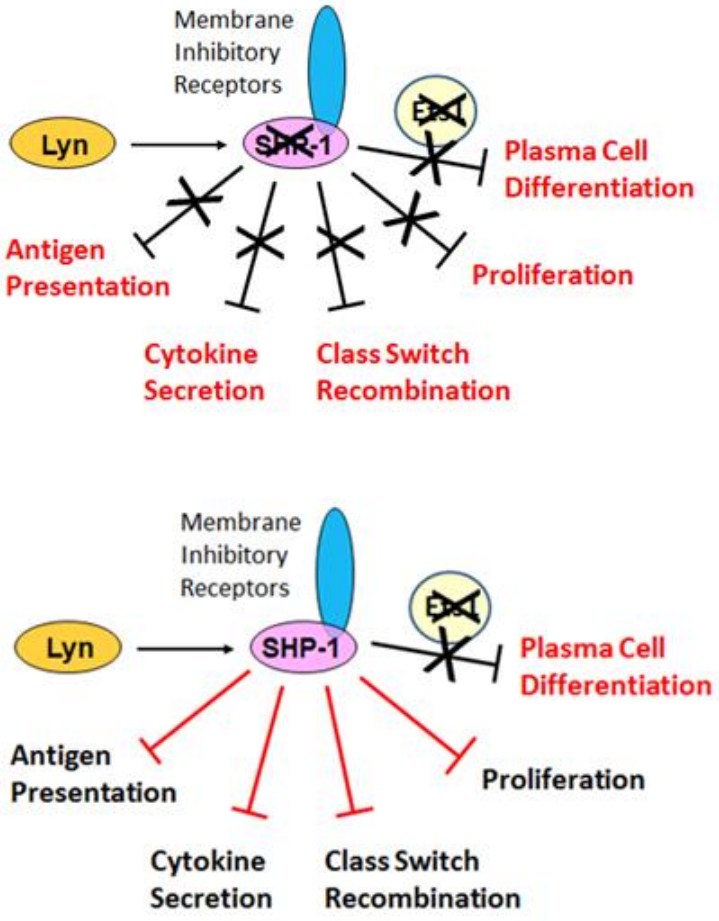


Figure 28. Model for more severe autoimmunity in Lyn SHP-1 compound heterozygotes compared to Lyn Ets1 compound heterozygotes. Lyn Ets1 compound heterozygotes did not develop as severe autoimmunity as the Lyn or Ets1 knockouts or the Lyn SHP-1 compound heterozygotes. This could be because SHP-1 is upstream of Ets1, and therefore loss of SHP-1 could be affecting more processes in B cells than loss of Ets1.

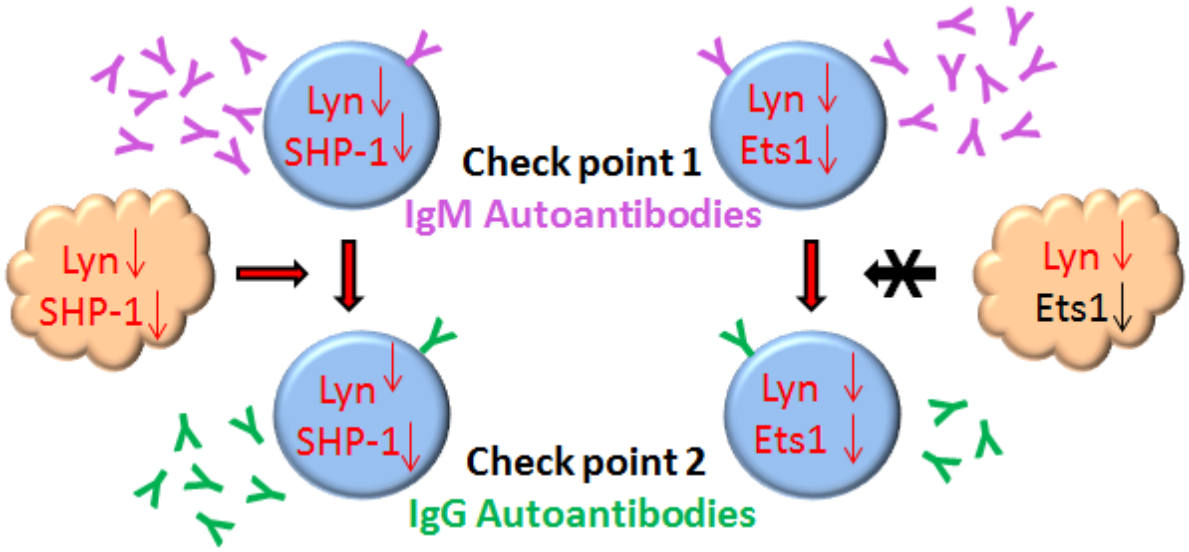


Figure 29. Break in tolerance checkpoints in Lyn SHP-1 and Lyn Ets1 compound heterozygotes. SHP-1 is expressed in T, B, and myeloid cells, while Ets1 is expressed in T and B cells. Both Lyn SHP-1 and Lyn Ets1 compound heterozygotes have a break in tolerance at checkpoint one, resulting in an accumulation of PCs and IgM autoantibodies. An accelerated accumulation of class switched, pathogenic IgG autoantibodies is seen in Lyn SHP-1, but not Lyn Ets1, compound heterozygotes. Loss of tolerance at checkpoint two may require additional hits in the myeloid compartment to break the tolerance at checkpoint two**.**

Future Directions

The autoantibody array done using young mice confirmed the findings that there is synergy between Lyn and Ets1 in the production of IgM autoantibodies, but this needs to be confirmed in older mice. We may get similar results to the ELISAs, where there are accelerated IgM autoantibodies in young mice, but in aged mice the IgM autoantibody levels catch up and there is no observed difference between the Lyn+/- Ets1+/- and the Lyn+/- mice. This would suggest that IgM autoantibody accumulation is accelerated in the compound heterozygotes, because we are observing them in the young mice earlier, but ultimately it does not result in higher levels of IgM autoantibodies. Alternatively, the autoantigen array could reveal differences in the levels or specificity of IgM or IgG autoantibodies in the old Lyn+/- Ets1+/- mice compared to Lyn+/- mice. Very little IgG and IgA anti-DNA Abs were observed by ELISA in old animals. However, IgG and/or IgA autoantibodies targeting other antigens might also be seen on the array because the array contains many more antigen specificities not tested by ELISA. If a difference was observed in the autoantibody profiles of old Lyn Ets1 heterozygotes, then we would conclude that Lyn and Ets1 synergize both to accelerate and exacerbate autoantibody levels.

Further studies into the kidney function of Lyn+/- Ets1+/- mice should be performed. Immunohistochemistry may reveal visible histological changes revealing tissue damage or Ig deposition. Loss of kidney function was not apparent in Lyn+/- Ets1+/- mice as measured by serum creatinine levels; however histological changes and Ig deposition would precede loss of kidney function.

Additional experiments can be done to determine the mechanism by which the interaction of Lyn and Ets1 are regulating T cell activation. Culturing Lyn heterozygous or deficient APCs with Ets1 heterozygous or deficient T cells in an in vitro assay could be used to determine whether T cells get activated better than if either the APCs or T cells were wildtype. Additionally, cell-type specific knockouts of Lyn and Ets1 can be used to determine which cells Lyn and Ets1 must act in to promote T cell activation and autoantibody accumulation. Is the mechanism by which Lyn-/- mice become autoimmune that they fail to express sufficient levels of Ets1 in B cells? To analyze Lyn Ets1 synergy in the B cell pathway, we could test whether forcibly maintaining Ets1 expression in B cells prevents autoimmunity in Lyn-/- mice. While B cell specific deficiency of Lyn is known to result in autoimmune disease (Lamagna, Hu et al. 2014), mice lacking Ets1 only in B cells have not been examined. If we analyzed mice that were B cell specific Ets1 knockouts, would they develop the lupus like phenotype? This

would answer whether complete loss of either Lyn or Ets1 in B cells alone is sufficient to cause autoimmunity.

As previously mentioned, T cell expression of Ets1 and the myeloid compartment's expression of Lyn could be limiting autoimmunity. Therefore a T cell specific knockout of Ets1 could be combined with a Lyn deficiency in macrophages, DCs, or B cells. Similarly, a B cell specific knockout of Ets1 could be combined with a Lyn deficiency in macrophages, DCs, or B cells. Analyzing the loss of Lyn and Ets1 in various combinations of cell types might reveal which cells Lyn and Ets1 are acting in to promote T cell activation and autoantibody production.

Does activation of human B cells downregulate Ets1? Are basal Ets1 levels reduced, or Ets1 downregulation impaired, in SLE B cells? Do SLE patients with low levels of Lyn in their B cells have lower levels of Ets1 (Flores-Borja, Kabouridis et al. 2005)? While these events are observed in mice, confirming these results in humans and in the context of human disease is important in order to implicate the dysregulation of this pathway in SLE pathogenesis and to consider targeting it for therapy. Do patients with both Lyn and Ets1, or both CSK and Ets1, risk alleles develop more severe disease? I aimed to determine whether partial loss of multiple components of a shared pathway

limiting PC accumulation exacerbates disease; however Ets1 protein expression was still relatively high in this model. What would be more relevant to human disease would be to determine whether individuals with polymorphisms in multiple components in this shared pathway develop a more severe disease.

CHAPTER FIVE
CONSEQUENCESES OF ETS1 DOWNREGULATION IN A NON-AUTOIMMUNE SITUATION

INTRODUCTION

I have previously discussed the consequences of Ets1 downregulation in the context of disease, when there is loss of Lyn-dependent inhibitory signaling. Here I explore the consequences of Ets1 downregulation under normal conditions, when inhibitory molecules are intact.

