MOLECULAR PLAYERS IN LUPUS—

LEADS FROM PROTEOMIC SCREENS

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DEDICATION

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LEADS FROM PROTEOMIC SCREENS

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MOLECULAR PLAYERS IN LUPUS—

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Systemic Lupus Erythematosus is a multifactorial systemic autoimmune disorder marked by anti-nuclear antibodies (ANA), rashes and photosensitivity, joint inflammation, nephritis, and other clinical criteria. SLE develops through the breakdown of three major checkpoints: adaptive immune tolerance, peripheral innate responsiveness, and end-organ inflammation. Adaptive immune dysfunction produces autoantibodies leading to immune complex formation and deposition in the

skin, joints, and kidneys. Innate immunity plays an important role in determining disease severity and progression.

Molecular markers in patient blood and urine improve diagnosis and treatment of SLE. Proteomic screens identify such markers and provide important clues about disease pathogenesis. We have discovered that soluble Axl receptor tyrosine kinase, the Wnt/β-catenin pathway-related factors, and rare fibrinogen alpha chain variant A-α-E are elevated in the serum of patients with SLE. Here I explore these factors and their contributions to disease. I find that Axl tyrosine kinase is sheared from the surface of lupus-prone and SLE CD19+ and CD11b+/CD14+ leukocytes by proteases ADAM10 and TACE (ADAM17) to abrogate macrophage anti-inflammatory signaling through Twist. I further find that β-catenin is dysregulated in SLE but the deletion of β-catenin in lupus-prone macrophages does not appreciably change disease course. Lastly, I find that fibrinogen alpha chain isoform Aα-E may be associated with aPL-negative thrombotic complications in SLE.

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Figure 31 – “Trans” sAxl inhibits Gas6-mediated *Twist* mRNA induction. (A) B6.Axl-/- and B6.Sle1.Yaa splenocytes were cultured for 48 hours in 1μg/ml LPS. Supernatants were isolated and used to treat fresh B6 splenocytes for 24 hours with or without 400ng/ml Gas6. The ratio of Gas6-treated to control is reported. B6.Sle1.Yaa splenocyte supernatant inhibits *Twist* expression in healthy B6 splenocytes (p=0.0017). (B) B6 and B6.Sle1.Yaa splenocytes were cultured in the presence or absence of ADAM10/TACE inhibitors in 1μg/ml LPS and supernatants for 48 hours, after which supernatants were isolated and used to treat healthy B6 splenocytes with or without 400ng/ml Gas6 over 24 hours. The ratio of Gas6-treated to LPS-only control is reported. Splenocytes not treated with supernatants were simultaneously treated with LPS with or without Gas6 and measured as a healthy baseline control (dotted line). Treatment of B6.Sle1.Yaa splenocytes with ADAM10/TACE inhibitors rescues supernatant-induced blockage of Gas6-induced *Twist* expression. This suggests that a factor cleaved by ADAM10 and/or TACE from B6.Sle1.Yaa splenocytes blocks Gas6 stimulation of the Axl receptor. (C) B6.Sle1.Axl+/+ and B6.Sle1.Axl-/- splenocytes were cultured in 1μg/ml LPS for 48 hours, after which supernatants were isolated and used to treat fresh healthy B6 splenocytes in the presence or absence of 400ng/ml Gas6 over 24 hours. The ratio of Gas6-treated to LPS-only control is reported. Splenocytes not treated with

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LIST OF DEFINITIONS

Acute phase proteins – a classification of proteins that are produced during inflammation, generally by the liver.

Adaptive immunity – an immune response characterized by specificity, memory, and improvement that generally occurs after the innate immune response.

ANA – antinuclear antibody.

Antibody – a soluble form of the B cell receptor secreted by plasma cells that recognizes specific motifs called epitopes on an antigen.

Antigen – any surface that may be recognized by an antibody.

Apoptosis – programmed cell death.

APS – antiphospholipid syndrome.

Arthritis – joint inflammation.

Autoantibody – an antibody which binds self antigens, *e.g.* double-stranded DNA.

Autoimmunity – an immune system reaction that targets self antigens.

Axl – Axl tyrosine kinase.

B6 – C57BL/6 mouse strain, generally used as a healthy control background.

B cell – a lymphocyte that may present antigen and which produces antigen-specific receptors.

BMDM – Bone marrow-derived macrophages

CAD – coronary artery disease.

CD (cluster of differentiation) – a nomenclature used to define immune cell receptors.

CFA – complete Freund's adjuvant, an adjuvant comprising killed mycobacterium used to potentiate an immune response.

Chemokines – a subset of cytokines that direct trafficking of immune cells.

Chemotaxis – the movement of cells along a gradient of attracting or repelling factors.

CVA – cerebrovascular accident.

Cytokines – soluble effector proteins that facilitate communication between immune cells.

DC – dendritic cell.

DVT – deep vein thrombosis.

ELISA – enzyme–linked immunosorbent assay, a plate assay using antibodies to determine a sample analyte concentration.

Epitope – a part of an antigen recognized by an antibody.

Inflammation – the immune system response involving increased access to a site or sites of potential injury.

Innate immunity – the immune response that generally first recognizes pathogens and does not exhibit specificity, memory, or improvement.

Interleukin (IL) – a nomenclature used to define cytokines produced by leukocytes.

iTRAQ – Isobaric tags for relative and absolute quantitation.

Leukocytes – white blood cells (WBCs), cells of the immune system comprising macrophages, lymphocytes, and others.

LPS – lipopolysaccharide.

Lymphocytes – a population of immune cells comprising B and T cells as well as natural killer cells (NK cells) and some dendritic cell (DC) populations.

Lymphoproliferation – an exuberant increase in lymphocyte (*i.e.* B cell, T cell) numbers *in vivo*.

Macrophage – a myeloid cell that acts to engulf particles, present antigens, and produce cytokines during immune responses and wound healing.

Mass spectrometry – an experimental method in which sample fragments are ionized and ejected across a magnetic field to be analyzed according to mass-to-charge ratios.

Myeloid – of or having to do with the lineage of immune cells that function in the innate immune response.

Natural killer (NK) cells – lymphocytes with invariant cell surface receptors which function in antiviral immunity and transplant rejection.

Nephritis – inflammation of the kidney

Neutrophil – a myeloid cell that acts immediately during an innate immune response and primarily acts to engulf particles and secrete cytokines.

NSAIDS – non–steroidal anti–inflammatory drugs.

Pathogen-associated molecular pattern (PAMP) receptors – receptors that recognize common bacterial, viral, and parasitic factors.

PBMC – peripheral blood mononuclear cell.

PCR – polymerase chain reaction, a method for amplifying nucleotide sequences.

pDC – plasmacytoid dendritic cell.

PE – pulmonary embolism.

Phagocytosis – the engulfment of extracellular material by a cell.

Platelet – a megakaryocyte fragment that forms important structural portions of clots.

Polymorphic genes – genes which vary considerably within a population and may result in differing phenotypes.

Proteomic screen – a method for discovering protein markers pertinent to disease.

Receptor – a protein on or in a cell that detects its substrate and transduces a downstream signal in the cell.

Renal – of or having to do with the kidney.

RT–PCR – reverse–transcriptase polymerase chain reaction, a method for determining the relative quantity of a given mRNA transcript in a sample.

SLE – Systemic Lupus Erythematosus, a multifactorial systemic autoimmune disorder that may involve kidney, joint, and skin immune complex deposition.

SLEDAI – SLE activity index, used to evaluate current SLE severity (see Table 2, p3).

Spontaneous lupus models – models of disease in which genetic background drives the development of disease without exogenous stimuli.

T cells – lymphocytes with specific receptors that recognize short (9-15AA) peptide sequences generated by proteolysis.

Thrombosis – the formation of a blood clot.

Thrombotic complication – an adverse clinical event such as deep vein thrombosis (DVT), pulmonary embolism (PE), and/or cerebrovascular accident (CVA) resulting from a clot.

TLR – toll-like receptors – a family of Pathogen-associated molecular pattern (PAMP) receptors that recognize common bacterial, viral, and parasitic products.

Tolerance – mechanisms that remove autoreactive B and T cells from activity *in vivo.*

Western Blot – a gel–based assay for measuring the content of a specific protein analyte.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

THE HEALTH BURDEN OF SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic Lupus Erythematosus (SLE) is a heterogeneous group of autoimmune disorders defined by a consensus of clinical and laboratory criteria. Approximately 1 in 2000 people in the United States suffer from some form of SLE, costing approximately $19B each year.[1,2]

SLE develops through the breakdown of three major checkpoints: adaptive immune tolerance, peripheral innate responsiveness, and end-organ inflammation.[3] Adaptive immune dysfunction produces autoantibodies leading to immune complex formation and deposition in the skin, joints, and kidneys. Innate immunity plays an important role in determining disease severity and progression.[4] Criteria for SLE diagnosis include malar rash, anti-nuclear antibodies (ANA), arthritides/arthralgias, photosensitivity, and renal involvement. These signs and symptoms result from immune recognition of nuclear antigens, deposition of immune complexes, and chronic inflammation. Factors and pathways that modulate immunity and related pathways will impact the course of disease, particularly in end organ systems where most major, life-threatening disease manifestations occur.

SLE has diverse manifestations that make it challenging to diagnose, follow, and treat. Just four of eleven American College of Rheumatology (ACR) criteria are required for a diagnosis of SLE (see Table 1).[5] These criteria illustrate the broad manifestations that occur commonly in SLE.

Criterion	Description
Malar rash	Fixed erythema the nasolabial folds
Discoid rash	Erythematous raised patches with adherent keratotic scaling
Photosensitivity	Skin rash in reaction to sunlight
Oral ulcers	Usually painless
Nonerosive arthritis	≥2 joints tender, swelling, or effusive
Pleuritis/pericarditis	By auscultation or EKG
Renal Disorder	Persistent proteinuria or urinary cell casts
Neurologic Disorder	Otherwise unexplained seizures or psychoses
Hematologic Disorder	Anemia, leukopenia, lymphopenia, or thrombocytopenia
Immunologic Disorder	Abnormal titer of anti-DNA, anti-Sm, anti-phospholipid antibodies
Positive ANA	Abnormal anti-nuclear antibody titre

Table 1 – Four of eleven accepted criteria from the American College of Rheumatology are considered sufficient for a diagnosis of Systemic Lupus Erythematosus.

While the ACR criteria as a group are highly sensitive and specific (96% sensitive, 96% specific) they represent an additional burden in clinical diagnosis. SLE is listed in differential diagnoses for many more common diseases such as acute pericarditis, antiphospholipid syndrome, fibromyalgia, Hepatitis C, infectious mononucleosis, infective endocarditis, Lyme disease, lymphomas, rheumatoid arthritis, and other autoimmune disorders. For this reason, most common complaints requiring a clinical

workup for SLE are caused by other etiologies.[6] SLE is often considered in the diagnosis of any new arthritis, kidney problem, rash, mental disorder, or blood abnormality. Further, some SLE criteria may be positive in otherwise-healthy individuals. For instance, antinuclear antibodies are present in up to 31% of healthy persons without SLE.[7] These confounding results add considerably to the overall clinical costs of SLE.

Once SLE is diagnosed, charting disease course is essential for adjusting medication type and dosing, calculating odds ratios, and recognizing disease-related complications. SLEDAI (SLE Disease Activity Index) is an exhaustive set of twenty-three weighted criteria used to monitor an individual patient's current SLE progression. Each criterion that has occurred in the ten days prior to a visit is counted and a weighted score added for each (see Table 2).

Symptom	Weight
Seizure	8
Organic Brain Syndrome	8
Visual Disturbance	8
Cranial Nerve Disorder	8
Lupus Headache	8
CVA	8
Vasculitis	8
Arthritis	4
Myositis	4
Urinary Casts	4
Hematuria	4
Proteinuria	4
Pyuria	4
New Rash	2
Alopecia	2
Mucosal Ulcers	2
Pleurisy	2
Pericarditis	2
Low Complement	2
Increased DNA binding	2
Fever	1
Thrombocytopenia	1
Leukopenia	1

Table 2 – SLE Disease Activity Index (SLEDAI) is used to estimate disease progression.

Increases of more than twelve points from the prior visit indicate a severe “flare” or exacerbation of disease.[8,9] SLEDAI scores correlate with independent clinician estimates, but both are confounded by factors such as unreported events and drug regimen. The many potential complications of SLE present additional costs as they require the consult of many specialists, including rheumatologists, cardiologists, pulmonologists, nephrologists, dermatologists, neurologists, and hematologists.

SLE is a chronic disease and treatments are palliative. Mild disease is treated symptomatically with NSAIDs (non-steroidal anti-inflammatory drugs), antimalarials, and topical corticosteroid creams or low-dose corticosteroids.[10] Severe disease is treated with cytotoxic drugs like azathioprine, mycophenolate mofetil, or methotrexate. These treatments carry severe side-effects. More targeted biologics like Rituxan show mixed results in SLE clinical trials.

Metabolic and proteomic markers aid in the diagnosis, treatment, and follow-up of SLE. These markers may predict the likelihood of an incomplete SLE patient developing fulminant disease, the occurrence of flares, and the effectiveness of a given treatment regimen. They may further predict the likelihood of specific complications, allowing efficient intervention and care. Lastly, markers may also offer important clues about disease pathogenesis. This may lead to new treatments.

SPONTANEOUS MODELS OF SYSTEMIC LUPUS ERYTHEMATOSUS

Multiple types of mouse models aid the exploration of human diseases like SLE: induced, spontaneous, and gene-targeted models. Induced mouse models occur as the result of direct intervention by the researcher. For instance, EAE (Experimental Autoimmune Encephalomyelitis) is a demyelinating disease caused by injection of immunogenic MOG protein and Freund's adjuvant.[11] This leads to a multiple sclerosis-like syndrome in susceptible mice. Similarly, anti-glomerular basement membrane disease (anti-GBM) is a lupus-like nephritis induced in mice by the transfer of rabbit serum and rabbit anti-basement membrane antibodies with Freund's adjuvant.[12] This model is especially useful for the study of end organ damage in SLE, as nephritis is its most common complication. Induced models are also advantageous in that they require less breeding than spontaneous models. Spontaneous mouse models, in contrast, occur over time without additional intervention due to their genetic background. Other models, like B6.Sle1 mice, develop a lupus-like syndrome as the result of one or more genetic susceptibility loci. SLE is strongly influenced by genetic polymorphisms and gene mutations, making spontaneous lupus-prone mouse models particularly useful for research. Similarly, gene-targeted models involve single-gene mutations that precipitate a lupus-like syndrome without further intervention.