Bruton's tyrosine kinase

Brutons tyrosine kinase (Btk) is a Tec family kinase expressed in B and myeloid cells that mediates BCR and Fc receptor signaling and modulates TLR induced cytokine profiles (Mohamed, Yu et al. 2009). Btk is a target of Lyn-dependent inhibitory pathways in B and myeloid cells (Satterthwaite, Lowell et al. 1998). Mutations in Btk lead to X-linked agammaglobulinemia (XLA), a primary immunodeficiency characterized by lack of mature B cells and serum Ig (Tsukada, Saffran et al. 1993). Btk is required for plasma cell accumulation and autoantibodies, in Lyn-/- mice (Satterthwaite, Lowell et al. 1998, Takeshita, Taniuchi et al. 1998, Gutierrez, Halcomb et al. 2010).

IKK2

IKK2, also known as IKBKB (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta) is part of the canonical IKK complex in the pathway of NF-kappa-B (NFκB) activation. IKK2 phosphorylates inhibitors of NFκB causing dissociation of the inhibitor and activation of NFκB. Subsequent translocation of NFκB into the nucleus results in the expression of hundreds of genes, many involved in homeostasis as well as anti-apoptotic molecules and pro-inflammatory cytokines. NFκB dysregulation and constitutive activation is seen in many autoimmune diseases such as RA, SLE, T1D, and Sjogrens. This is because NFκB plays a role in both positive and negative selection of lymphocytes, B cell homeostasis and survival in the periphery, driving Th17 differentiation, and the secretion of inflammatory cytokines (Brown, Claudio et al. 2008).

Btk and IKK2 are required for Ets1 downregulation

In collaboration with Wei Luo and Lee Ann Garrett-Sinha, Lyn and Ets1 were identified in a pathway limiting PC accumulation. BCR crosslinking was found to downregulate Ets1 via Btk in wildtype B cells, and Btk is required for Ets1 downregulation in Lyn-/- mice (Luo, Mayeux et al. 2014). Finally, IKK2 and JNK were found to regulate Ets1

levels downstream of BCR and TLR signaling (Figure 30). Primary B cells expressing constitutively active (CA) IKK2 are more efficient at downregulating Ets1 in response to activating signals, although their basal Ets1 levels are normal (Luo, Mayeux et al. 2014).

Summary

In a normal, non-autoimmune scenario, BCR crosslinking downregulates Ets1 expression through Btk in an IKK2 dependent manner. Btk-/- mice have reduced basal IgM levels (Satterthwaite and Witte 1996). Is this because they fail to downregulate Ets1? In other words, is Btk-mediated downregulation of Ets1 required to maintain basal Ig levels? To answer this, Btk-/- Ets1-/- mice will be used to determine whether deficiency of Ets1 restores Ig levels in Btk-/- mice. Additionally, I asked whether efficient downregulation of Ets1 upon BCR stimulation is enough to cause PC accumulation or autoimmunity. To answer this, mice expressing IKK2 CA in their B cells will be assayed for the accumulation of PCs and autoimmune disease.

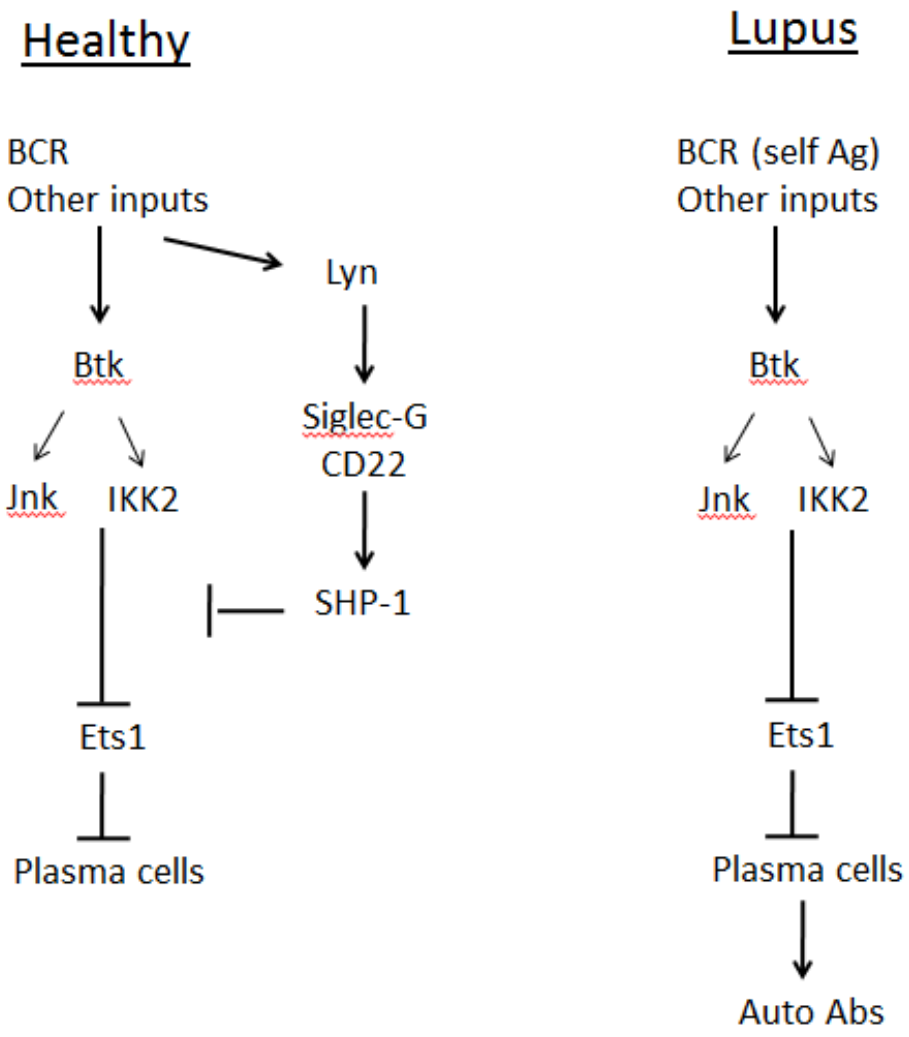


Figure 30. BCR crosslinking downegulates Ets1 via Btk in an IKK2-dependent manner. As previously mentioned, the following biochemical pathway was identified in which Lyn maintains Ets1 expression, thus limiting PC differentiation was defined. Ets1 was found to be downregulated in B cells,through BCR or TLR stimulation in a pathway dependent on PI3K, Btk, IKK2, and JNK. Ets1 levels are downregulated in response to activating signals, allowing for PC differentiation, and inhibitory signals maintain Ets1 levels, limiting PC accumulation

RESULTS

Deficiency of Ets1 is sufficient to restore antibody secreting cells in Btk deficient mice

To determine whether reduced Ig levels in Btk deficient mice is due to an inability to downregulate Ets1, the number of antibody secreting cells in Btk-/- Ets1-/- mice were analyzed by ELISPOT. The reduced number of IgM secreting cells seen in Btk-/- mice was rescued to wt levels in Btk-/- Ets1-/-mice, with both wt and Btk-/- Ets1-/- mice having significantly higher number of IgM secreting cells than Btk-/- mice (Figure 31.A). Additionally, there was a significant increase in the number of IgG secreting cells in Btk-/- Ets1-/- mice compared to wt and Btk-/- mice (Figure 31.B).

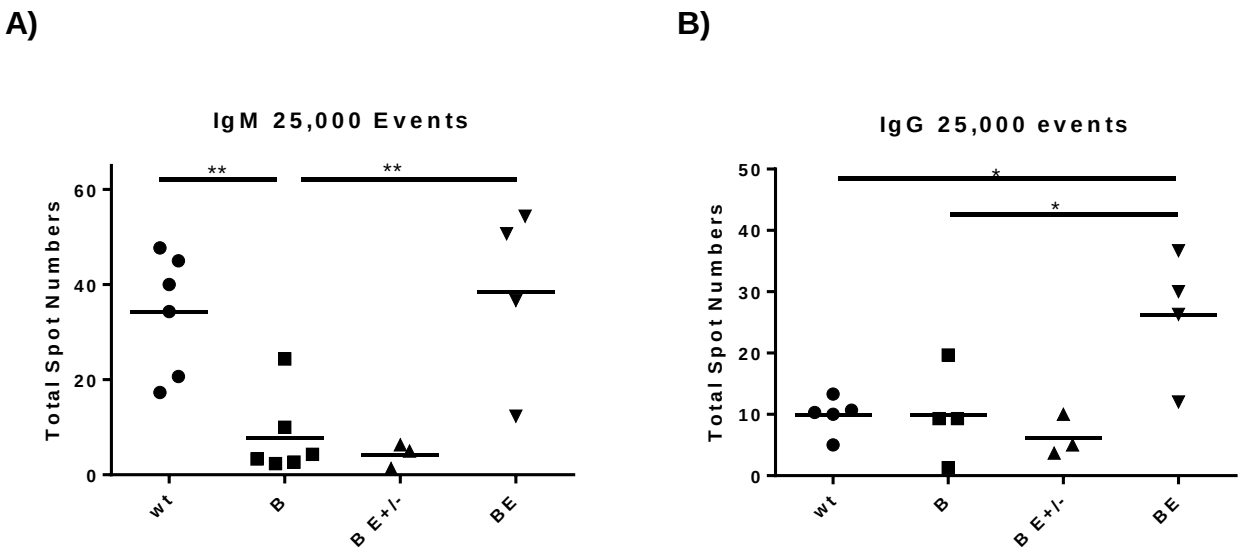


Figure 31. Ets1 deficiency is sufficient to rescue reduced antibody secreting cells in Btk-/- mice. Total splenocytes in young (2-3 months) mice were analyzed by ELISPOT for A) IgM secreting cells in wildtype (wt, n=6), Btk-/- (B, n=6), Btk-/- Ets1+/- (B E+/-, n=3), Btk-/- Ets1-/- (BE, n=4), and B) IgG secreting cells in wildtype (wt, n=5), Btk-/- (B, n=4), Btk-/- Ets1+/- (B E+/-, n=3), Btk-/- Ets1-/- (BE, n=4). P values were obtained using a two-tailed, unpaired student's t test (*p≤0.05 and **p≤0.005).