Some lupus-prone gene mutations and polymorphisms have human analogs in SLE. Toll-like receptor 7 (TLR7), for instance, is overly-expressed in Mrl-lpr mice and also in many SLE patients.[13-15] While it is not clear whether Fc receptor gene sequences in the Sle1 susceptibility locus contribute to the lupus-like syndrome in B6.Sle1 mice, Fc receptor polymorphisms are known to contribute in human SLE and Wistar rat nephritis.[16-18]

The similarity of models to human disease varies, but most models include similar gender bias (F>M), signs (*e.g.* antinuclear antibody, renal manifestations), and polymorphisms. Mouse models are usually designated as lupus-prone, whereas only human disease is referred to as SLE. A few of the most pertinent spontaneous models are discussed below and outlined in Table 3.[19,20]

Strain	Background	Features	Important References
NZM2410 (BWF1)	NZB x NZW F1	Antinuclear antibodies Glomerulonephritis	21
Sle1	C57BL/6 (B6)	B & T cell activation Loss of tolerance Anti-nuclear antibodies	22
Sle2	C57BL/6 (B6)	B cell hyperactivity IgM autoantibodies	23
Sle3	C57BL/6 (B6)	T cell tolerance loss Renal manifestations	24,25
Mrl-Fas^{lpr}	Mrl	Lymphoproliferation Loss of T cell selection Antinuclear antibodies	26
BXSB/yaa	B6 x SB/Le	TLR hyperexpression Lymphoid hyperplasia Antinuclear antibodies	27,28

Table 3 – Commonly-used spontaneous mouse models of SLE, their features, and important references. Parts of this table were adapted from *Morel 2010* and others.

NEW ZEALAND CROSS AND ITS DERIVATIVES

A cross of New Zealand Black (NZB) and White (NZW) mice by Heyler and Howie in 1963 produced a small percentage of F1 mice that died of renal failure over the course of 8-10 months.[21] Interbreeding of these mice produced, among others, the NZM2410 and other models that consistently develop a lupus-like disease. These strains are sometimes referred to as BWF1. They exhibit a lupus-like kidney pathology—including nephritis, fibrinoid necrosis, and stereotypical wire loop formation—as well as antinuclear antibodies associated with human disease.

The Wakeland group at the University of Florida and later at the University of Texas Southwestern Medical Center discovered several NZM2410 loci that contribute independently to disease.[29] Three of the most important loci—designated *Sle1*, *Sle2*, and *Sle3*—have been backcrossed onto the C57BL/6 (B6) background. These mice exhibit consistent, unique phenotypes.

Sle1, found on chromosome 1, drives anti-nuclear antibody production indicative of a loss of tolerance to chromatin. B6.Sle1 mice develop mild SLE symptoms after 9-12 months. This locus contains a number of genes that may contribute to a loss of tolerance. Fc receptor genes, for instance, are found in the *Sle1* locus. While it is uncertain whether these genes are part of *Sle1* susceptibility, targeted deletion of B cell FcγRIIB alone in B6 mice causes a frank lupus-like syndrome including autoantibody production.[30] Conversely, upregulation of activating FcγR genes in mice favors inflammatory activity.[31] B6.Sle1 B and T cells show respective upregulation of activation markers B7-2 and CD69.[32] In B and T cells, the Cr2 gene in this locus contributes to T cell interaction abnormalities.[33,34] SLAM family members, receptors known to regulate immune cell function, are an essential part of the *Sle1* locus and contribute to disease pathology. A polymorphism of the SLAM family member Ly108 found in the *Sle1* locus contributes to loss of B-cell tolerance due to impaired negative selection of autoreactive B cells at the immature B stage.[35] Other SLAM family members CD84 and Ly9 in the *Sle1* locus have also been shown

to contribute to tolerance loss.[36] Thus the Sle1 locus appears to set the stage for other loci to produce overt disease (see the model in Figure 1).[37]

Sle2, found on chromosome 4, drives B cell hyper-reactivity.[23] This locus causes a frank lupus-like syndrome only when combined with other loci such as *Sle1* (*e.g.* in B6.Sle1.Sle2 mice). The *Sle2* locus likely confers this phenotype due to its inclusion of genes encoding TLR4, type I interferon, and lymphocyte signaling cascade-mediating tyrosine kinase Lck.[38] TLR4 is the receptor for lipopolysaccharide (LPS) and B6.Sle2 mice are hyper-responsive to LPS stimulation.[39] Lck dysregulation is known to contribute to lymphocyte hyperactivation in human disease.[40] Mice with the *Sle2* locus exhibit reduced levels of type I interferon, which leads to the accumulation of B1a B cells in B6.Sle2 mice.[41,42] B1a B cells, which are marked by CD5 and are derived from the fetal liver, produce natural IgM antibodies and act as antigen presenting cells (APCs) in the peritoneal and pleural cavities. This B cell subset is predicted to contribute to lupus development through increased aberrant antigen presentation, cytokine production, and natural autoantibody secretion.

The *Sle3* locus is found on chromosome 7. B6.Sle3 mice exhibit immune dysregulation that—particularly in concert with the Sle1 locus—can lead to severe disease.[25] *Sle3* causes major dysregulation of T cells, and B6.Sle3 mice show increased T cell activation, proliferation, and cytokine production with reduced T cell

apoptosis due to dendritic cell hyperactivation.[24,43,44] In combination with the *Sle1* locus, *Sle3* helps act as an accelerator to transition from a loss of tolerance to full autoimmunity. Transplanted dendritic cells with *Sle3* also break immune tolerance when transplanted.[44] In addition, B6.Sle3 mice lack normal segregation of B and T cells in secondary lymphoid organs. Some of these effects may be mediated by TGFβ underexpression from this locus, as TGFβ-knockout mice exhibit similar T phenotypes.[45,46] B cells with this *Sle3* exhibit increased cell survival through increased *Bcl3* expression.[47] These B cells also express elevated *Rag* genes that may contribute to SLE by increasing B cell receptor editing.[48]

OTHER ADDRESSED LUPUS-PRONE STRAINS

SLE is a diverse disease and is best addressed with multiple models. Mrl-lpr mice rapidly develop a lupus-like syndrome by 3-4 months of age. These mice carry a defect in the *Fas* gene at the *lpr* locus. Fas receptor signaling mediates negative selection through programmed cell death. Loss of Fas in this model leads to lymphoproliferation, autoreactivity, and a severe lupus-like syndrome. The lpr locus is insufficient for autoimmunity in B6 mice.[49]

BXSB/Yaa mice are a C57BL6/J and SB/Le cross that has been backcrossed to SB/Le.[28] These mice exhibit leukocyte hyperproliferation and proliferative glomerulonephritis. Part of this defect is chromosome 1-dependent, but other loci

also contribute. Unlike most lupus-prone models and human SLE, this strain exhibits male bias due to Y-linked Yaa locus.[27,28] BXSB/Yaa mice die at approximately 5-6 months.

The *Yaa* locus mainly act to accelerate disease progression through the overexpression of TLR7.[14,50] TLR7 polymorphisms are also associated with human disease.[15] B6.Sle1.Yaa mice develop fully-penetrant lupus nephritis as a result of TLR7-mediated acceleration.[51]

SINGLE-GENE SPONTANEOUS LUPUS-PRONE MODELS

While SLE is a complex, multifactorial disease, single-gene spontaneous lupus-prone models aid the study of individual gene contributions to SLE. Striking examples include previously-mentioned *Fas* defects discovered in Mrl-lpr mice and *Tlr7* upregulation at the Yaa locus.

Single-gene changes altering signal transduction often lead to autoimmune syndromes in mice. Lyn is an important B cell receptor-associated Src-family tyrosine kinase. Lyn inhibits activation of cells by phosphorylating ITIM-containing inhibitory receptors, thus recruiting the phosphatases SHIP and SHP-1. In the absence of Lyn, mice develop high circulating antibody levels, splenomegaly, and glomerulonephritis.[52,53] Human *Lyn* polymorphisms predispose to SLE.[54] Much of

this effect is due to the role of Lyn in B cells, where it acts to dampen B cell signaling.[55] Lyn deficiency also increases inflammatory markers on macrophages.[56] Disruption of Lyn target SHP-1 also produces a lupus-like syndrome, as does PD-1 deficiency.[57,58]

Single-gene mutations limiting the clearance of apoptotic cells offer another single-gene mechanism for lupus development in mice. Mutations in TREX1, which degrades single-stranded DNA, lead to a defect of ssDNA clearance that contributes to autoantigen availability and produces an inflammatory lupus-like syndrome in mice.[59] TREX1 polymorphisms cause human SLE as well as other syndromes.[60] DNase deficiency appears to cause SLE through a similar mechanism.[61] Mer-deficient cells also fail to recognize and clear apoptotic cells, and Mer-knockout mice develop a lupus-like phenotype.[62] Complement deficiency causes SLE and a lupus-like syndrome in humans and mice. C1q-deficient mice develop autoantibodies and 25% of littermates develop glomerulonephritis at least partially as a result of failed apoptotic cell clearance.[63] This effect is common among various early components of the complement cascade (i.e. C3, C4,) in both humans with SLE and lupus-prone mice and appears to include failure to clear apoptotic cells.[64]

Lastly, genes influencing lymphocyte survival are sometimes sufficient to cause autoimmunity. This includes the *Fas* defect previously discussed, Fas ligand knockouts, and transgenic Bcl-2 mice.[65,66] These are also involved in human disease.[67] BAFF transgenic mice, which exhibit heightened B cell survival, also develop systemic autoimmunity.[68]

LUPUS-PRONE MICE POSE A MULTI-STEP MODEL OF SLE PATHOGENESIS

Mouse models are tools for exploring human disease. As SLE is strongly influenced by a patient's genetic background, spontaneous lupus-prone mouse models are essential. Each model has similarities with human disease, and helps uncover genes and markers with clinical significance. Single-gene mutation models help uncover specific aspects of disease pathogenesis.

Combinations of the reviewed loci and genes highlight the separate roles of tolerance loss in lymphoid cells and dysregulation of immunity in innate cells and antigen-presenting B cells. The *Sle1* locus alone causes a loss of tolerance that only leads to severe disease in the presence of loci or mutations affecting immune dysregulation. For instance, *Sle3* may help accelerate *Sle1* through its effects in hyperinflammatory dendritic cells. *Yaa*, on the other hand, may help accelerate *Sle1* by increasing the sensitivity of innate cells to single-stranded RNA. Such combinations of these loci pose a multi-step model for SLE development (see Figure

1).[69] In humans, an analogous process arises as many healthy people have positive ANA and many patients who develop lupus have been shown to have pre-existing background ANA titres.[7,70] Identifying individual genes in these loci increases our understanding of disease. Unsurprisingly, polymorphisms in some of these genes cause lupus susceptibility in human patients.

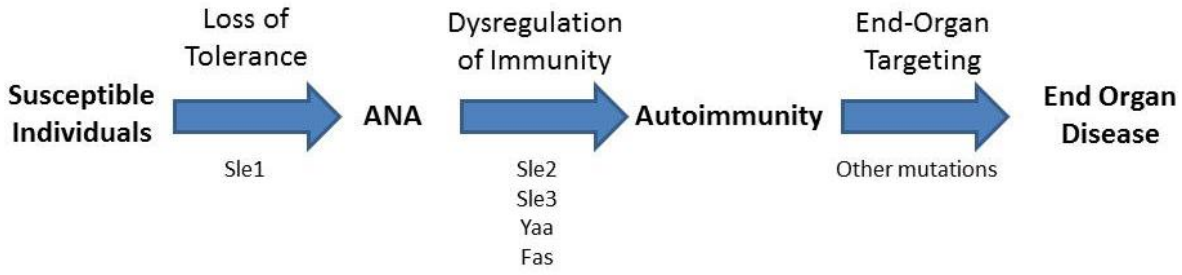


Figure 1 – The development of SLE in mice and humans is believed to be a multistep process that involves many loci. Adapted from Nguyen *et al* 2002.

Mouse models established the paradigm outlined in Figure 1 (see p14). In brief, SLE develops through the breakdown of three major checkpoints: adaptive immune tolerance, peripheral innate responsiveness, and end-organ inflammation. GWAS (Genome-Wide Association) studies have validated this three-step model by finding similar gene changes in human SLE as well as by showing the importance of combined risk alleles in SLE development.[64] A basic discussion of how each checkpoint contributes to disease is outlined below, with a focus on the roles of innate immunity.

LOSS OF TOLERANCE

Central and peripheral tolerance are a key component of adaptive autoimmunity

Adaptive immunity provides meticulous protection from pathogens not cleared by innate immunity. Autoreactive B and T cells are byproducts of adaptive immunity, comprising an estimated 75% of the initial cell populations.[71] These cells are normally eliminated by negative selection or peripheral tolerance mechanisms. In SLE, autoreactive T helper cells stimulate autoreactive B cells to produce anti-nuclear antibodies that bind and aggregate self antigens. These aggregates deposit in joints, skin, and kidney to produce major SLE symptoms such as arthritis, rashes, and nephritis. These processes are potentiated by dysregulated apoptosis, systemic inflammation, and other factors. This is the subject of frequent review and is very briefly outlined below.[72,73]

Tolerance is the removal of autoreactive B and T cells from the pool of active leukocytes *in vivo*. B and T cells have specific antigen receptors (B cell receptors and T cell receptors, respectively). These receptors are generated randomly from the rearrangement, combination, and mutation of specific genes. Each nascent pre-B and pre-T cell may thus theoretically mature to produce receptors with specificity toward any epitope. Tolerance prevents autoimmunity by removing receptors with specificity to self epitopes. Central tolerance occurs in the bone marrow (for B cells) and the thymus (for T cells). Stromal cells in these locations express self antigens

and induce programmed cell death in B and T cells with receptors that react strongly to these epitopes. B cells also have the mechanism of receptor editing to rescue autoreactive receptors. This method produces a broad repertoire and removes most self-reactive cells.

Peripheral tolerance occurs outside the bone marrow and thymus to deactivate (*i.e.* anergize) self-reactive cells. This anergy appears to be influenced by antigen dose, location, and timing. It also appears to operate through multiple mechanisms. In T cells, these mechanisms are generally divided into *clonal anergy* and *adaptive tolerance*.[74] Clonal anergy in T cells occurs when mature T cells are incompletely activated. These cells exist an arrested state due to Ras/MAP kinase inhibition, but can be rescued by soluble IL-2 or anti-OX40 antibody stimulation *in vitro*. Adaptive tolerance, in contrast, occurs in naive cells lacking sufficient costimulation or in the presence of exuberant CTLA-4 co-inhibition. These cells are unresponsive to IL-2, but this state reverses in the absence of antigen.[75]

Anergic B cells may develop through related and unrelated mechanisms. Overstimulation is a common theme in the B cell anergy literature, leading both to apoptosis and to an unresponsive state. Autoreactive B cells stimulated with high antigen titres express higher levels of pro-apoptotic BIM than non-reactive control cells.[76] Autoreactive B cells also compete poorly for BAFF versus normal cells,

leading to a short 4-5 day half-life.[77] A non-apoptotic mechanism related to overstimulation appears to be induced by innate overstimulation. Macrophages and dendritic cells can also induce an anergic, non-apoptotic state through secreted IL-6 and CD40L in a contact-dependent manner.[78] This mechanism appears to be reversed in the presence of copious interferon, as occurs in SLE.[79,80] A separate mechanism is due to location. Self-reactive B cells are excluded from germinal center reactions when competing with non-autoreactive B cells.[81] This may explain how autoreactive B cells are less likely to differentiate into antibody-secreting plasma cells.[82] Both of these phenotypes are reversible by copious BAFF.[83] These and other peripheral tolerance mechanisms are an area of ongoing research.

B cells circumvent selection in SLE

B cell dysfunction represents a common pathway among lupus-prone mouse strains. B cells become plasma cells and excrete soluble forms of the B cell receptor (BCR) called antibodies. While most SLE criteria are neither necessary nor sufficient for SLE diagnosis, anti-nuclear antibody (ANA) is always present in SLE. Autoreactive B cells must evade both negative selection and peripheral tolerance mechanisms to contribute to disease.

Defects in central tolerance contribute in such models as Mrl-lpr which, as previously described, contains a defective *Fas* gene that is important in negative selection.

Negative selection is crucial in avoiding systemic autoimmunity, as the majority of naturally-occurring B cell receptors produced by intrinsic diversity mechanisms are autoreactive.[71] Deletion, anergy, and receptor editing are the major mechanisms for central tolerance in B cells.[84-86] The Ly108 polymorphism in the *Sle1* locus leads to loss of B-cell tolerance due to impaired negative selection of autoreactive B cells at immature B stage, confirming that central deletion mechanisms contribute to SLE.[35] The Nussenzweig group profiled the B cell compartment in three SLE patients and found evidence that these central mechanisms were also breached in human patients, leading to high circulating levels of autoreactive B cells that may have provided the background conditions for fulminant SLE development.[87]

Many lupus-prone models have intrinsically hyperactive B cells that evade peripheral tolerance mechanisms, and adoptive transfer of hyperactive donor B cells is sufficient to produce mild nephritis in a significant portion of recipient mice.[88,89] B cells responding to copious B-cell activation factor (BAFF) avoid peripheral tolerance, suggesting that both hyperactivity and sheer numbers of autoreactive cells contribute to disease.[68] Normal anergic self-reactive B cells express very low levels of BAFF receptor, highlighting the importance of this mechanism.[77] BAFF-blocking antibody pharmaceutical belimumab (Benlysta®) is the first new FDA-approved SLE therapy in fifty years.[90] In humans, autoreactive B cells in SLE may not be properly excluded from germinal center reactions and appears to be a major

failure of peripheral tolerance.[91] As previously reviewed, other mechanisms likely apply in SLE.

B cells produce pathogenic autoantibodies in SLE

B cells which have circumvented central and peripheral tolerance may secrete autoantibodies of many varieties, including anti-Ro, La, RNP, phospholipid, and DNA immunoglobulins. Antibodies against these antigens correlate with many SLE disease manifestations and lupus-related syndromes.[92] These include thrombotic complications in patients with anti-phospholipid antibodies (aPL) in anti-phospholipid syndrome (APS), anti-centromere antibodies in systemic sclerosis with manifestations like Raynaud's and CREST phenomenon, and anti-Ro and La antibodies associated with Sjoegren's Syndrome and photosensitivity.

Antinuclear antibodies (ANA) are the most commonly-referenced SLE autoantibodies. These include anti-DNA, anti-histone, and anti-nucleosome immunoglobulins that deposit through immune complex formation in various organs. Here, these autoantibodies are recognized through their Fc receptor to activate inflammatory cells like macrophages and to initiate the complement fixation cascade. These processes cause the major end-organ damage leading to morbidity and mortality in SLE pathogenesis, including glomerulonephritis.[93]

B cells contribute to SLE pathogenesis in antibody-independent ways

B cells contribute to lupus-prone mouse models even in the absence of soluble antibody.[94] B cell depletion in SLE patients has been shown to improve symptoms even in the absence of autoantibody level reduction.[95] B cells likely contribute to SLE development in the absence of antibodies through antigen presentation and cytokine production.

B cells act as antigen presenting cells to present general and cognate antigens and associated proteins on cell-surface MHC receptors to induce T cell activation.[96] As previously mentioned, the presentation of antigens is largely altered in SLE. Highly-concentrated antigen presentation by autoreactive B cells may thus contribute to T cell activation and disease pathogenesis in a vicious cycle.

B cells also participate in cytokine production.[97] In Mrl-lpr mice, for instance, B cells contribute to CD8+ T cell activation in the absence of antigen presentation.[98] B cells can produce a number of cytokines that contribute to SLE, including IL-10, IL-6, LTα, and TNFα.[99] IL-10 is normally an anti-inflammatory cytokine, but paradoxically promotes inflammation in SLE.[100] B cells in SLE patients also produce large amounts of BAFF, which contributes to disease progression.[101]

T helper cells drive B cell activity

T cells have many classes with general functions having to do with T cell receptor interactions. T cell receptors (TCRs) recognize short peptide sequences presented on the surfaces of all nucleated cells on MHC Class I and MHC Class II receptors on professional antigen-presenting cells (APCs). CD8+ T cells, known as cytotoxic T cells, recognize foreign peptides presented on MHC Class I receptors and induce cell death. CD4+ T cells are divided into T_H1, T_H2, T_H17, Treg, T_{FH}, and other subtypes.[102] T_H1 cells secrete cytokines like interferon gamma (IFN /gamma) and IL-2 to activate macrophages and are generally considered anti-viral and anti-bacterial. T_H17 cells perform similar functions but are particularly important in mucosal immunity and also express IL-22. T_H2 are anti-parasite T cells that secrete IL-4, IL-5, and IL-13 to activate eosinophils, mast cell, and basophils. These cytokines also influence B cell production of antibody subtypes that facilitate the degranulation of these cells. T follicular helper (T_{FH}) cells perform a very direct role in B cell biology. This T helper subset are the primary organizers of the germinal center reaction in which antigen-specific B cells multiply, undergo affinity maturation, perform class-switching, and differentiate into memory and plasma cells.[103] As previously mentioned, exclusion of autoreactive B cells from the germinal center reaction is central to SLE pathogenesis. T_{FH} dysfunction also contributes to SLE by allowing B cell affinity maturation and class-switching to produce higher-affinity, class-switched autoantibodies.[104,105]

As T cell help promotes the survival and activation of autoreactive B cells, this is an important checkpoint failure in SLE. Potentially autoreactive T cells must escape central selection mechanisms. Abnormal T cell survival due to Bcl2 overexpression, for instance, contributes to SLE.[67] Further, binding specificities of different T cell receptors may predispose to faulty self recognition. Specific MHC polymorphisms increase the likelihood of developing SLE, highlighting the importance of aberrant T cell help.[106,107] Many lupus-associated T cell defects, including aberrant CD40L, cause overexuberant activation of cognate B cells.[108]

As mentioned previously, T cells influence the type of antibody B cells produce. This is important, as IgG autoantibodies are the major pathogenic subtype in SLE.[109] T_H1 CD4+ T cells in Mrl-lpr mice produce copious interferon gamma (IFN-γ), which drives the production of IgG2a antibodies.[110] While B cells may class-switch to IgG in the absence of T cell help, these responses are generally short-lived and less potent.[111,112] Thus T cell help is an essential part of the adaptive checkpoint failure in SLE.

Th17 cells are also implicated in SLE. Th17 cells generally provide epithelial barrier immunity and secrete copious inflammatory cytokines. Mrl-lpr and Ets1-/- lupus-prone mice each have increased Th17 cell numbers, as do many SLE patients.[113-115]

Th17 cells may contribute to SLE pathogenesis through enhanced inflammatory mediator production and end organ damage.[116]

DYSREGULATION OF INNATE IMMUNITY

Adaptive immunity is necessary but not sufficient for SLE pathophysiology. Up to 31% of healthy persons without SLE have detectible levels of antinuclear antibody (ANA). Further, even highly-stringent ANA titres are only 95% specific and exhibit poor sensitivity in SLE diagnosis.[7] Dysregulation in innate immune components is the next checkpoint in the pathogenesis if SLE.

PLASMACYTOID DENDRITIC CELLS AND TYPE I INTERFERON (IFN I)

The blood of SLE patients contains high amounts of type I interferon (IFN I).[117-119] IFN I is usually associated with anti-viral and anti-inflammatory activity. [120-122] In SLE, plasmacytoid dendritic cells (pDCs) produce IFN-α and other cell subtypes respond by producing interferon-induced genes to create the "interferon signature."[123,124] Plasmacytoid dendritic cells can be induced to produce IFN in response to apoptotic/necrotic cells, autoantibody crosslinking of FCγIIa receptors, unmethulated CpG sequences through TLR9, other toll-like receptors (TLRs), and interferon α itself.[125] Many of these are known to be predisposing factors to SLE development.[126] IFN-α acts to promote the maturation of dendritic cells (DCs), promote plasma cell development, induce BAFF to maintain mature B cells, and upregulate IRF7 in plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs), and monocytes.[79,127,128]

CONVENTIONAL DENDRITIC CELLS

Conventional dendritic cells (DCs) also contribute to SLE pathogenesis. Dendritic cells are increased in lupus-prone BWF1 mice,[129,130] which may be due in part to the abundance of type-I interferon in SLE.[131] Much of the effect of the *Sle3* locus is caused by dendritic cell hyperactivation.[44] These dendritic cells also express abundant inflammatory markers[132] and infiltrate into the kidney to cause end-organ damage in nephritis. This occurs both by antigen presentation and by cytokine secretion.[133-135] Dendritic cells in B6.Sle1.Sle2.Sle3 mice fail to induce regulatory T cells (Tregs), which further contributes to lupus development.[136] Dendritic cell activation state is an important modulator of tolerance, as the transfer of activated dendritic cells from normal donors to normal recipient mice causes loss of tolerance in the host and the development of anti-DNA antibodies.[137] Sustained dendritic cell dysregulation is also an important part of disease.

MACROPHAGES AND NEUTROPHILS

Myeloid cells like neutrophils and macrophages—part of the innate immune system—also contribute to SLE pathogenesis. I go into detail here on these cells because they have been a focus of my research. These cells produce important inflammatory mediators and appear to contribute to periodic worsening of symptoms

termed “flares.”[22,119,138] The following is a brief summary of innate abnormalities contributing to SLE. A complete reference is in Appendix A, p144.[4]

	SLE		Notes
	Mϕ	PMNs	
Chemotaxis	↑[31]	↑[139,140]	Neutrophil migration is normal in treated patients[139]
Immune complex (IC) clearance	↓[141]		
Phagocytosis	↓[142,143]	↓[144]	
Apoptosis	↑[145]	↑[142]	
Superoxide production	↓[145]	↑ ↓[146] ↓[147] ↑[148,149]	↑ in immune complex deposition ↓ in asymptomatic ANA

Table 4 – A literature summary of functional alterations in macrophages and PMNs in SLE, adapted from *Orme and Mohan* 2012.