More efficient downregulation of Ets1 in IKK2 CA mice is not enough to induce autoimmunity

Ets1 deficiency results in PC accumulation and autoimmunity, and B cells from IKK2 CA mice more efficiently downregulate Ets1 (Luo, Mayeux et al. 2014). To determine whether more efficient downregulation of Ets1 in response to activating signals is sufficient to cause autoimmunity, IKK2 CA mice were analyzed for autoantibody production, changes in immune cell subsets, and kidney function,

Immune cell subsets in IKK2 CA mice

It has previously been found that CD19 cre IKK2 CA mice more efficiently downregulate Ets1 following BCR stimulation. To determine whether more efficient downregulation of Ets1 is sufficient to cause autoimmunity, I first analyzed various cell populations within the spleen by flow cytometry. There was a significant decrease in CD4+ T cells and CD4+CD69- T cells in IKK2 CA mice compared to controls (Figure 32.A & C). As previously reported, there was a significant increase in marginal zone (MZ) B cells in IKK2 CA mice compared to controls, however follicular (FO) and PC frequencies were normal (Figure 32.B). There was no observed increase in the frequency of CD11b+ or CD11c+ cells of either the B or myeloid lineage (Figure 32.D & E).

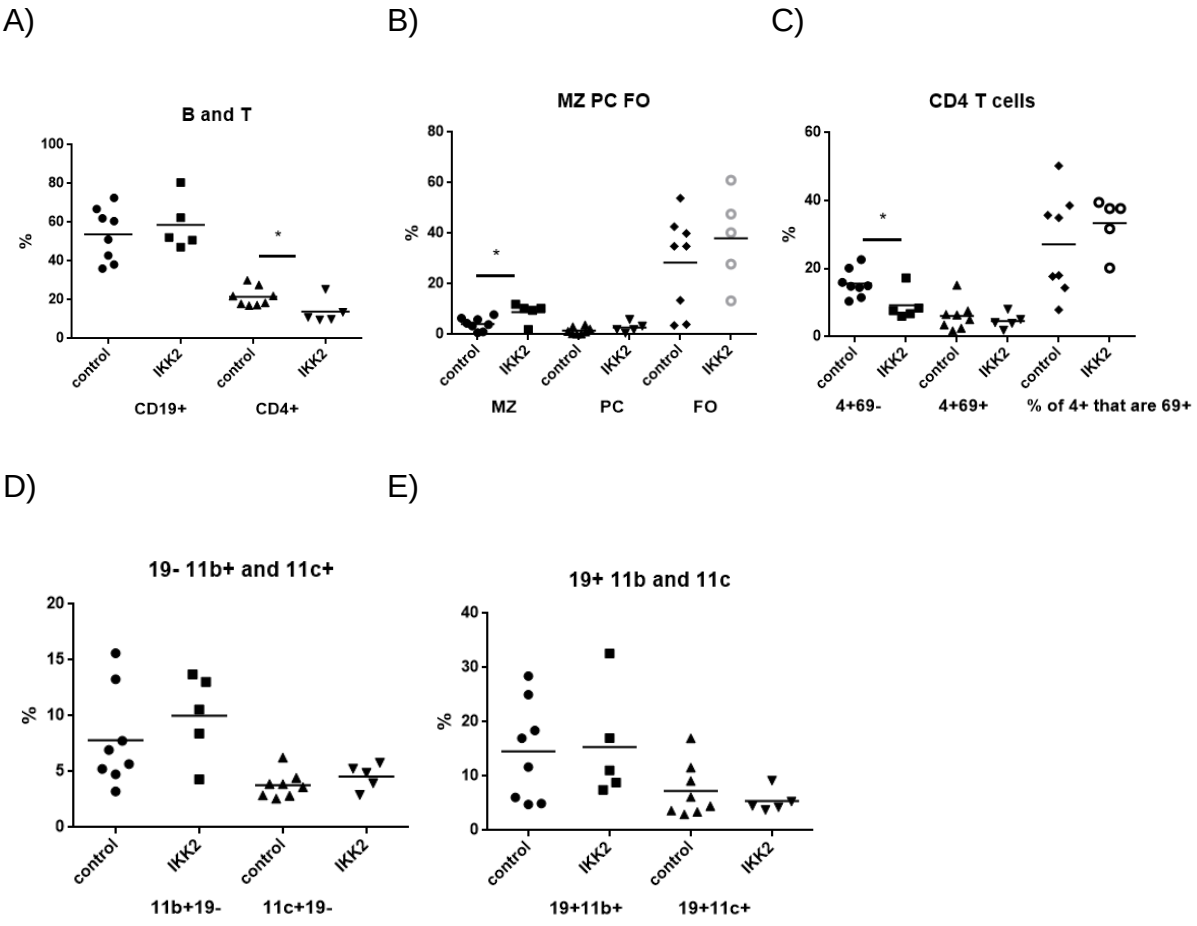


Figure 32. Immune cell subsets within the spleen of IKK2 CA mice. Total splenocytes from old (15-18 months) mice were analyzed for A) CD19+ B cells and CD4+ T cells, B) Marginal zone (MZ) B cells, Plasma cells (PCs), and Follicular (FO) B cells, C) non-activated CD4+ CD69- and activated CD4+ CD69+ T cells, D) CD19- CD11b+ and CD19- CD11c+ cells, and E) CD19+ CD11b+ and CD19+ CD11c+ cells. Wt, CD19 cre, and IKK2 CA (IKK2*) without cre mice were used as controls.

IKK2 CA mice have normal levels of autoantibodies

To determine whether more efficient downregulation of Ets1 results in elevated autoantibody production, IKK2 CA mice were analyzed for anti-ssDNA and anti-dsDNA IgM and IgG by ELISA. Autoantibody levels were not increased in IKK2 CA mice compared to control mice (Figure 33.A, B, C, & D).

Normal levels of serum creatinine in IKK2 CA mice

To determine whether IKK2 mice had increased kidney damage, serum creatinine levels were analyzed as a measure of kidney function. IKK2 mice had similar levels of serum creatinine compared to control mice (Figure 34).

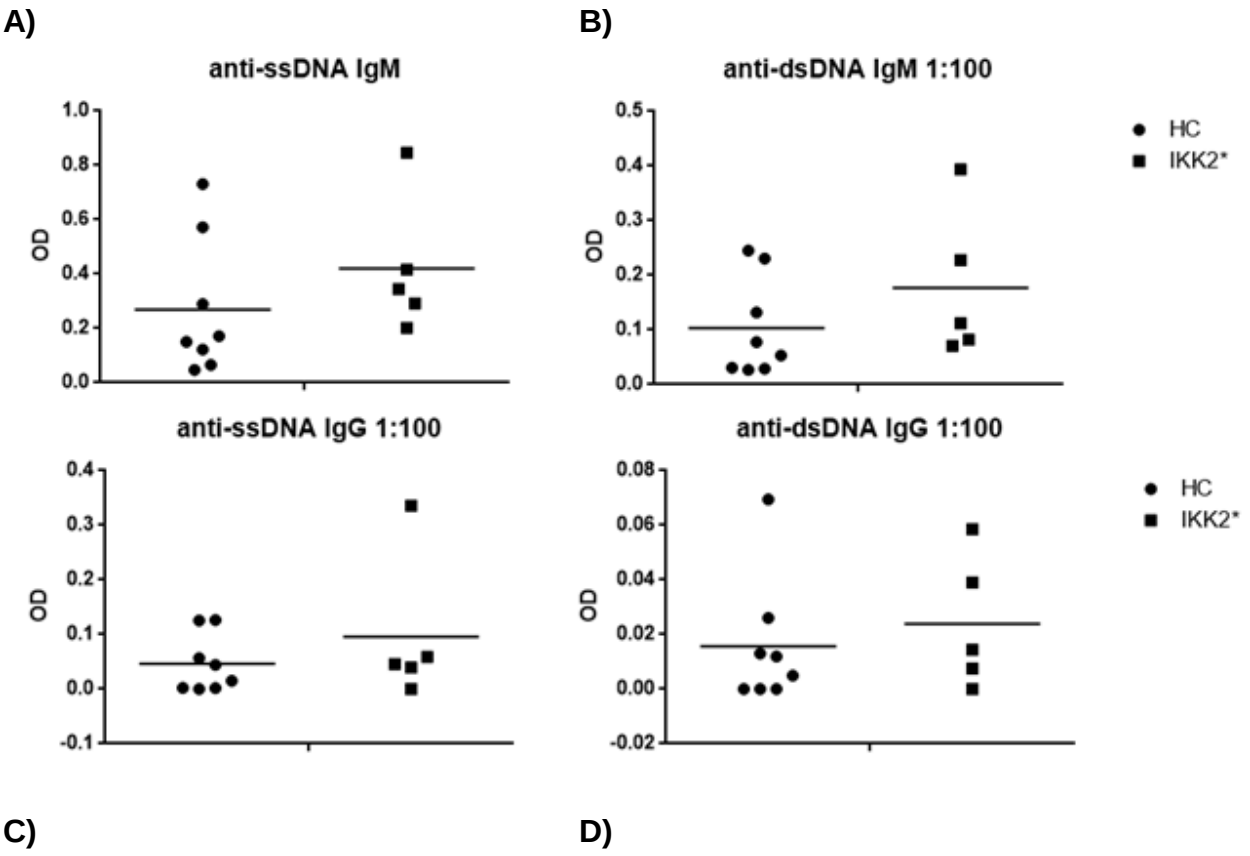


Figure 33. Normal autoantibody production in IKK2 CA mice. Serum (1:100) from old (15-18 months) control (HC n=8) and IKK2 CA (IKK2* n=5) was used to perform ELISAs for A) anti-ssDNA IgM, B) anti-dsDNA IgM, C) anti-ssDNA IgM, and D) anti-dsDNA IgG. Serum was diluted at 1:100. Wt, CD19 cre, and IKK2* without cre mice were used as controls.