INNATE ACTIVATION CHANGES IN SLE

Classically, SLE macrophages and neutrophils have been described as impaired. However, recent findings also implicate hyperactive macrophages and neutrophils in SLE. Activation markers such as multi-drug resistance factors and costimulatory factors are increased on these cells in SLE.[150-153]

Myeloid cells engulf and phagocytose debris and some phagocytic receptors modulate cell activation. SLE macrophages and neutrophils express high levels of inflammatory $F_C\gamma R1$ (CD64)[31,141,154] and complement receptor CR3 (CD11b/ITGAM).[155-157] In contrast, these cells express reduced anti-inflammatory

$F_C\gamma$R2B (CD32).[17,141,147,158-160] Loss of CD32 predisposes mice to lupus.[161,162] Additional phagocytic receptors vitronectin and phosphatidylserine (PS) receptors detect apoptotic bodies and initiate pro- and anti-inflammatory pathways, respectively.[163] Lupus-prone macrophages express increased proinflammatory vitronectin. This may explain the proinflammatory effect of apoptotic bodies in SLE.[164] Accordingly, ITGAM, $F_C\gamma$R2B, and $F_C\gamma$R3 polymorphisms predispose to human SLE (see Appendix A, p144).[17,18,165]

Lupus-prone macrophage adhesion and chemotaxis his defective and diminished in some ways.[166,167] However, these cells overexpress ICAM-1 to potentiate tissue recruitment and priming.[152,168,169] Interestingly, the loss of endothelial ICAM-1 attenuates disease in mouse models.[170,171] These cells further express increased Siglec-1 (sialoadhesin, CD169) which strengthens inflammatory macrophage-neutrophil and macrophage-CD8+ T cell adhesion.[172-174] Lupus-prone macrophages and neutrophils also exhibit hyperactive chemotaxis due to increased MCP-1,[31,175,176] MIP-1α, CCL5,[177] CXCR4, CXCL12,[178] and corresponding receptors.[179-181] Anti-chemotactic drugs are effective in attenuating disease in lupus-prone mouse models, including BX471—a short peptide CCR1 antagonist—and CXCR4 and CXCL12 blockers.[182,183]

NEUTROPHIL NETS

One peculiar aspect of neutrophil immunity is the formation of neutrophil extracellular traps (NETs). Neutrophils given appropriate stimuli undergo a modified form of apoptosis to produce extracellular fibers comprising DNA, histones, and antimicrobial peptides.[184] These NETs act to trap and lyse bacteria, but they also contribute to SLE. In SLE, NETs activate complement and immunostimulatory pathways,[185,186] induce plasmacytoid dendritic cell (pDC) type I interferon production,[187] and present nuclear antigens in an inflammatory environment.[188] Each of these factors contributes to SLE pathogenesis. NETs may persist abnormally in patients with SLE due to DNase1 impairment, exacerbating disease.[189]

INFLAMMATORY MEDIATOR PRODUCTION

Myeloid cells secrete inflammatory mediators in SLE. Neutrophils secrete increased matrix metalloproteases and both macrophages and neutrophils secrete excessive elastase in SLE.[156,190] IL-10 is generally considered an anti-inflammatory cytokine. Elevated IL-10 from macrophages and type I interferon from plasmacytoid dendritic cells in SLE, however, contribute to inflammation and IL-10-blocking antibodies were effective in one clinical trial.[100,191-193]

Myeloid-derived prostaglandins may also worsen disease. Cyclooxygenase and prostaglandins are elevated on SLE and mouse models.[194-196] Mrl-lpr mice treated

with COX-2 inhibitor SC-236 or omega-3 fatty acids show improvement.[197,198] A long-term case series study of celecoxib use in SLE show disease improvement and few side-effects.[199] Relatedly, reactive oxygen species (ROS) correlate with disease activity in SLE.[146,200] SLE neutrophils generate high ROS and SLE serum induces ROS from healthy PMN.[146,149,201-204] Multiple clinical trials target ROS in SLE.[205-207] ROS contribute to pathology neoantigen production, direct damage, and cell modulation.

Neoantigens are newly-exposed or modified epitopes that may be immunogenic. Neoantigens in SLE are generally produced by hydroxyl or other free radical modification of self.[208] SLE sera exhibit autoantibodies against ROS-altered albumin, mitochondrial DNA, and nuclear dsDNA.[209-211] Similar antibodies can also be induced experimentally. These antibodies serve as both pathogenic mediators and as clinical markers of disease.

ROS from macrophages activates cells. Nitric oxide (NO) stimulates T cell misfiring.[212] Oxidative stress also activates macrophages. Anti-phospholipid antibody (aPL)-positive patients treated with vitamin E in a randomized clinical trial showed decreased macrophage activation markers than placebo-treated controls.[213] Vitamin E is an anti-oxidant and may act to neutralize macrophage-activating ROS.

MACROPHAGE PHENOTYPES

A major paradigm in adaptive immunity is the $T_H1/T_H2/T_H17/T_{Reg}$ schema. In this view, broad phenotypes of CD4+ T cells are delineated in these functional groups. T_H1 cells, for instance, are viewed as mainly concerned with the elimination of intracellular viruses, bacteria and protozoa. They are induced by Interleukin 12 (IL-12), rely on transcription factors STAT4 and T-bet, and produce Interferon gamma (IFN-γ) to induce activation of macrophages and CD8+ T cells, among others.[214] T_H2 cells, on the other hand, are viewed as mainly concerned with the elimination of parasites. They are induced by IL-4, rely on transcription factors STAT6 and GATA3, and produce IL-4, -5, and -13 to activate granulocytes and induce B cell class switching to IgE. This broad categorization of T cells is a useful paradigm in T cell immunity.

Do myeloid cells like macrophages exhibit similar categorical phenotypes? Macrophages have recently been subdivided and categorized based on activity, location, and cell surface marker expression. Mantovani *et al* have subdivided activated macrophages into M1, M2a, M2b, and M2c varieties, which provides a useful classification paradigm.[215] In a prior review we collected data from Mantovani and others to compile these phenotypes, an adapted version of which is found in Table 5.[216-220]

While M1 and M2 macrophages in many ways correspond to respective T_H1 and T_H2 phenotypes, they differ in several ways. Importantly, the subdivisions listed in Table 5 are completely fluid and reversible and respond to microenvironmental milieu. They do not necessarily represent distinct populations of cells, but they do represent a useful functional nomenclature by which broad insights may be made into their function in diseases like SLE.

	Inducers		Markers and Products		Suggested Roles	Changes in SLE and evidence
M1 (Classically–activated)	GM-CSF IFNγ LPS TNF-α	CCL2 CCL5 (RANTES) CCR2 CCR7 CD14 CD86 CXCL8 CXCL8, 16	CXCL9, 10, 11 $FC\gamma R^{hi}$ IFN IL-1 MHC II TLR^{hi} TNF-α iNOS (ROS)	IL-6 $IL\text{-}10^{lo}$ $IL\text{-}12^{hi}$ IL-23 $IL\text{-}R_1$ $Ly6C^{hi}$ CCL2, 3, 4	Type I Immunity Bacteria, Viruses, Protozoa Type 4 Hypersensitivity Tumor resistance	Likely to be increased Inducers are increased (IFNγ, TNF-α, GM-CSF, possibly CSF-1) Products are elevated (CD86, IFNγ, CCL2, CXCL10, IL-6, TNF-α) STAT4 polymorphisms predispose to SLE
M2a (Alternatively-activated)	IL-4 IL-10 IL-13 PPARγ-agonists	Arginase CCL2 CCL16, 18 CCL17, 22 CD163 $CXCR1, 2^{hi}$ CCL24 (MPIF2)	Dectin-1 $F_C\varepsilon R$ IL-4R Mannose R Scavenger R P2Y12 P2Y13	$IL\text{-}10^{hi}$ $IL\text{-}12^{lo}$ $IL\text{-}R_2$ (decoy) Lectins $MHC\ II^{hi}$ MSR1	Type II Immunity Parasites Allergies Profibrotic	Likely to be decreased Products are depressed (MHC II, MSR1, mannose receptor, P2Y12) PPARγ agonists have shown promise in trials IL-4 and IL-13-stimulted macrophages transplanted into nephritic SCID mice ameliorates renal pathology
M2b	ICs (FCR agonists) IL-1R agonists LPS TLR agonists	CD86 CCL1 (MCP-1) IL-1	IL-6 TNFα	IL-10hi IL-12lo $MHC\ II^{hi}$	Immune regulation T_H2 activation	Likely to be increased Inducers are increased (ICs,TLR agonists) Products are elevated (IL-10, TNF-α, IL-6, CD86) Notch-1 and NFκB signaling are overactive in SLE PPARγ agonists have shown promise in trials
M2c (Deactivated)	Glucocorticoid IL-10 TGF-β	Arginase CCL16, 18 $CCR2^{hi}$ $CD14^{hi}$ CD150 (SLAM) CD163 ECM $IL\text{-}12^{lo}$	IL-4R IL-10R $IL\text{-}10^{hi}$ Scavenger R TGFβ Vitronectin R	IL-Ra $Ly6C^{lo}$ CXCL13 $MHC\ II^{lo}$ Mannose R P2Y14 RAGE	Immune suppression Tissue repair Matrix remodeling	Likely to be decreased Products are depressed (CD14,mannose receptor, MHC II) Scavenger receptor-blocking antibodies worsen disease
Mac-regs	CpG LPS TGF-β VEGF	Arginase CCL4 $CD11B^{lo}$ (ITGAM) $CTLA4^{hi}$	CXCL1 & 2 Foxp3 PDGF PGE_2	$GITR^{hi}$ IL-1α $IL\text{-}4^{hi}$ VEGF	IL-4R-mediated immune suppression	Likely to be increased but not effective Inducers are increased (TGFβ, PDGF, Cpg) Products are elevated (PDGF, PGE_2)

Table 5 - Macrophage subsets and their inducers, markers, targets, and putative roles using a classification nomenclature set out by Mantovani *et al* and others.

APPARENT M1 DOMINANCE IN SLE

As outlined in Table 5, M1 macrophages are classical phagocytic, inflammatory macrophages that have roles in delayed-type (type IV) hypersensitivities, tumor resistance, and type I inflammation. Could M1 macrophages, like T_H1 T cells, participate directly in SLE pathogenesis? Several markers of M1 macrophages are elevated in SLE macrophages, including CD86,[221] which correlates with the severity of renal pathology IFN-γ ,[222] IL-6,[192] CCL2,[31] and CXCL10[223] from circulating macrophages; CXCL10 from neurological lupus macrophages;[224] and CCL2 from intrarenal macrophages.[175,176] These markers are important in macrophage activation state, chemotaxis, and general pro-inflammatory activity.

As noted, macrophage phenotype is plastic and microenvironment-dependent. SLE sera contain large amounts of M1 inducers like TNF-α,[119] GM-CSF,[225] and IFN-γ.[119] TNF-α in particular is one of the “danger signals” popularized by Polly Matzinger[226] that fundamentally alters macrophage cell signaling integration.

Predisposing genetic factors also support the M1 dominance hypothesis. IFN-γ production by M1 cells utilizes the STAT4 pathway and is inhibited by factors predisposing to the M2a phenotype.[227] STAT4 polymorphisms have been linked to SLE and appear to increase M1 sensitivity to cytokines in these patients.[180,228,229] Circulating CSF-1, which is elevated in SLE patient serum, appears to induce a

Ly6C^{high} M1 phenotype. Cutaneous manifestations of lupus in Mrl-lpr mice exposed to sunlight are also influenced by CSF-1 and may thus be M1-mediated.[230] Indeed, recent work in models of atherosclerosis—which occurs frequently in patients with SLE—show the importance of the M1 subtype in instigating inflammation as well as M2 macrophages in anti-inflammatory activity.[231] While HMG-CoA reductase inhibitors may have multiple unrelated effects, they have also been shown to improve lupus symptoms by unknown mechanisms.[232]

SHORTCOMINGS OF THE M1 DOMINANCE MODEL

The M1 versus M2 paradigm may be oversimplified. M1 macrophages are unlikely to produce the large amounts of IL-10 seen in SLE; this expression pattern is a hallmark of all M2 subtypes.[192,215,233] Immune complexes and other TLR agonists, which are abundant in SLE serum, are further expected to favor the M2b subtype. M2b macrophages secrete IL-6 that is elevated in peripheral SLE macrophages and the subtype has been induced in mouse macrophages using anti-dsDNA antibodies.[192,234] Further, CCL5 antagonists—which might be expected to blunt renal injury because they block M1 actions on cytotoxic T and NK cells—actually worsen mouse renal damage even in the absence of lymphocyte infiltration.[235] These findings paint a more nuanced picture of macrophage subpopulation contribution to SLE. A recent review by Anders and Ryu suggested that increased M1 as well as

M2 macrophage subpopulations in various kidney pathologies could explain findings and influence disease course.[236]

POTENTIAL LOSS OF M2A AND M2C SUBPOPULATIONS IN SLE

M2a, b, and c macrophages perform separate tasks in inflammation. M2a macrophages are also known as alternatively activated or profibrotic; M2b as regulator or T_H2-related; and M2c as deactivated, remodeling, or anti-inflammatory. Each may have its own role in SLE. As discussed above, all M2 macrophages produce an elevated IL-10:IL-12 ratio, unlike M1 macrophages.[215] This inverted ratio is found on both peripheral and renal SLE macrophages.[192,233,236-239]

M2a macrophages appear to be downregulated in SLE. M2a markers MHC II, MSR1 (CD204) type A scavenger receptor, mannose receptor, and possibly P2Y12 are uniformly decreased in human SLE peripheral macrophages.[141,240-243] Pharmacological expansion of M2a macrophages with PPARγ agonists or a combination of IL-4 and IL-13 has shown promise in mouse trials.[219,244]

M2c macrophages also appear to be downregulated in SLE, though this subset is less well-characterized than M2a macrophages. M2c macrophages are referred to in the literature as deactivated, remodeling, or anti-inflammatory macrophages, reflecting the various roles these macrophages are thought to play in immunity.

Interesting findings regarding M2c macrophages in lupus include the fact that serum antibodies against scavenger receptors—expressed largely on the M2c subtype—worsen lupus.[242] Further, CD14 levels—enriched on the M2c subtype—are low on peripheral monocytes and macrophages isolated from patients with SLE.[241,245] Taken together, these might be seen as evidence of a reduction of M2c macrophages in SLE.

One promising role of M2c macrophages is their anti-inflammatory effect. High IL-10 levels seen in SLE might normally be expected to lead to M2c macrophage phenotype and decreased inflammation. However, high type I interferon (IFN-1) has been shown to alter macrophage response to IL-10.[100] High IFN-1 is a hallmark of SLE, and its ability to subvert IL-10 responses toward inflammation is highlighted by improvement in human SLE trials with IL-10-blocking antibodies.[191,193]

M2c macrophages also play a role in matrix deposition and tissue remodeling. TGF-β and IL-10—each increased in both peripheral and renal SLE macrophages[176,192,233]—lead to this phenotype. How their absence or presence might contribute to SLE pathology in this way has yet to be intensively studied. Mrl mice show wound healing without fibrosis, which may be due to a lack of M2c macrophages.[246]

POTENTIAL GAIN OF M2B SUBPOPULATION IN SLE

In contrast to M2a and c macrophages, M2b macrophages are likely increased in SLE. In an activated lymphocyte-derived DNA (ALD-DNA) induced mouse model of lupus, Zhang *et al* showed that increased Notch-1 signaling caused M2b macrophage differentiation. Notch-1 signaling further caused a lupus-like phenotype.[247] NF-κB p50 is an important part of M2b macrophage differentiation and has been shown to be increased in expression in kidneys of SLE patients with glomerulonephritis.[154,155] SLE serum samples are characterized by an increased ratio of IL-10 to IFNγ secretion, which could be a direct result of M2b activation.[192] Indeed, the surplus of unphagocytosed immune complexes (ICs) that occur in SLE are inducers of M2b macrophages. M2b macrophages produce nonspecific inflammatory factors that are elevated in peripheral SLE macrophages like IL-10, TNF-α, and IL-6.[192,233,241,248]

PPARγ knockout mice, an interesting SLE model, develop high serum anti-nuclear antibody (ANA) and a glomerulonephritis syndrome that is similar to human SLE.[249] The M2b macrophage phenotype predominates in these mice and has deficiencies in phagocytosis and apoptotic cell clearance. The use of a PPARγ agonist rosiglitazone has been proposed to divert macrophage differentiation from M2b toward an M2a phenotype.[219,250] Both rosiglitazone and pioglitazone have shown short-term therapeutic efficacy in murine lupus nephritis, though the mechanisms are

not known.[244,251] This evidence is indirect, as thiazolidinediones exhibit PPAR-γ-independent anti-inflammatory effects.[252] Nevertheless, M2b macrophage levels directly correlate with relapse (increasing and stimulating autoimmune response) and remission (decreasing along with a lower autoimmune response) in murine lupus nephritis.[253]

The NFκB pathway is a central regulator for both M1 and M2b macrophage subtypes. Many mediators of SLE pathogenesis lead to NFκB-driven transcription, including toll-like receptors and ligands, inflammatory Fc receptors and autoantibodies, and reactive oxygen species (ROS). Interestingly, cell-penetrating anti-dsDNA antibodies produced by Jang *et al* induce TNF-α production and activate the NFκB pathway in RAW264.7 mouse macrophages, potentially highlighting a novel mechanism for NFκB induction in SLE.[234] Similarly, paracrine LTB_4—produced by non-macrophage cells in SLE[254,255]—amplifies NFκB-mediated transcription.[256]

REGULATORY MACROPHAGES MAY BE INSUFFICIENT IN SLE

Long the subject of speculation, regulatory macrophages—termed Mac-regs or M_{Regs}—express the canonical regulatory T cell transcription factor Foxp3.[220] These cells repress inflammation much like their regulatory T cell counterparts and secrete large amounts of PGE_2, consistent with a finding in several mouse lupus models.[194,257] These cells also secrete PDGF, which has been shown to be

increased on macrophages from polycytidylic acid-accelerated lupus in BWF1 mice.[237] Thus it is possible that M_{Regs} are induced in SLE but are insufficient to halt disease progression.

END-ORGAN DAMAGE

End-organ damage represents the final checkpoint in SLE pathogenesis. It takes several forms, including nephritis, hematologic complications, heart and lung complications, skin disease, and neurologic manifestations. Their associated signs and symptoms represent the morbidity mortality risks in SLE. While each complication results from prerequisite adaptive and innate dysfunction, not all etiologies are clear.

Nephritis

Inflammation in the kidney (nephritis) is the most common serious sequela of SLE and the second most common cause of death in SLE (26.5%).[258] Nephritis begins with immune complex deposition in the glomerular basement membrane and worsens due to subsequent innate cell infiltration and inflammation. This can involve Fc receptor recognition of the immune complex and/or fixation of compliment due to the presence of IgG glycosylated constant domains. This process is variable and causes a range of kidney dysfunction. Common pathological findings include infiltrating mononuclear cells, mesangial cell proliferation, and glomerular wire loop deformity.[259] Cell casts are often found in the tubules of nephrotic kidneys in advanced disease.

Hematologic Complications

Vascular involvement is common in SLE. Anemia is a somewhat mild complication affecting approximately half of SLE patients.[260] This may be due to the long duration of disease-related inflammation (anemia of chronic disease),[261] to hemolysis by anti-cardiolipin antibodies (hemolytic anemia),[262] or to bleeding secondary to thrombocytopenia. Relatedly, thrombocytopenia is another common hematological problem in SLE patients and is usually caused by anti-cardiolipin antibodies.[262]

Anti-phospholipid antibodies—including anti-cardiolipin antibodies—are commonly associated with a separate disease called Anti-Phospholipid Syndrome (APS) or Hughes Syndrome. APS patients experience a hypercoagulable state marked by thrombotic events related to the binding of autoantibody to cell membranes. Thrombotic events are a common complication in SLE and include deep vein thrombosis (DVT), pulmonary embolism (PE), cerebrovascular accident (CVA), transient ischemic attack (TIA), and pregnancy complications. Thrombosis is the most common cause of death in patients with SLE (26.5%).[258]

Thrombotic complications in SLE are usually ascribed to anti-phospholipids (APL) or anti-phospholipid syndrome (APS). Many cases of thrombotic events in SLE, however, occur in the absence of APL; further, patients with APL do not always experience thrombotic events.[263] Sometimes termed “sero-negative” APS, this

phenomenon has yet to be adequately explained.[264] Each of the aforementioned hematologic complications of SLE poses a threat to the life of the patient. Further, risk of thrombotic events is increased in pregnant patients with SLE and causes high miscarriage rates.[265]

Other Manifestations

Arthritides and arthralgias are common in SLE, with joint pain and swelling common in the fingers, hands, wrists, and knees. They tend to migrate over the course of disease and are generally non-deforming.[266] Joint deformation does occur in some patients and generally involves either ulnar deviation of hand joints or corticosteroid-induced large joint deformities.

Cardiac manifestations are also common in SLE, affecting the heart in several ways. Endocarditis is common, usually manifesting as Liebman-Sacks endocarditis, a non-infectious grouping of vegetations typically affecting the mitral valve containing clots and leukocytes.[267] More commonly, pericarditis—inflammation of the heart sac—occurs when immune complexes deposit to cause inflammation. This is a very common complication of SLE, although usually mild relative to other disease manifestations and requires no separate treatment strategy.[268] More severed pericarditis may lead to cardiac tamponade and require direct treatment.[269] Direct inflammation of the heart muscle, termed myocarditis, is very uncommon but

reported in SLE. SLE patients are also more prone than matched controls to develop atherosclerotic plaques and coronary artery disease (CAD). Some of these complications may be due to treatments.

Heart and lung involvement are commonly linked in SLE. Pleuritis occurs by similar disease processes and leads to a high incidence of pleural effusion in SLE patients. This often manifests with sharp pleuritic pain due to the rubbing of irritated serosal surfaces. Pneumonitis can also develop in SLE patients but is rare.[270]

Rashes comprise a hallmark feature of SLE patients and appear to be due to increased photosensitivity. The malar rash, which appears over the face in sun-exposed areas and spares the nasolabial folds, is most commonly observed. Discoid (round, sometimes-painful erythematous plaques) and other erythematous rashes also occur. The precise pathophysiology of these rashes is unclear.[271]

Neurologic involvement in SLE is common and the subset of SLE patients with neurologic symptoms is termed ‘neuropsychiatric lupus.’ Symptoms of neurological dysfunction can be caused by many unrelated disorders, making it a less-specific finding. These symptoms include emotional changes, anxiety or depression, headaches, and sensory disturbances.[272] At least some of these symptoms are correlated with anti-phospholipid antibodies and includes ischemic signs on MRI

images.[273] This suggests that microthrombi are a major cause of neurologic manifestations in SLE. In addition, anti-NMDA receptor antibodies exist in the CSF of patients with neuropsychiatric lupus and appear to mediate some of these symptoms.[274,275]

Patients with SLE also have a higher likelihood of developing lymphoid malignancies like Hodgkin's disease and non-Hodgkin's lymphoma.[276]

SUMMARY—THE SEARCH FOR MARKERS AND PATHWAYS IN SLE

The foregoing reviews help highlight four important concepts in the field:

1. SLE is a complex, heterogeneous disease with strong genetic influences.
2. Mouse models further our understanding of SLE.
3. Three major checkpoint failures— adaptive immune tolerance, peripheral innate responsiveness, and end-organ damage—occur in SLE pathogenesis.

The purpose of the present work is to discover and explore the dysregulation of myeloid cells in SLE. In the coming chapters I will highlight how we discover new biomarkers and discuss my efforts to characterize three signaling pathways we found to be aberrant in SLE—Axl tyrosine kinase receptor, Wnt/β-catenin, and Fibrinogen alpha chain isoform Aα-E.

CHAPTER TWO

DISCOVERING ADDITIONAL MARKERS IN SLE

OVERVIEW OF PROTEOMIC SCREENING METHODS

Disease markers are proteins or other metabolites in the urine or serum that aid in the diagnosis and/or tracking of disease. Proteomic screens use multi-patient sample sets and cutting-edge technologies to uncover clinically-relevant protein markers. SLE is heterogeneous and comprises different syndromes, making these markers essential in evaluating disease. Markers also offer clues about disease pathogenesis that may lead to new treatment options.

Two proteomic biomarker discovery methods are now common (see Table 6). In "targeted" or "focused" proteomics, antibodies against known antigens are used for detection. Our laboratory used this method to identify SLE markers VCAM-1, angiostatin, and ferritin.[277-279] This method is powerful but limited by the breadth and quality of available antibodies. These antibodies are not quantitative and do not differentiate between posttranslational modifications. In "unbiased proteomics," labeled protein fragments are analyzed by mass spectrometry. Parent proteins are inferred from protein fragment databases. This method theoretically interrogates the entire proteome. It includes technologies such as SILAC (Stable isotope labeling by amino acids in cell culture), ICAT (Isotope-coated affinity tag), and iTRAQ (Isobaric

tags for relative and absolute quantitation). A major benefit to these methods is that they also detects rare variants and posttranslational modifications not typically detected by targeted screens.

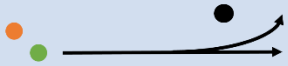
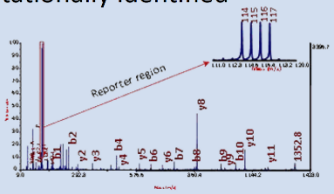
	Targeted/Focused Screens	Unbiased Screens
Method	 antibodies capture and detect antigens in tiny wells, concentration measured by binding resonance	 samples are digested, labeled isobarically, detected by mass spec, and computationally identified 
Pros	Technically simple	Theoretically unlimited repertoire Direct comparison of samples Pick up rare variants and modifications
Cons	Repertoire limited by antibodies Sample comparison only indirect No detection of modifications	Technically difficult

Table 6 – At-a-glance comparison of targeted and unbiased proteomic screening methods.

RESULTS FROM A TARGETED PROTEOMIC SCREEN

Tianfu Wu and others in the laboratory previously performed a large targeted proteomic microarray. Serum and urine samples from healthy controls and patients with active or inactive SLE were obtained and depleted of high-abundance proteins. These samples were then probed using described methods in a targeted, biased approach. Background for two hits in this proteomic screen are detailed below.

AXL TYROSINE KINASE

Axl receptor tyrosine kinase (Axl, UFO, JTK11) is elevated in active SLE patient serum. Axl is a member of the TAM family of receptor tyrosine kinases.

TAM Family Receptor Tyrosine Kinases

The TAM family of tyrosine kinase receptors comprises Tyro-3, Axl, and Mer. Each has a role in cancer biology and other overlapping functions.[280,281] Mer (Stk) is best-studied for its role in apoptotic cell clearance.[282,283] Axl and Tyro3 (Dtk, Sky) are best studied for their roles in cancer survival and proliferation.[284,285] This shared survival signaling is mainly through the PI3K/Akt/mTor pathway.[286-288] All three family members are expressed differentially throughout embryogenesis and each has distinct but overlapping functions including cell survival, coagulation, and regulation of inflammation.[280,289] TAM family members also act as non-specific virus receptors.[290,291]

TAM family members share approximately 72% intracellular and 52% extracellular homology.[292] As shown in Figure 2, each TAM family receptor comprises extracellular Ig-like and fibronectin (FN) III domains (ectodomain) and an intracellular kinase domain. Homology among family members is most striking in the kinase domain, comprising a unique family KW(I/L)A(I/L)ES motif.[293] Gas6 and/or Protein S bind to two extracellular Ig-like domain loops to cause downstream signaling in this kinase domain.[294]Two FN III repeats bind DNA and heparin and may function in cell-cell adhesion. While Axl has been shown to be cleaved by protease ADAM10 at the membrane edge of its ectodomain ([432]QPLHHLVSEPPPRA[446]), this sequence is not conserved in other TAM family members.[295]

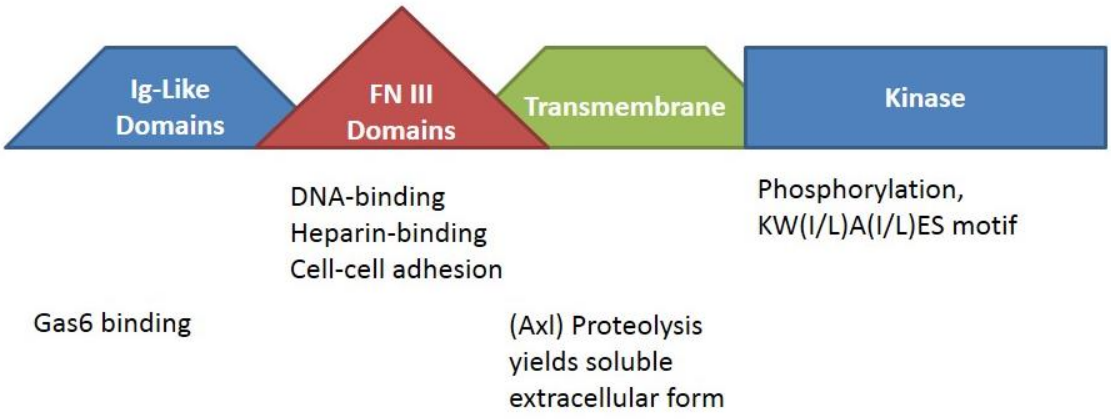


Figure 2 – Homology of TAM family receptor tyrosine kinases Axl, Tyro3, and Mer. Two N-termonal Ig-like domains and two fibronectin (FN) III repeats comprise the extracellular ectodomain. A distinctive KW(I/L)A(I/L)ES motif distinguishes the intracellular kinase domain.