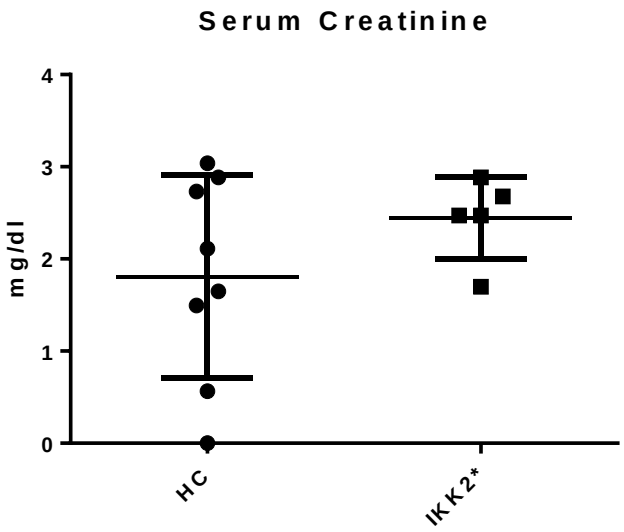


Figure 34. Normal serum creatinine levels in IKK2 CA mice. Serum (1:10) from old (15-18 months) control (HC n=8) and IKK2 CA (IKK2* n=5) was analyzed using a serum creatinine colorimetric assay to determine kidney function. Wt, CD19 cre, and IKK2* without cre mice were used as controls.

Summary

Ets1 downregulation, following BCR crosslinking, is mediated by Btk in an IKK2 dependent manner. To determine whether Btk-mediated downregulation of Ets1 is required to maintain basal Ig levels, antibody secreting cells were analyzed in Btk-/- and Btk-/- Ets1-/- mice. It was determined that Ets1 deficiency is sufficient to restore the number of IgM secreting cells in Btk deficient mice. Additionally, IKK2 CA mice were used to determine whether more efficient downregulation of Ets1 is sufficient to cause autoimmunity. As previously reported, IKK2 CA mice had significantly higher percentages of MZ B cells. There were significantly lower percentages of CD4+ and CD4+ CD69- T cells in IKK2 CA mice compared to controls, however there was no difference observed in autoantibody or serum creatinine levels. This suggests that more efficient downregulation of Ets1 is not sufficient to cause autoimmunity.

CHAPTER SIX
DISCUSSION

INTRODUCTION

SLE is characterized by the loss of tolerance to self antigens and the production of autoantibodies. Autoantibodies can be used in the diagnosis of SLE and to determine prognosis and predict specific disease manifestations. Using a protein array, I identified novel autoantibodies and autoantigens in SLE patients. Anti-STIP1 antibodies were elevated in a subset of SLE patients, and anti-STIP1 IgG was associated with reduced disease activity, suggesting a protective effect. Furthermore, the steps leading to the production of these autoantibodies are dysregulated in SLE patients and murine models of lupus. To determine which molecules are contributing to the accumulation of antibody secreting plasma cells, I focused on known regulators of BCR signaling and plasma cell accumulation, Lyn and Ets1, to determine their interaction and the downstream effects on disease. Lyn Ets1 compound heterozygotes were used to determine whether partial loss of both Lyn and Ets1 would result in accelerated autoimmunity. Synergy was observed for Lyn and Ets1 in the activation of T cells and the accumulation of neutrophils, mDCs, ABCs, and IgM autoantibodies, although there was no acceleration of the production of IgG autoantibodies.

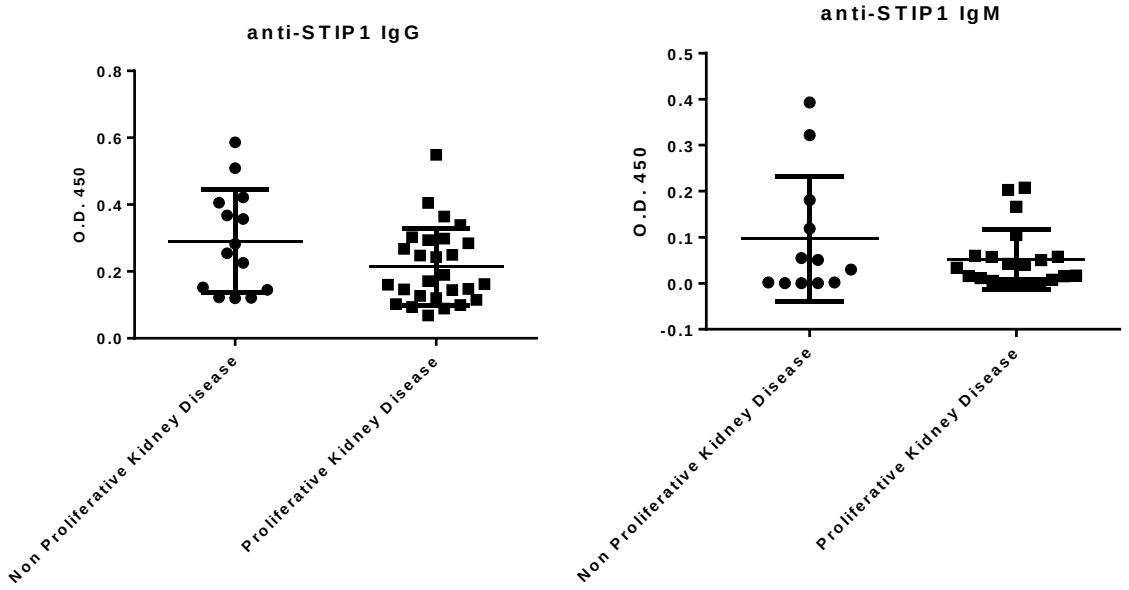
Diagnosis of SLE can be difficult and often patients see multiple doctors and go years from onset of disease before a diagnosis is made. Autoantibodies are used as a diagnostic tool and a way to identify patients that are more likely to manifest with certain symptoms. Depending on the antigen targeted by the autoantibody, patients may be more likely to present with damage to their kidneys, heart, or CNS. While some autoantibody specificities have been identified and are associated with certain manifestations, currently known biomarkers are not very specific and many autoantigens that can mediate damage have not been identified. Because SLE patients have a general loss of immune tolerance and display B cell hyperactivity, it is likely that many important autoantibodies remain undiscovered. Indeed, the protein array bearing 9,000 non-redundant self proteins was successfully able to identify novel autoantibodies elevated in the serum of SLE patients compared to HCs. This serves as an invaluable tool in the lab to aid researchers in discovering novel antibody specificities which may reveal potentially pathogenic and/or protective autoantibodies and pathways targeted in SLE. Thus these studies have important implications beyond our discovery of anti-STIP1 as a novel, and possibly protective, autoantibody in SLE.

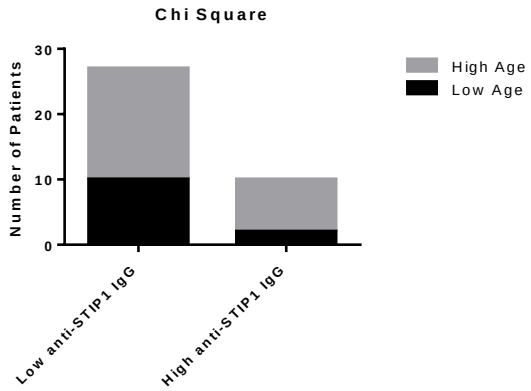
In addition to identifying novel biomarkers, it is crucial to continue to try to understand the mechanisms of disease pathogenesis. B cell dysregulation and autoantibody

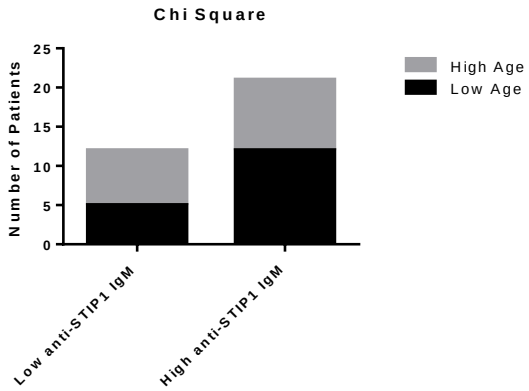
accumulation are hallmark features of SLE. Using mouse models, two checkpoints have been identified in the loss of tolerance. Checkpoint 1 involves polyclonal B cell activation and is followed by checkpoint 2, in which a subset of autoreactive B cells class switch to pathogenic IgG. The protein array studies suggest this is also the case in human SLE patients. Roughly 1200 IgM autoantibodies were identified as significantly different in SLE patients compared to HCs, revealing a global loss of tolerance and accumulation of autoantibodies against many autoantigens that occurs before class switching. Future therapeutic targets may be identified by studying regulators of BCR signaling and PC accumulation, as we have begun to do here with Lyn and Ets1. It will also be important to consider how to target the production of pathogenic autoantibodies while leaving protective B cell functions intact.

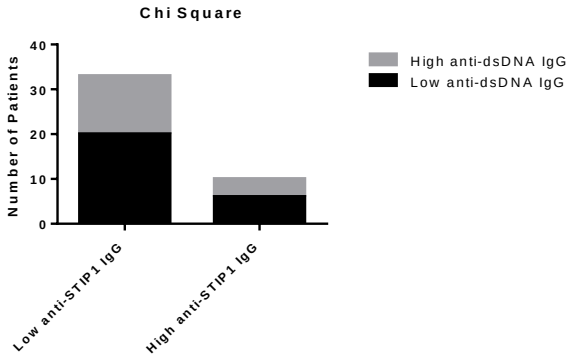
APPENDIX A
CATALOG OF SLE PARAMETERS ANALYZED IN LOW AND HIGH ANTI-STIP1 PATIENTS

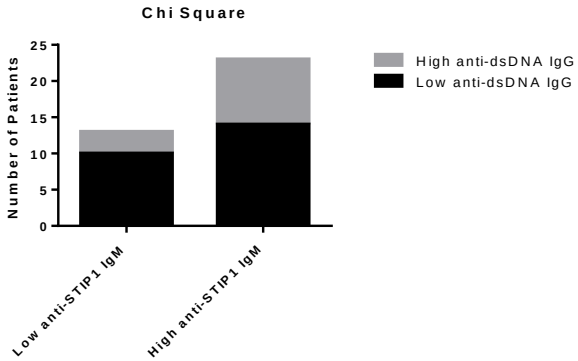
As described in Figure 10, low (less than the mean of HC + 3 SD) and high (greater than mean of HC + 3SD) serum anti-STIP1 IgG and IgM SLE patients were grouped and compared for kidney disease class (Non Proliferative = I, II, & V Proliferative = III & IV), age, anti-dsDNA IgG, Urine Creatinine, Urine protein, UProtein/UCr, BUN, HCO3, ESR, CRP, C3, gender, and ethnicity. $p > 0.05$ for all as measured by two-tailed student's t-test or Fisher's exact test.

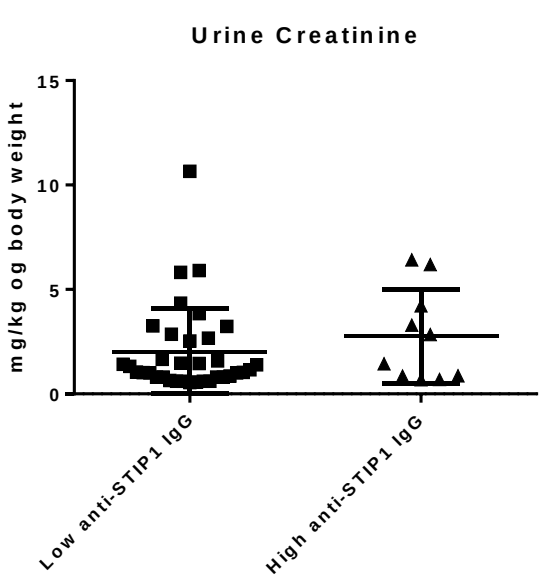


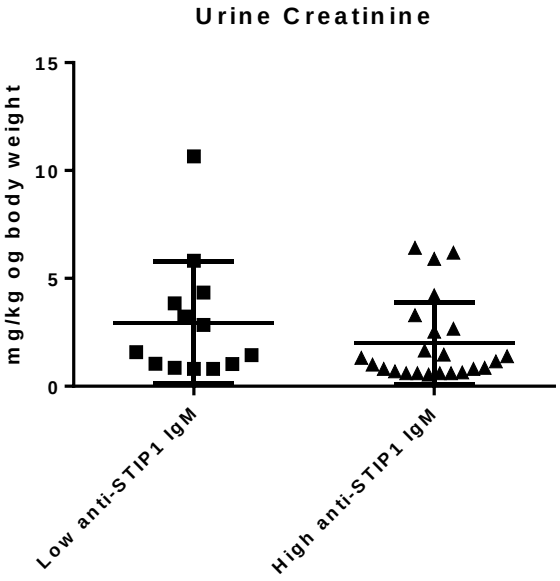

Chi Square
Number of Patients
30
20
10
0
High Age
Low Age
Low anti-STIP1 IgG
High anti-STIP1 IgG

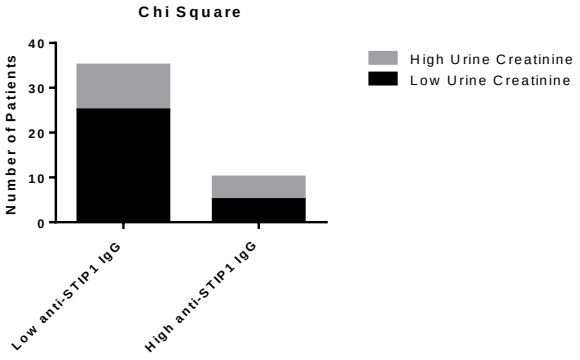

Chi Square
Number of Patients
25
20
15
10
5
0
High Age
Low Age
Low anti-STIP1 IgM
High anti-STIP1 IgM

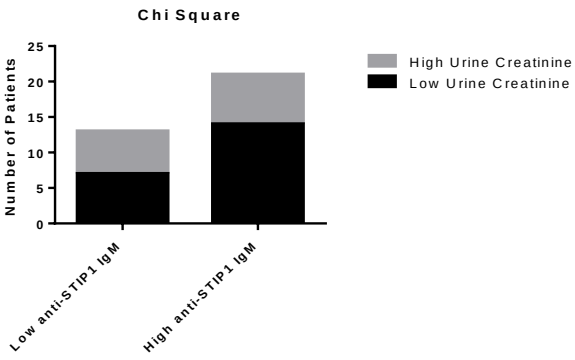

Chi Square
Number of Patients
40
30
20
10
0
High anti-dsDNA IgG
Low anti-dsDNA IgG
Low anti-STIP1 IgG
High anti-STIP1 IgG

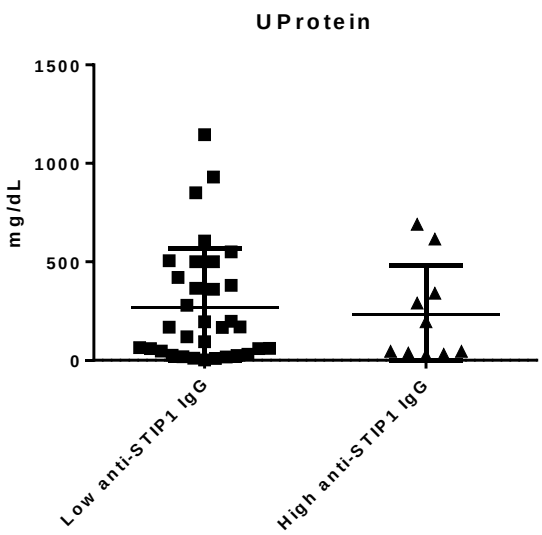

Chi Square
Number of Patients
25
20
15
10
5
0
High anti-dsDNA IgG
Low anti-dsDNA IgG
Low anti-STIP1 IgM
High anti-STIP1 IgM

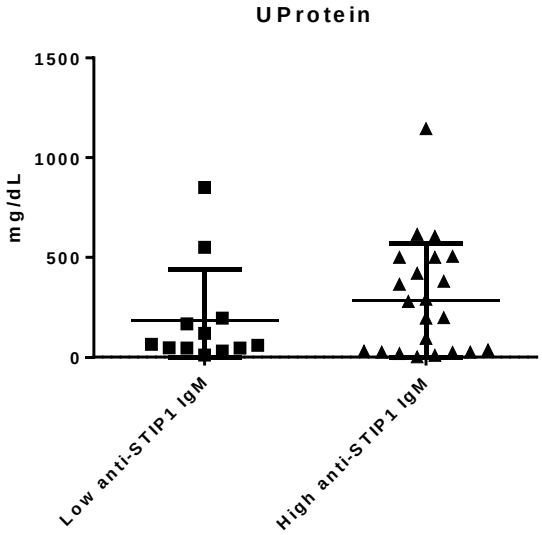

Urine Creatinine
mg/kg og body weight
15
10
5
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

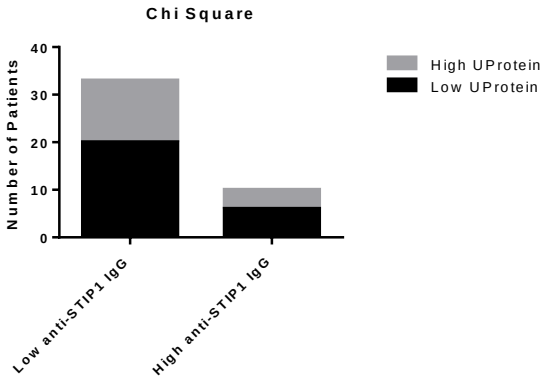

Urine Creatinine
mg/kg og body weight
15
10
5
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

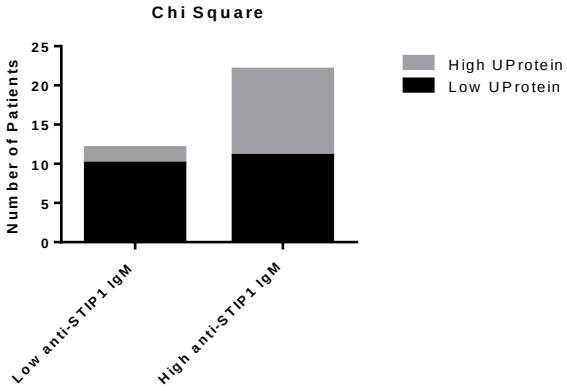

Chi Square
Number of Patients
40
30
20
10
0
High Urine Creatinine
Low Urine Creatinine
Low anti-STIP1 IgG
High anti-STIP1 IgG

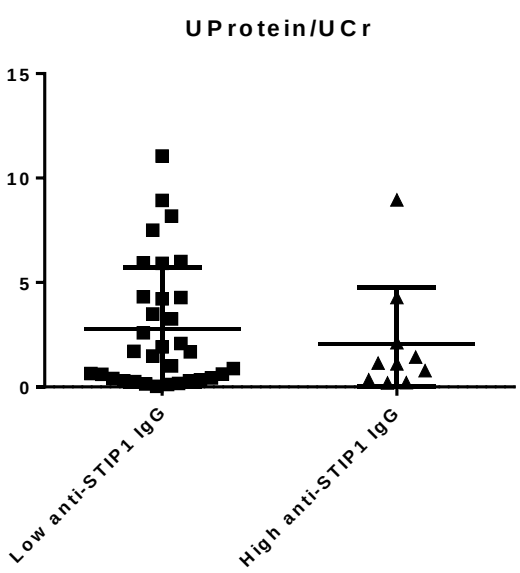

Chi Square
Number of Patients
25
20
15
10
5
0
High Urine Creatinine
Low Urine Creatinine
Low anti-STIP1 IgM
High anti-STIP1 IgM