Growth arrest-specific 6 (Gas6) and Protein S, highly-homologous gamma-carboxylated acute-phase proteins, are the known ligands activating this family of

receptors. Tyro-3 and Mer are activated by either Gas6 or Protein S, whereas Axl responds only to Gas6 and has much greater affinity for Gas6 than do other family members.[280,296] While one group has reported IL-15R/Axl heterodimers and IL-15 activation of Axl, no subsequent report has confirmed this finding.[297]

While TAM family members share broad similarities, each receptor has unique downstream signaling. Dimerization of Axl leads to autophosphorylation and docking in the cytoplasmic C-terminal protein with sites for PLCγ, Grb2, p85 (of PI3K), c-src, Lck, SOCS-1, Nck2, RanBPM, and C1-TEN.[298,299] Tyro-3 is known only to bind p85 (of PI3K) and RanBPM, whereas Mer may activate PI3K, PLCγ, Src, Grb2, and Vav1.[284] Vav1 likely mediates much of Mer's phagocytic activity.

Axl and its close relatives—Tyro-3 and Mer—are implicated in autoimmunity. TAM family triple knockout mice develop a severe autoimmune syndrome.[300] Soluble Mer, Axl, and Gas6 have been shown to be elevated in SLE serum, though the significance and consistency of Gas6 levels is mixed.[301-303] Further, a recent study of intra-articular injection of Protein S and Gas6 reduced inflammation and cytokine secretion in the collagen-induced arthritis (CIA) mouse model.[304] Axl knockout mice fare more poorly than controls in experimental autoimmune encephalomyelitis (EAE).[305] Axl expression is normally induced by thrombin and angiotensin II,[306]

oxidative stress,[307] statins,[308] and—perhaps most relevant to SLE—type I interferons.[309]

Axl in Malignancy and Proliferation

Axl is a tyrosine kinase receptor initially discovered in myeloid tumor cells.[310] Since its discovery Axl has been shown to contribute to malignant cell survival in myeloid leukemia,[311] colon cancer,[312] esophageal cancer,[313] thyroid cancer,[314] lung adenocarcinomas,[315] bone cancer,[316] malignant gliomas,[317] skin cancer,[318] gastric cancer,[319] renal carcinoma,[320] metastatic breast cancers,[321,322] bone-invading prostate cancer,[323] and pancreatic cancer.[324] The acute phase protein ligand Gas6 was discovered to signal through Axl.[325,326] Interestingly, the tumorigenic properties of Axl appear to be independent of Gas6.[327-329]

The proliferative signaling of Axl is not strictly oncogenic. Axl mediates post-injury proliferation in injured blood vessels,[306] renal mesangial cells,[330-332] and endothelial cells.[288,333] Of interest in SLE, Axl signaling is necessary for mesangial cell proliferation and nephritis. Gas6-null rats do not experience nephritis in the Wistar nephritis model.[330] In a therapeutic vein, Axl appears to be responsible for the protective effects of statins in injured vascular smooth muscle.[308]

Axl in Immunity

Among leukocytes Axl is reported mainly on macrophages but also dendritic cells, γδT cells, CD25+ T cells, and B1a B cells.[334] In macrophages, Axl recognizes extracellular Gas6, dimerizes, and causes downstream anti-inflammatory signaling through Twist, which is a negative feedback regulator of NFκB activity as a transcription factor (Figure 3).[309,335] It is unknown which Axl signaling pathway leads to *Twist* induction.

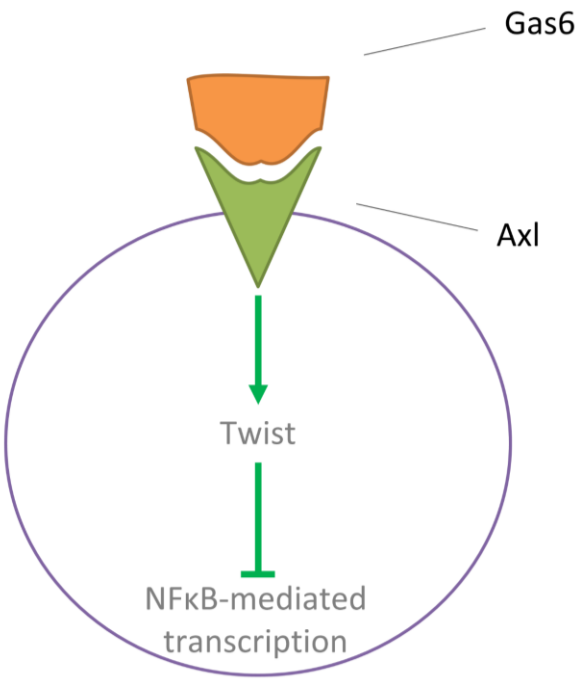


Figure 3 – Normal Axl Function in Immune Cells. In normal, healthy immune cells (*e.g.* macrophages), Axl recognizes extracellular Gas6, dimerizes, and causes downstream anti-inflammatory signaling through Twist.

Axl is regulated posttranslationally by proteolytic cleavage to produce soluble Axl (sAxl) and an inactive stump receptor.[336] Axl is normally cleaved by cell surface matrix metalloprotease ADAM10.[295,337] Our group previously showed that ADAM17 (TACE), a close family member, is elevated in SLE leukocytes. I hypothesized that serum sAxl is cleaved from leukocytes by both ADAM10 and TACE and abrogates an important anti-inflammatory pathway in SLE macrophages.

CTNNB1

I further parsed data from the previous screen using Ingenuity™ canonical pathways. I discovered significant upregulation of Wnt/β-catenin-associated factors (Table 7 and Figure 4). The β-catenin pathway produces serpins, metalloproteases, and defensins that are also known to be elevated in SLE.[190]

Factor	β-catenin relationship
DKK-1	↓/↑
Fas Ligand	↑
SAA	↑
TGF alpha	↑
TGF beta 2	↑
MMP-7	↑
TIMP-3	↑

Table 7 - Factors found to be elevated in SLE that are related to β-catenin.

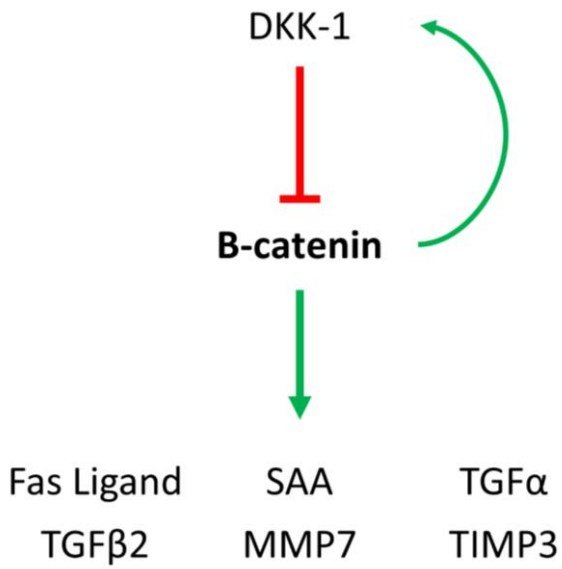


Figure 4 – β-catenin transcriptional products are elevated in SLE serum.

Dual roles of β catenin

β-catenin is known for two very different roles, *transcriptional signaling* and *adhesion.* In signaling, β-catenin translocates to the nucleus to transcribe TCF/LEF genes.[338] Soluble factor Wnt signals through seven-membrane-spanning surface raceptor Frizzled to release Disheveled (Dsh).

Dsh inhibits the formation of the catenin destruction complex that comprises Axin-2, GSK3 (Glycogen synthase kinase 3), and APC (Adenomatous polyposis coli) (Figure 5).[339,340] In the absence of Wnt signaling, however, the destruction complex phosphorylates β-catenin at Ser33, Ser37, and Thr41 to promote degradation.[341] Wnt/β-catenin signaling is essential in embryogenesis, where it determines early cell fates and dorsal cell characteristics.[342]

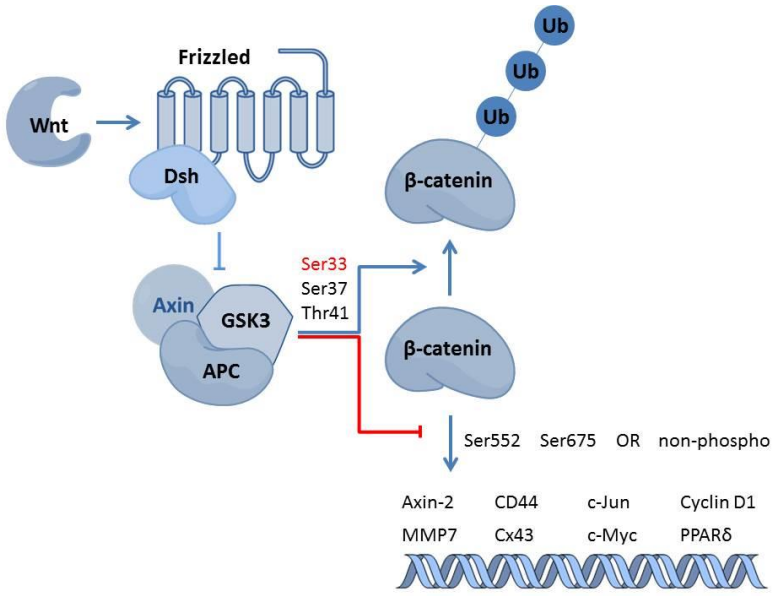


Figure 5 – The canonical Wnt/β-catenin pathway is regulated by β-catenin phosphorylation.

A separate pool of unphosphorylated β-catenin exists near the cell surface and is involved in cell-cell adhesions termed *adherens junctions.* Adherens junctions are important in maintaining the integrity of epithelial layers, blood vessels, and cardiac muscle fibers. In the adherens junction, β-catenin attaches loosely to actin filaments

inside the cell as well as cadherin proteins spanning the membrane and interacting with adjoining cells. Without β-catenin, these junctions are compromised.[343]

Individual β-catenin molecules cannot participate in both transcription and cell-cell adhesion due to spatial exclusion.[344] The location of an individual β-catenin molecule is regulated at least in part by phosphorylation at a number of residues, but is not entirely understood.[345] Chaperone proteins can favor localization at the cell membrane.[346]

B-catenin in immunity

Wnt-β-catenin activity is an important determinant of dendritic cell function in inflammatory bowel disease (IBD).[347] β-catenin transcription induces anti-inflammatory mediator expression and T-cell suppression surface markers in intestinal dendritic cells. Wnt/β-catenin signaling is elevated in lupus-prone mouse serum and kidneys.[348] BioGPS confirms β-catenin expression in leukocytes, particularly in B cells, but little is known about the direct functions of β-catenin in immunity. I hypothesized that leukocyte β-catenin dysfunction may contribute to innate dysregulation in SLE.

RESULTS FROM AN UNBIASED ITRAQ PROTEOMICS STUDY

As previously discussed, both biased (targeted) and unbiased proteomic screening methods are commonly used. Unbiased screens are technically difficult but essential for discovering markers outside of the established proteome.

Isobaric tags for relative and absolute quantitation (iTRAQ) is a leading unbiased metabolic discovery method. iTRAQ quantifies proteomic differences in small samples. In brief, samples from up to eight subjects are digested and labeled with unique isobaric tags and analyzed by LC-MS/MS. Peptide reconstruction software identifies parent proteins and correlates isobaric tag quantities.[349] This method is ideal because it allows direct comparison between groups and recognizes posttranslational modifications.

We prepared sera from SLE patients and healthy controls, labeled with 4- or 8-plex iTRAQ reagents, and subsequently performed MALDI-TOF/TOF analysis to compare a total of five control and nine SLE subjects.

FIBRINOGEN Aα-E

This screen discovered a number of up- and down-regulated metabolites. One novel upregulated factor in SLE sera is Fibrinogen alpha chain isoform alpha-E (Aα-E). As shown in Table 8, Aα-E is the *less* common of two alternatively spliced variants of the fibrinogen alpha chain and represents 1-2% of fibrinogen alpha chains in a healthy bloodstream.[350] The more common Aα (98-99%) is 610 amino acids in length whereas Aα-E is a much larger 846 amino acids long due to the inclusion of exon 6. This larger Aα-E is more stable than the common Aα chain and may lead to weaker clots.[351]

	Aα	**Aα-E**
% of Fibrinogen A chain in normal blood	98-99%	1-2%
Length (AA)	610	846
Stability	Unstable	Stabilized by add'l E domain

Table 8 – Comparison of Fibrinogen Aα and Aα-E chains.

Fibrinogen in the clotting cascade

Clotting is an essential and well-regulated process that controls blood loss at an injured site in the body. Clotting is initiated either by surface exposure to anionic surfaces in the intrinsic factor pathway or by injury in the extrinsic factor pathway (see Figure 6).[352] In both pathways, a series of serine proteases designated by

upper-case roman numerals cleave and activate additional serine proteases that converge to form a clot-producing complex. Activated serine proteases are designated with a lower-case “a.” Thrombin is the final active serine protease in the pathway and cleaves fibrinogen and factor XIII to produce fibrin monomers and crosslinking XIII. Fibrinogen functions to provide fibrin monomers (N-terminal end, common to both chains) and to act as a cofactor for platelet aggregation. A complete fibrinogen complex comprises three pairs of α, β, and γ chains.[353] Platelet aggregation is enhanced by Aα-E. Aα-E also has chaperone activity.[351]

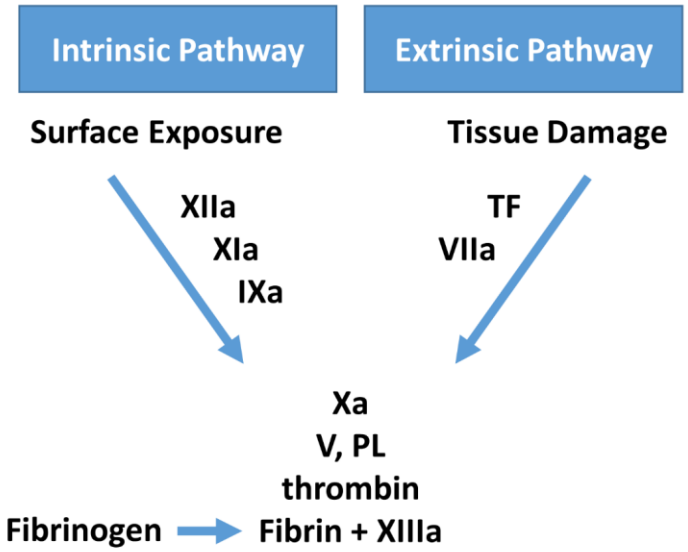


Figure 6 – Fibrinogen is cleaved to Fibrin at the end of the coagulation cascade and is essential for the production of clots.