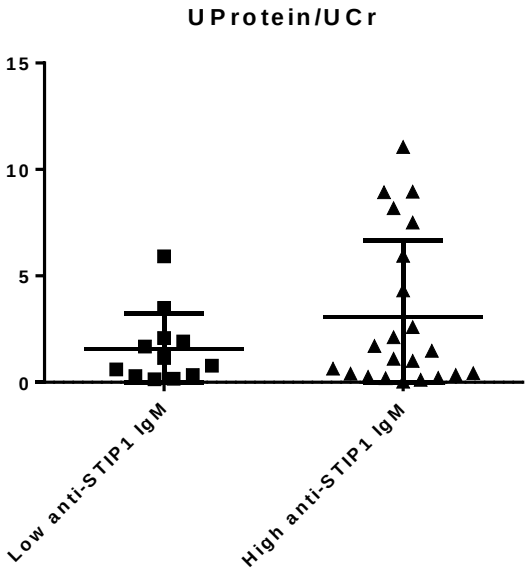

UProtein
1500
1000
500
0
mg/dL
Low anti-STIP1 IgG
High anti-STIP1 IgG

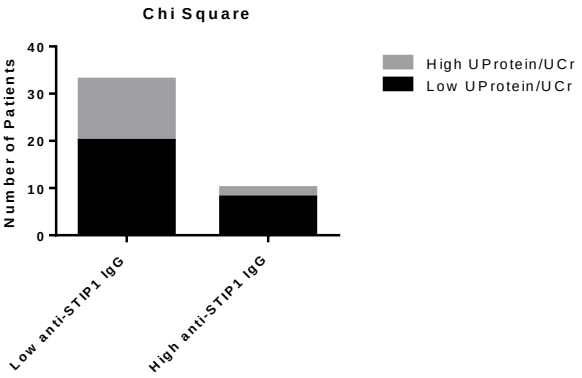

UProtein
1500
1000
500
0
mg/dL
Low anti-STIP1 IgM
High anti-STIP1 IgM

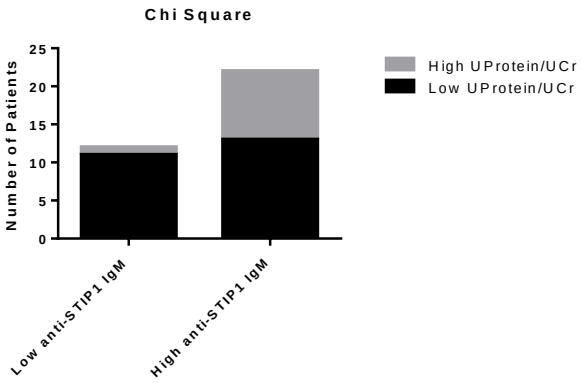

Chi Square
40
30
20
10
0
Number of Patients
High UProtein
Low UProtein
Low anti-STIP1 IgG
High anti-STIP1 IgG

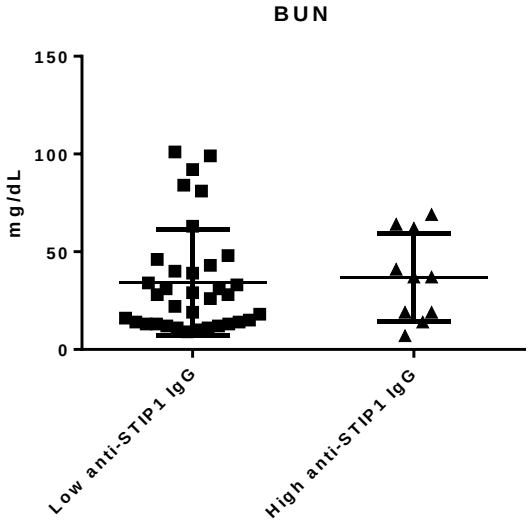

Chi Square
25
20
15
10
5
0
Number of Patients
High UProtein
Low UProtein
Low anti-STIP1 IgM
High anti-STIP1 IgM

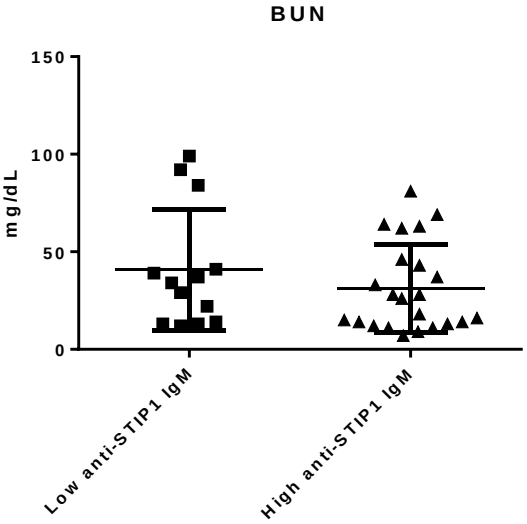

UProtein/UCr
15
10
5
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

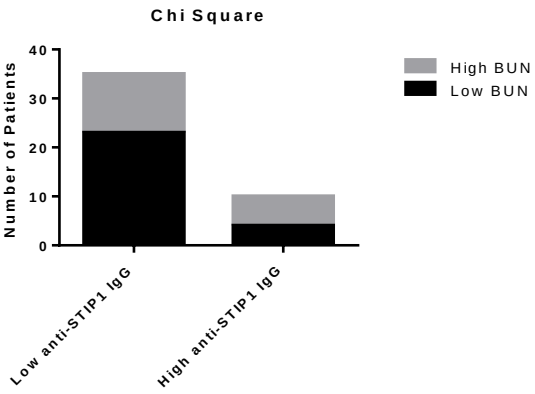

UProtein/UCr
15
10
5
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

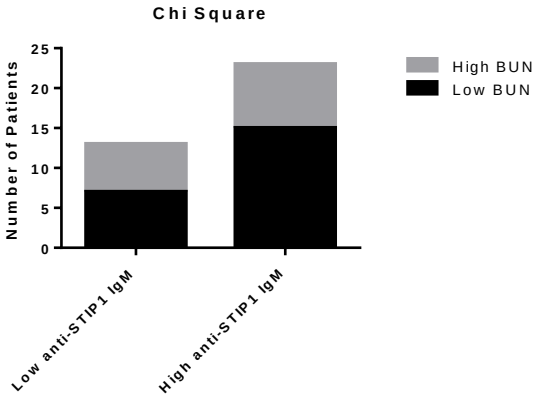

Chi Square
Number of Patients
40
30
20
10
0
Low anti-STIP1 IgG
High anti-STIP1 IgG
High UProtein/UCr
Low UProtein/UCr

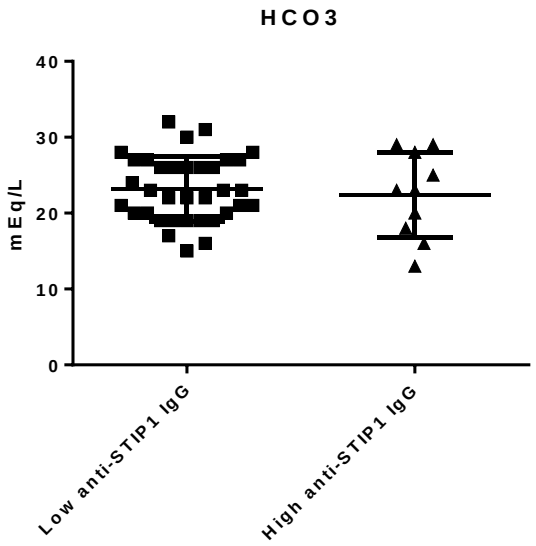

Chi Square
Number of Patients
25
20
15
10
5
0
Low anti-STIP1 IgM
High anti-STIP1 IgM
High UProtein/UCr
Low UProtein/UCr

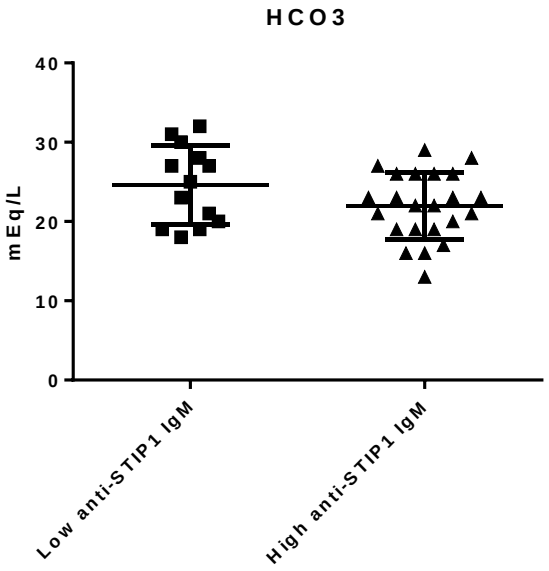

BUN
150
100
50
0
mg/dL
Low anti-STIP1 IgG
High anti-STIP1 IgG

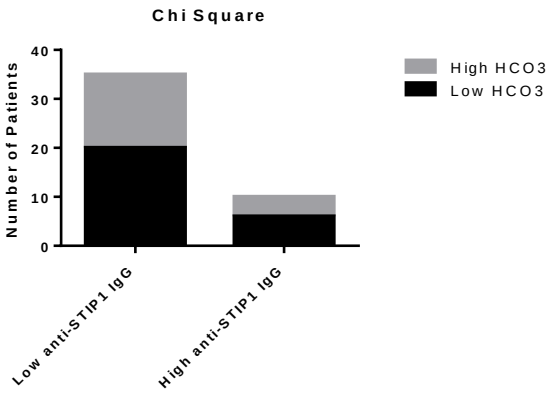

BUN
150
100
50
0
mg/dL
Low anti-STIP1 IgM
High anti-STIP1 IgM

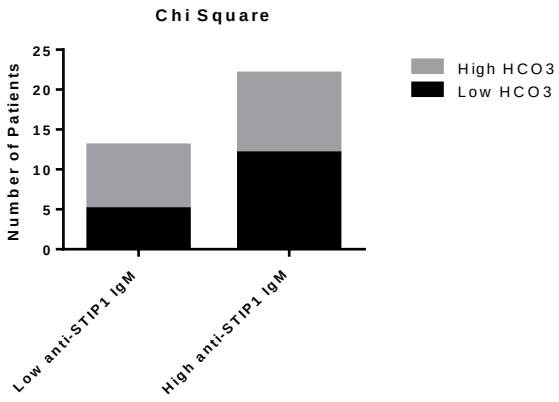

Chi Square
40
30
20
10
0
Number of Patients
High BUN
Low BUN
Low anti-STIP1 IgG
High anti-STIP1 IgG

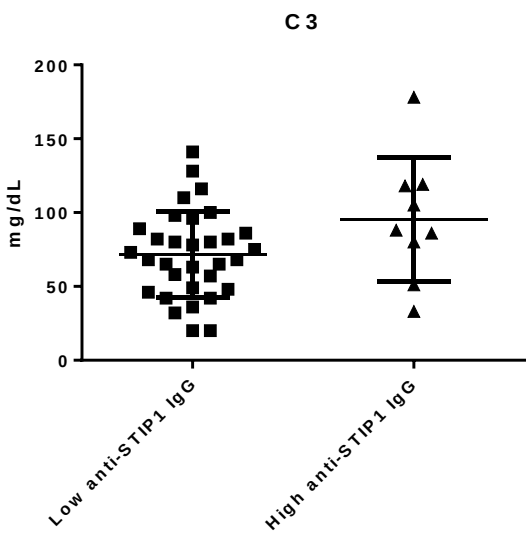

Chi Square
25
20
15
10
5
0
Number of Patients
High BUN
Low BUN
Low anti-STIP1 IgM
High anti-STIP1 IgM

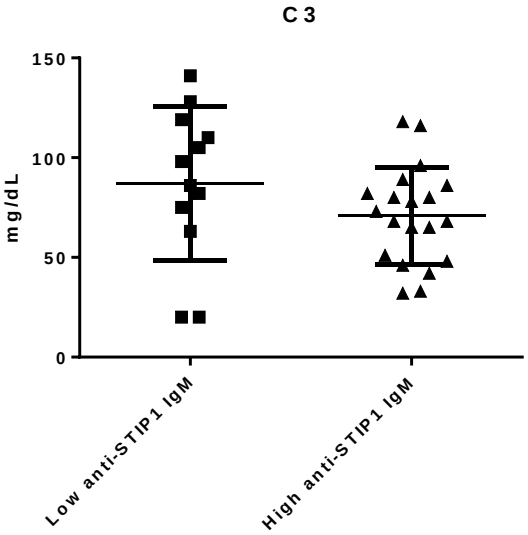

HCO3
mEq/L
40
30
20
10
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

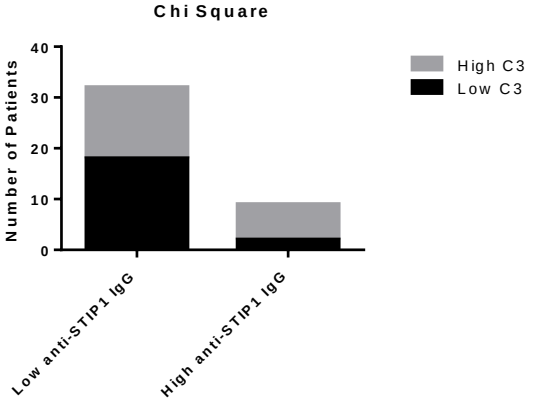

HCO3
mEq/L
40
30
20
10
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

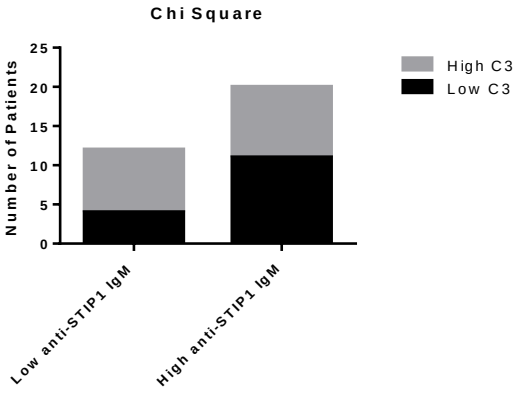

Chi Square
Number of Patients
40
30
20
10
0
High HCO3
Low HCO3
Low anti-STIP1 IgG
High anti-STIP1 IgG

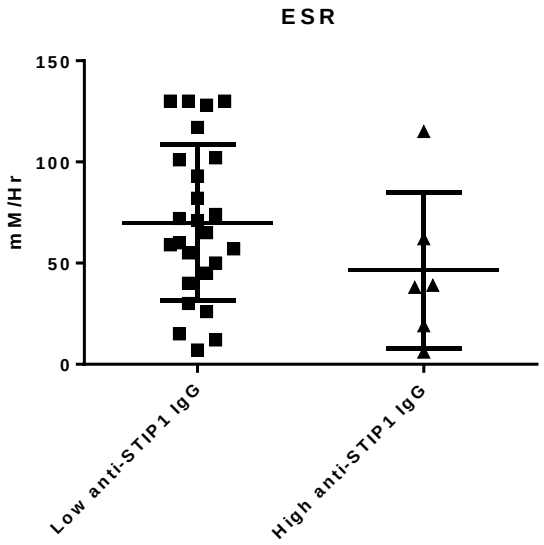

Chi Square
Number of Patients
25
20
15
10
5
0
High HCO3
Low HCO3
Low anti-STIP1 IgM
High anti-STIP1 IgM

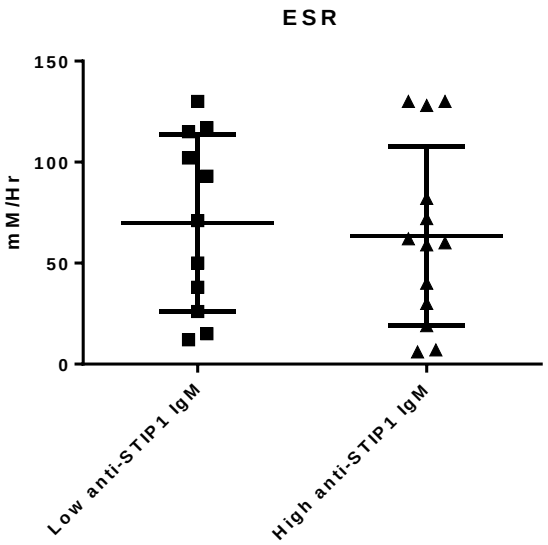

C3
mg/dL
200
150
100
50
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

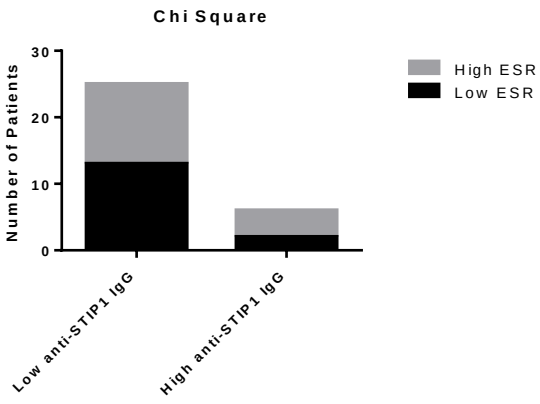

C3
mg/dL
150
100
50
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

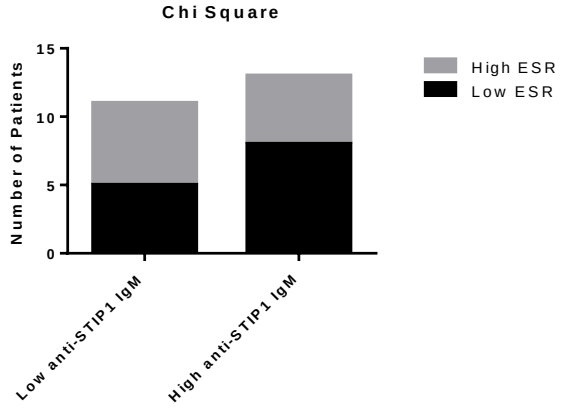

Chi Square
Number of Patients
40
30
20
10
0
High C3
Low C3
Low anti-STIP1 IgG
High anti-STIP1 IgG

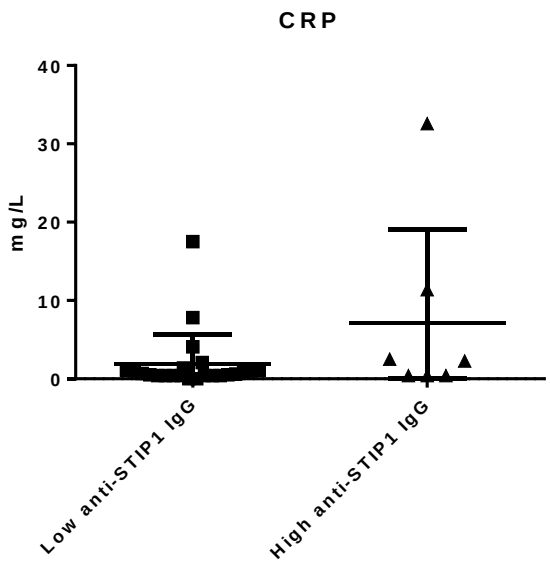

Chi Square
Number of Patients
25
20
15
10
5
0
High C3
Low C3
Low anti-STIP1 IgM
High anti-STIP1 IgM