Fibrinogen in SLE

Fibrinogen is elevated in SLE, but the Aα-E isoform has not been explored.[172,248] Thrombotic complications in SLE are usually ascribed to anti-phospholipid antibodies (APL) or anti-phospholipid syndrome (APS). Many cases of thrombotic

events in SLE, however, occur in the absence of APL.[264,354] Aα-E elevation in SLE may explain these events and act as a clinical indicator for anticoagulation therapy. I hypothesized that Fibrinogen Aα-E may be a useful new marker for thrombotic complications in SLE.

CHAPTER THREE

MATERIALS AND METHODS

STATISTICS AND COMPUTATION

Literature Search Strategy

I compiled SLEBASE, an online resource to summarize cell changes reported in SLE.[4] To establish the database, I searched MEDLINE using OvidSP and a rational search strategy.[355] In this Boolean protocol (depicted in Figure 7) I searched for peer-reviewed publications on SLE published since 1985 in which neutrophils or macrophages featured prominently. From the resulting five hundred articles I gleaned data on the expression of over eighty markers, chemokines, and other inflammatory mediators that are pertinent to SLE pathogenesis. Further searches regarding these mediators turned up other publications that were subsequently added to the search.

# ▲	Searches	Results
1	exp Lupus Erythematosus, Systemic/	42488
2	limit 1 to yr="1985 -Current"	29707
3	exp Macrophages/	123433
4	exp Neutrophils/	67519
5	3 or 4	184804
6	2 and 5	503

Figure 7 – OvidSP search of MEDLINE to generate SLEBASE. From Orme and Mohan 2012.

Student's T Test

P values where not otherwise noted were determined using a student's t test using GraphPad® Prism software. Significance cutoffs for p values was set at 0.05.

ANIMAL METHODS

Animals

Congenic control (C57BL/6, WT) and Mrl/lpr, BXSB, B6.Axl-/- and NZM2410 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Hudson, NY). B6.Sle1.Sle3,[51] B6.Sle1,[37,356] B6.Sle1.Yaa,[14] B6.Sle1.Yaa.Axl-/-, B6.LyzM-cre.Ctnnb-fl,[357] B6.Sle1.LyzM-cre.Ctnnb-fl, B6.Lyn-/-,[55] and BWF1[21] mice were bred in our mouse colony. Mice used for this study were 3 to 13 month males and females maintained in a stress-free environment. The Animal Care and Use Committee at the University of Texas Southwestern Medical Center approved all experiments on mice.

Mouse Genotyping

Mice were genotyped using the Type-it™ Mutation Detect PCR Kit from Qiagen (Cat #206343). Tails were suspended in 100µl tail digestion buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS solution at pH 8.0) and 10µl Proteinase K (0.5mg/ml) and incubated at 55˚C overnight. Suspension was subsequently boiled 10 minutes and spun down to remove debris. 2µl of the resultant extracted DNA was then subjected to PCR with 7.5µl master mix, 0.38µl of each primer (10µM aliquots), and 5.7µl RNAse-free H_2O per reaction. The thermocycler was set to the following:

	Temperature	Time	
1	94 ˚C	10:00	
2	94 ˚C	00:30	32x
3	58 ˚C	1:00	
4	72 ˚C	1:00	
5	72 ˚C	10:00	
6	4 ˚C	∞	

Mice were genotyped as follows using the indicated PCR primers, listed 5’ to 3’.

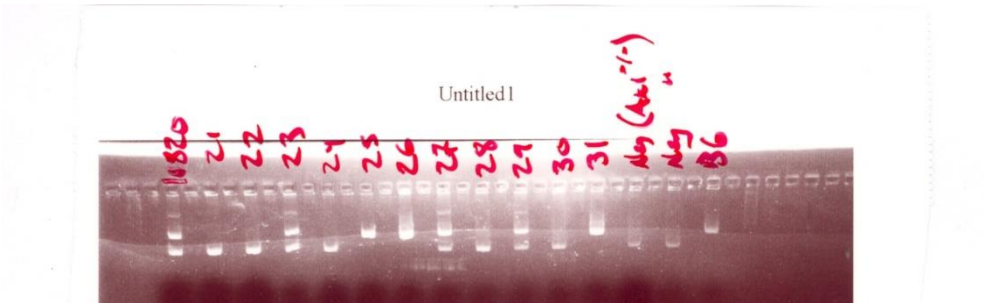
Axl^{wt}, Axl^{het}, and $Axl^{-/-}$ mice were genotyped using the following primers:

Axl 9713: AGA AGG GGT TAG ATG AGG AC

Axl 9714: GCC GAG GTA TAG TCT GTC ACA G

Axl 9715: TTT GCC AAG TTC TAA TTC CAT C

Mutants produce a band at ~275bp, heterozygote at ~300bp and ~275bp, and wild-type at ~368bp. An example follows:



B6.Sle1 mice were genotyped using the following primers in pairs:

Sle1 D1Mit15 up: TCC ACA GAA CTG TCC CTC AA

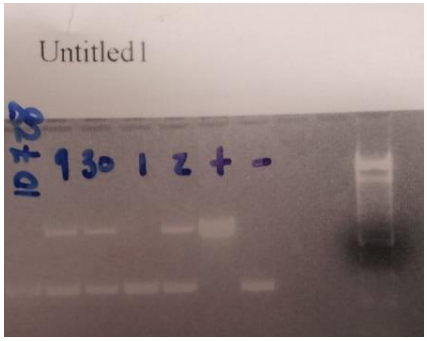
Sle1 D1Mit15 down: ATA CAC TCA CAC CAC CCC GT

Sle1 D1Mit17 up: GTG TCT GCC TTT GCA CCT TT

Sle1 D1Mit17 down: CTG CTG TCT TTC CAT CCA CA

Sle1 D1Mit47 up: CTG ACC TCC ACA CGA CCC

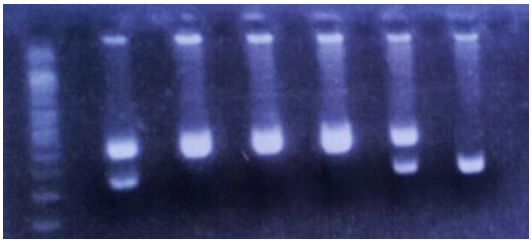
Sle1 D1Mit47 down: GCT TGG GAA ACT GGA TGA AA



β-catenin$^{fl/fl}$ mice were genotyped using the following primers:

Ctnnbfl-for: AAG GTA GAG TGA TGA AAG TTG TT

Ctnnbfl-rev: CAC CAT GTC CTC TGT CTA TTC



A higher band indicates the wild-type allele, whereas a lower band indicates a floxed allele.

Lyz-M-cre mice were genotyped using the following primers:

IMR 3066: CCC AGA AAT GCC AGA TTA CG

IMR 3067: CTT GGG CTG CCA GAA TTT CTC

IMR 3068: TTA CAG TCG GCC AGG CTG AC

A 700bp band indicates LysM-cre is present, whereas a 350bp band indicates that a wild-type allele is present.

Anti-GBM Disease Model

Anti-glomerular basement membrane (GBM) disease is a common, effective, and short model of glomerulonephritis, one of the most common end-organ manifestations of SLE.[358]

At Day -5, mice are inoculated intraperitoneally with 225µl of a mixture of 100µl Complete Freund's Adjuvant (CFA, Sigma #F5881), 100µl PBS, and 25µl rabbit IgG (Sigma I5006, 10mg/ml in PBS). The components are mixed in Micro-Mate® interchangeable hypodermic syringes (Popper & Sons, NY, #C5000-2) with Discofix® 3-way stopcock (B. Braun, PA, #D300). On Day 0, mice are inoculated intravenously (tail vein injection) with mouse anti-rabbit glomerular basement membrane serum. This inoculation is dosed by weight with 150µl per 20g animal. In a variant of anti-GBM disease, 10^7 bone marrow-derived macrophages (BMDM) are introduced by tail vein injection at Day 0 and anti-GBM serum is injected on Day 1.

Analysis of Kidney Function

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

measured in a 96-well plate assay using Pierce® BCA Protein Assay (Thermo Scientific, Rockford, IL) per the manufacturer's protocol.

Production of anti-Fibrinogen Aα-E rabbit antiserum

I provided GenScript™ with the non-overlapping Aα-E sequence. They used proprietary software to generate seven potential peptide sequences for use in producing anti-Aα-E serum (Table 9). Criteria included drivers of immunogenicity such as charge and uniqueness as well as non-homology to the rabbit proteome. The epitope RGSVLRVELEDWAG was selected and peptide CRGSVLRVELEDWAG was synthesized and conjugated to key limpet hemocyanin (KLH) at the N terminal cysteine. The cysteine was necessary for conjugation. A rabbit was injected three times with the peptide and

No	Start	Antigenic Determinant	Mus_musculus | Oryctolagus_cuniculus blast
1	195	CSYDPRNNSPYEIEN	92% | 92%
2	131	CAGDALIEGSVEEGA	78% | 100%
3	152	CMQFSTFDRDADQWE	100% | 92%
4	61	YKRGFGSLNDEGEGC	92% | 92%
5	46	QRMDGSLNFNRTWQC	100% | 100%
6	89	CRGSVLRVELEDWAG	100% | 85%
7	215	CFRGADYSLRAVRMK	100% | 100%

Table 9 – Potential antigenic peptides for immunization to generate anti-Aα-E antiserum. Peptide #6 was selected for its immunogenicity and non-homology to the rabbit proteome. Peptides are colored for their hydrophobic and hydrophilic residues.

carrier, raised over 75 days, and exsanguinated. Serum was isolated by centrifugation.

PROTEOMIC METHODS

Depletion of high-abundance proteins

First, 14µl of serum sample was added to 184µl of buffer A (Agilent) and loaded into a 0.22µm spin cartridge (Agilent 5185-5990) to remove debris before loading. The cartridge was centrifuged at 14k rpm for 5 minutes.

Eluent was then spun on a high-capacity multiple affinity removal spin cartridge (MARS, Agilent #5188-5341) per manufacturer instructions. This column depletes six abundant proteins: albumin, IgG, antitrypsin, IgA, transferrin, and haptoglobin. Each cartridge was equilibrated with 4ml buffer A (Agilent) using a luer lock syringe. This buffer was used to prepare the resin bed and remove trapped air bubbles. 200µl of pre-cleansed serum sample was next added and the column was centrifuged. This wash was repeated thrice and flow-throughs were collected, comprising low-abundance proteins. Columns were reused for additional samples after reequilibration with buffer A.

Desalting depleted samples

Low-abundance flow through from the previous protocols were added to spin concentrators (Agilent 5185-5991) and centrifuged at 3800g for 20 minutes. The columns were washed three times with 50mM $NaHCO_3$ and the concentrated samples were recovered from the column itself using a pipette.

iTRAQ labeling

Protein concentration was measured in each depleted sample using the BCA assay (Fisher #23225) per the manufacturer's instructions. 100µg of each sample was reconstituted to 5µg/µl using the dissolution buffer (0.5M triethyl ammonium bicarbonate, pH 8.5). 1 µl denaturant (2% SDS) was added and each sample was vortexed. 1µl reducing agent (100mM TCEP, tris(2-carboxyethyl)phosphine) was added and each sample was vortexed. Tubes were incubated at 60° C for one hour.

Samples were then spun shortly and 1µl fresh 84mM iodoacetamide was added. Samples were again vortexed and spun down, then incubated in the dark at room temperature for 30 minutes.

A vial of sequencing grade trypsin (Promega # V5111) was reconstituted to 1mg/ml with 21µl 50mM acetic acid. 10µl was added to each sample to digest the proteins. Samples were incubated overnight at 48 °C.

Samples were then spun down and brought to room temperature. iTRAQ tag vials were reconstituted with 70µl EtOH. 4-plex tags included 114, 115, 116, and 117, respectively. 8-plex tags included additional tags at 113, 118, 119, and 121,

respectively. Each separate tag was added to individual samples, vortexed, spun, and incubated at room temperature for one (4-plex) or two hours (8-plex).

All sample tubes were then pooled, vortexed, and spun. Tubes were dried and subsequently reconstituted with 100µl water, a process that was repeated 3-4 times to remove denaturant and reducing agents that would interfere with mass spectrometry.

Mass Spectrometry Analysis

Mass spectrometry comprises ionization of peptide fragments and querying peptides across a magnetic field. Frequency of mass-to-charge ratio (m/z) hits is tallied in histogram format. Isobaric tags cause these m/z ratios to diverge slightly, allowing the differentiation of individual samples within the sample pool.

Fragment m/z ratios are compared against a database of known peptide fragment ratios in order to reverse-engineer the identity of the fragment and parent peptide. These data are compiled and the relative quantities of detected proteins within samples are determined.

WET LABORATORY METHODS

Patient Samples

Collection of peripheral blood from consented human subjects was overseen and approved by the University of Texas Southwestern Medical Center Institutional Review Board (IRB). Controls were matched where possible by age, gender, and ethnicity. Exclusion criteria were only applied as described in each experiment.

Cell Acquisition and Sorting, Serum Acquisition

Peripheral blood was collected in heparinized tubes and processed. Cells were centrifuged out and serum was extracted from the supernatant. Peripheral blood mononuclear cells (PBMCs) were isolated with density-gradient centrifugation over Ficoll. Splenocytes were harvested by homogenization and extrusion through a filter and lysed with 1.5ml RBC lysis buffer for thirty seconds followed by centrifugation.