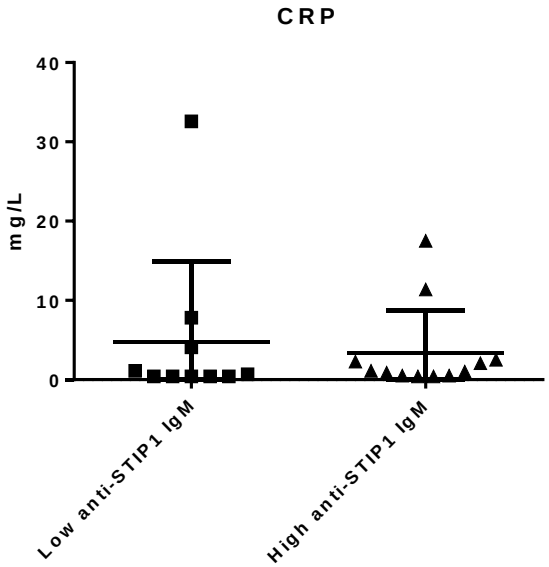

ESR
150
100
50
0
mM/Hr
Low anti-STIP1 IgG
High anti-STIP1 IgG

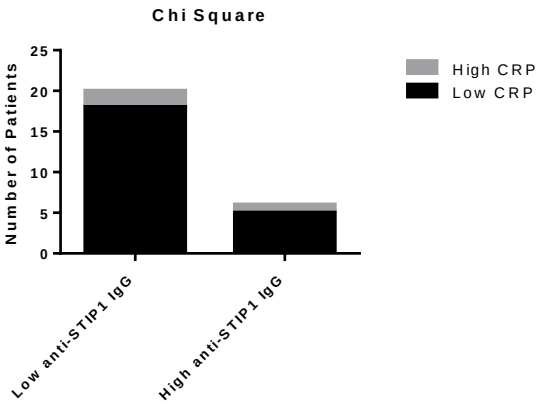

ESR
150
100
50
0
mM/Hr
Low anti-STIP1 IgM
High anti-STIP1 IgM

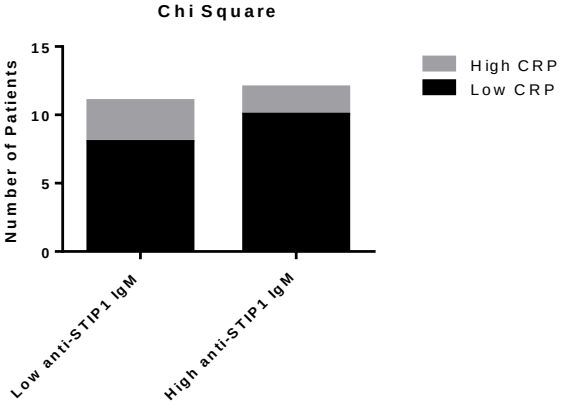

Chi Square
30
20
10
0
Number of Patients
High ESR
Low ESR
Low anti-STIP1 IgG
High anti-STIP1 IgG

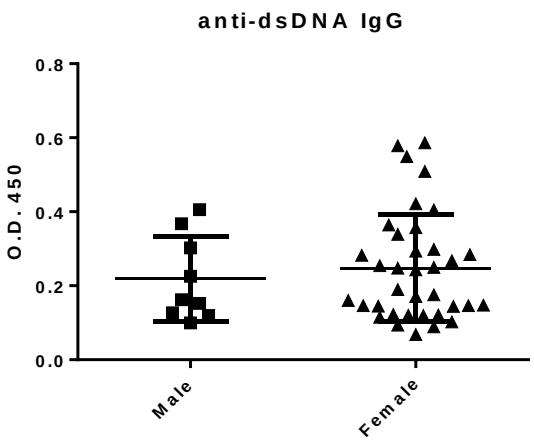

Chi Square
15
10
5
0
Number of Patients
High ESR
Low ESR
Low anti-STIP1 IgM
High anti-STIP1 IgM

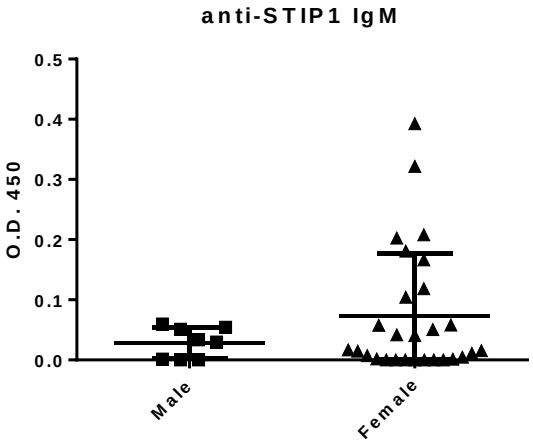

CRP
mg/L
40
30
20
10
0
Low anti-STIP1 IgG
High anti-STIP1 IgG

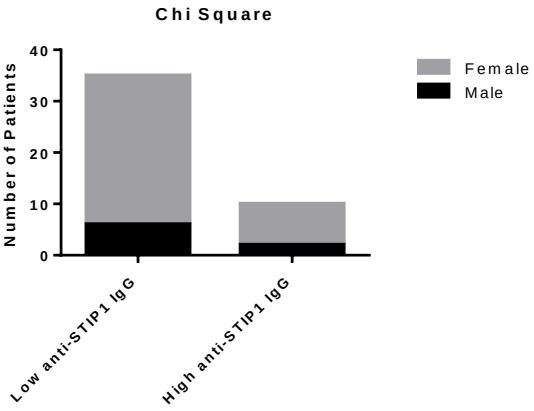

CRP
mg/L
40
30
20
10
0
Low anti-STIP1 IgM
High anti-STIP1 IgM

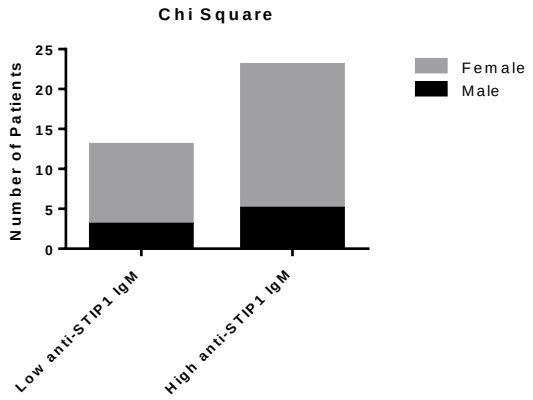

Chi Square
Number of Patients
25
20
15
10
5
0
High CRP
Low CRP
Low anti-STIP1 IgG
High anti-STIP1 IgG

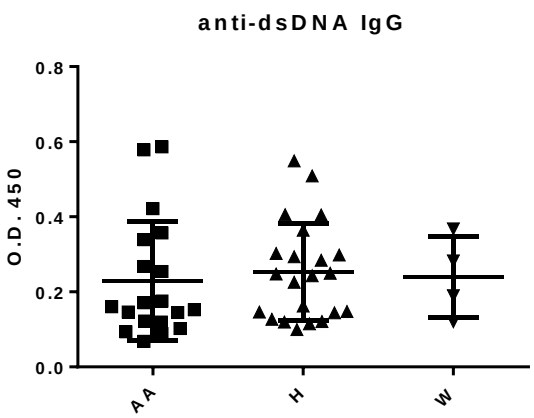

Chi Square
Number of Patients
15
10
5
0
High CRP
Low CRP
Low anti-STIP1 IgM
High anti-STIP1 IgM

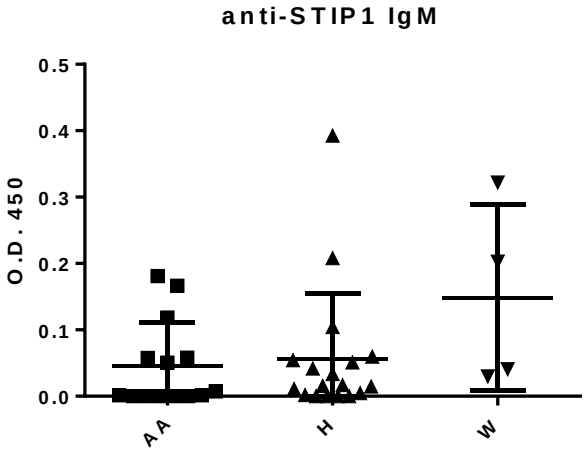

anti-dsDNA IgG
O.D. 450
0.8
0.6
0.4
0.2
0.0
Male
Female

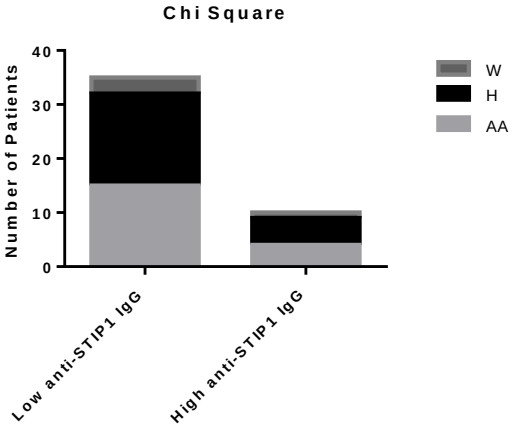

anti-STIP1 IgM
O.D. 450
0.5
0.4
0.3
0.2
0.1
0.0
Male
Female

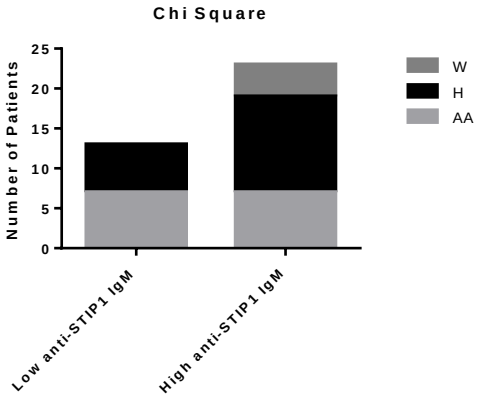

Chi Square
Number of Patients
40
30
20
10
0
Female
Male
Low anti-STIP1 IgG
High anti-STIP1 IgG


Chi Square
Number of Patients
25
20
15
10
5
0
Female
Male
Low anti-STIP1 IgM
High anti-STIP1 IgM


anti-dsDNA IgG
O.D. 450
0.8
0.6
0.4
0.2
0.0
AA
H
W


anti-STIP1 IgM
O.D. 450
0.5
0.4
0.3
0.2
0.1
0.0
AA
H
W


Chi Square
Number of Patients
40
30
20
10
0
W
H
AA
Low anti-STIP1 IgG
High anti-STIP1 IgG


Chi Square
Number of Patients
25
20
15
10
5
0
W
H
AA
Low anti-STIP1 IgM
High anti-STIP1 IgM

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