Cells were lysed for Western analysis, treated as described, or stained for flow cytometry assays. Antibodies used in flow cytometry are listed in Table 10:

Ag	Company	Catalog #	Notes
APC CD11c	Becton Dickenson	554680	12µl/test
APC-Cy7 CD11b	Becton Dickenson	559877	5µl/test

Axl	LifeSpan BioSystems	LS-B7213	A555 conjugated, Invitrogen Cat #A10470 2µl/test
	R&D	FAB154P	10µl/test (human), PE
	R&D	FAB8541P	10µl/test (mouse), PE
FITC CD3	eBiosciences	17-0038-73	12µl/test
P-Axl (Y779)	R&D Systems	AF2228	2µl/test
PE mouse IgG_1 κ iso	Becton Dickenson	554680	0.25µl/test
Rabbit iso	Becton Dickenson	550875	A555 conjugated, Invitrogen Cat #A10470 2µl/test
FITC CD14	Becton Dickenson	555397	20µl/test
PE-Cy5 CD4	Becton Dickenson	555348	20µl/test
APC CD19	Becton Dickenson	555415	20µl/test
FITC CD4	Becton Dickenson	BD 553729	20µl/test
PerCP CD11b	Becton Dickenson	BD 550993	20µl/test
PE-Cy7 B220	Becton Dickenson	BD 552772	20µl/test

Table 10 – A catalog of antibodies used in the present work for Western flow cytometry.

Flow wash buffer was 1% BSA in 1x PBS. Cells were run on LSR II and FacsCalivur ® machines (BD). Data were analyzed using FlowJo (TreeStar).

Cell Line and PBMC Treatment Regimen

For ADAM10 and TACE inhibition studies, cells were treated with DMSO vehicle control, 50µM GI254023X (OKeanos, China), 50µM TAPI-0 (Santa Cruz Biotech, #sc-203410), or both 50µM GI254023X and 50µM TAPI-0. For Gas6 stimulation studies, cells were treated with low-dose 1ng/ml LPS (Sigma L4391) with or without mouse (R&D #986-GS-025) or human (R&D #885-GS-050) recombinant Gas6 (400ng/ml) over 24 hours. Resultant cultures were spun down and prepared for Western analysis or RT-PCR.

Bone Marrow-Derived Macrophages

L-cell supernatant is prepared by growing L929 cells in flasks (~20mls DMEM with 10% FBS, 1% glutamine, 1% HEPES, 1% Pen/Strep) to confluency (3-5 days). Cultures are then split 5x, grown 7 days, and supplemented with 10ml DMEM with 10% FBS, 1% glutamine, 1% HEPES, and 1% Pen/Strep. After 7 additional days supernatant is filtered and frozen (0.4µm filters).

Bone marrow-derived macrophages (BMDM) are prepared by dissection and flushing of tibias using ~3ml BM25 media (DMEM with 10% FBS, 25% L

supernatant, 5% horse serum, 1% glutamine, 1% sodium pyruvate, and 1% Pen/Strep) into 10cm untreated petri dishes (final volume 10ml). Cultures are incubated at 37° C in 5% CO_2 for 3 to 4 days and 5ml media is replenished with new BM25. At day 7 or 8, media is aspirated and replaced with 5ml BM15 media (DMEM with 10% FBS, 15% L supernatant, 5% horse serum, 1% glutamine, 1% sodium pyruvate, and 1% Pen/Strep). Plates are then scraped and split for propagation and supplemented with additional BM15 media. Cells are allowed 2-3 days reequilibration before any treatment or freezing.

Axl Mutagenesis

An Axl vector (pCMV_SPORT6.1 Axl) was obtained from the Harvard/Dana Farber PLASMID core (Cat #MmCD00319729). A complete plasmid map is available in Appendix B on p178. Mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Cat #200521) per the manufacturer's instructions. In brief, isolated plasmids were replicated by high-fidelity polymerase chain reaction (PCR) in the presence of mutant primers. Parent DNA was subsequently digested using restriction enzyme Dpn I, which specifically degrades methylated DNA. As the PCR reaction did not include a methylation step, only mutant DNA remained. Bacteria were then transduced with the newly-synthesized plasmids for amplification. Bacterial samples were isolated, digested, and extracted. Resultant plasmids were then sequenced at the UT Southwestern Sequencing Core and

suitable mutant samples were collected. This process was repeated twice with primers listed in Table 17 on p183 first to produce wild-type and then to produce uncleavable Axl expression vectors.

Cell transduction

Transduction of cells was performed using the Amaxa® Nucleofector II and Mouse Macrophage Nucleofector® Kit (Lonza, VPA-1009) per manufacturer's instructions. In brief, 10^7 cells were resuspended in 100µl nucleofection solution with 5µg plasmid DNA and subjected to nucleofection program Y-001. Cells were then further suspended in 1.5ml equilibrated FBS-supplemented media and incubated at 37° C for 24 hours to allow recovery and protein expression prior to analysis or treatment.

Antibody Isolation

Fibrinogen Aα-E antibodies were isolated from immune rabbit serum using Protein G Sepharose®, Fast Flowbeads (Sigma #P3296-1ML) per manufacturer instructions. In brief, beads were incubated at room temperature on an oscillator with serum. Beads were washed with 20 mM sodium phosphate buffer (pH 7.0). Antibodies were eluted using 100 mM glycine HCl (pH 2.7). Of note, these antibodies were never affinity-purified.

Antibody biotin conjugation

Anti-Fibrinogen Aα-E antibody (isolated as described above) was conjugated with biotin for use as a detection antibody in Aα-E-specific ELISA assay (p76) using the EZ-Link™ Sulfo-NHS-Biotinylation Kit (Thermo Scientific #21425) per manufacturer instructions. In brief, 400µl purified antibody (3mg/ml, total 1.2mg, ~8nmol antibody) was incubated with 35.44µl Sulfo-NHS-Biotin (4.4mg.ml, mw 443, 160nmol biotin) and 500µl PBS at room temperature for 60 minutes. Desalting columns (Thermo Scientific) were repaired by repeated washes and spins with 2.5ml PBS at 1,000g for 2 minutes each. After incubation, the biotin-antibody solution was placed on the desalting column and allowed to absorb (~5 minutes). The columns were then centrifuged and flow-through represented purified antibody. The resultant detection antibody was used at a dilution of 1:5000 in ELISA (see below).

Enzyme-Linked Immunosorbent Assay (ELISA)

Human and mouse sera were probed for sAxl with the Human Axl DuoSet from R&D Systems (#DY154) and the Mouse Axl ELISA kit from Raybiotech (#ELM-AXL-001), respectively, per the manufacturers' protocols. All sera were diluted 1:100 in a serum diluent (2% BSA, 3mM EDTA, 0.05% Tween-20). Media samples were diluted 1:5 or 1:1 in assay buffer.

Total IgM and IgG (Bethyl Laboratories, Montgomery, TX) as well as anti-dsDNA, anti-ssDNA, and anti-histone antibodies (Alpha Diagnostics International, San Antonio) were detected by ELISA per the manufacturers' protocols. In brief, wells are pre-coated with mBSA, coated with dsDNA from calf thymus, blocked with 3% BSA and 3mM EDTA in 0.1% gelatin PBS, probed with sera, and analyzed using HRP-conjugated anti-mouse IgG or IgM antibodies.

Fibrinogen ELISAs were performed per the manufacturer's instructions. In brief, anti-fibrinogen capture antibodies (whether from the kit or from anti-Aα-E rabbit antiserum) were resuspended 1:400 in 100mM bicarbonate (pH 9.6) and added to wells in 100µl aliquots and left overnight at 4° C. Wells were washed 3 times with 200µl 0.05% Tween-20 PBS wash solution (pH 7.4). Wells were then blocked with 200µl 50mM Tris, 14M NaCl, 1% BSA (pH 8.0) at room temperature for one hour. Wells were then washed three times. Samples were then diluted 1:100 in 50mM Tris, 14M NaCl, 1% BSA, 0.05% Tween-20 (pH 8.0) and added to wells in 100µl aliquots. Standards were diluted from 400ng by serial dilution. Samples and standards were incubated at room temperature 60 minutes. Wells were then washed five times with the previous wash solution. Detection antibody was diluted 1:6500 in in 50mM Tris, 14M NaCl, 1% BSA, 0.05% Tween-20 (pH 8.0) and added in 100µl aliquots to each well and incubated 60 minutes. Wells were then washed five times

as previously. 1x TMB was then added to each well and incubated at room temperature for 20 minutes. Wells were then read by spectrophotometer at 450nm.

Histopathology

Kidneys and spleens were isolated and prepared for histological analysis by being cut in half laterally and fixed in OCT medium and subsequently frozen for immunofluorescence (IF) or in 10% formalin and subsequently embedded in paraffin for immunohistochemistry (IHC). Specimens were sectioned (3-5mm) and stained with appropriate antibodies. These antibodies included the following:

Antibody	Dilution	Host	Catalog #	Source
α-Axl	1:400	Rabbit	#LS-B7213	LifeSpan BioSystems
A-Y779-P-Axl	1:200	Rabbit	#AF2228	R&D Systems
α-pan-Cytokeratin	1:500	Mouse	#sc-8018	Santa Cruz Biotechnology
α-Rabbit IgG (Alexa 488)	1:500	Goat	#7074S	Cell Signaling
α-Rabbit IgG (Alexa 488)	1:500	Goat	#A-11034	Life Technologies

A-Mouse IgG (Alexa 555)	1:500	Donkey	#A-21422	Life Technologies
α-F4/80-APC	1:500		#FAB5580A	R&D Systems
α-β-catenin	1:200	Rabbit	PA5-16429	Fisher
α-phospho-β-catenin	1:200	Rabbit	PA1-14447	Fisher

Table 11 – Antibodies used in immunohistochemistry (IHC) and immunofluorescence (IF).

IHC slides were boiled in citrate solution prior to staining. IHC slides were counterstained with hematoxylin and, as noted, IF slides were fixed with DAPI-containing or DAPI-free fixative prior to imaging.

RT-PCR

RNA was extracted from tissue and reverse-transcribed to produce cDNA using standard protocols. In brief, splenocytes were harvested, filtered through a fine mesh, depleted of red blood cells by hypotonic solution, and pelleted. Each sample was then resuspended and sonicated in 1ml TRIzol® Reagent (Life Technologies #15596-026) and left at room temperature 5 minutes. 200µl chloroform was then added to each sample and each sample was vortexed 15 seconds and left at room temperature for 3 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes and the top (aqueous) layer was transferred to a new tube. 500µl isopropanol were added to each tube and left at room temperature for 10 minutes.

Samples were then centrifuged at 12,000 x g for 10 minutes and supernatant was aspirated. Pellets were washed with 500µl 80% ethanol (w/v) and centrifuged at 7,500 x g for 5 minutes. Supernatant was removed carefully by pipette. Samples were left to air dry 2-3 minutes followed by incubation at 70° C for 2-3 minutes. Resultant RNA was resuspended in 80µl and quantified using NanoDrop® 1000 (Thermo Fisher).

cDNA was produced from the foregoing RNA using a High Capacity cDNA Kit (Applied Biosystems #4368814). In brief, a master mix was prepared with 2µl 10X RT-PCR buffer, 2µl10x RT primers, 0.8µl 100µM dNTP mix, and 1µl transcriptase per sample. Water and isolated RNA were added to 14.2µl and 1µg RNA for a final volume of 20µl in each sample. Samples were treated with thermocycler at 25° C for 10 minutes, 37° C for 120 minutes, and 85° C for 5 minutes.

The resultant cDNA was then detected with SybrGreen® using the following probes:

Target	Sequence (5' to 3')
Cyclophilin A for	cattatggcgtgtaaagtcacc
Cyclophilin A rev	gcagacaaagttccaaagacag
Axl for	aaccttcaactcctgccttctcg
Axl rev	cagcttctccttcagctcttcac
Twist for	ggacaagctgagcaagattca

Twist rev	cggagaaggcgtagctgag
IL-6 for	ctgcaagagacttccatccagtt
IL-6 rev	gaagtagggaaggccgtgg
TNFa for	ttctgtctactgaacttcggggtgatcggtcc
TNFa rev	gtatgagatagcaaatcggctgacggtgtggg
Ctnnb for	aaaatggcagtgcgtttag
Ctnnb rev	tttgaaggcagtctgtcgta
Axin2 for	ctggctccagaagatcacaa
Axin2 rev	aggtgacaaccagctcactg
MMP7 for	gtatggggaactgctgacatcatg
MMP7 rev	ctgaatgcctttaatatcatcctg

Table 12 – Primers used for RT-PCR quantification of gene transcription.

In brief, 10µl iTaq Universal SYBR® Green Supermix (BioRad #172-5121), 0.1µl of each probe (100µM), 5µl of the above cDNA, and 5µl water are added to each well for a total of 20µl per well. Samples were sealed, spun down, and subjected to the following temperature cycles by thermocycler:

Step	Time
1. 95° C	30 seconds
2. 95° C	15 seconds
3. 60° C	60 seconds
4. Plate read	n/a
5. Melt curve analysis	2-5 sec/step

Table 13 – RT-PCR thermocycle steps

Results were analyzed by comparing fluorescence cycle thresholds of control (*i.e.* Cyclophilin A) and test gene wells by the following formula:

$$2^{(Ct_{test\ gene} - Ct_{control\ gene})}$$

Western Analysis

Human PBMC and mouse spleen and kidney samples were isolated, lysed, and prepared for Western Blot analysis in sample buffer by boiling for 10 minutes. Western blot sample buffer (5x) was prepared as follows:

3.125ml Tris (pH=6.8)

5ml glycerol

1g SDS

1ml BME

0.25g bromophenol blue

Up to 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 primary and subsequent secondary antibodies against antigens as listed in Table 14.

	Antigen	Dilution	Host	Cat #	Company
1˚	Axl	1:1k	Rabbit	#LS-B7213	LifeSpan BioSystems
	P-Axl	1:1k	Rabbit	#AF2228	R&D Systems
	Axin-2	1:600	Rabbit	#32197	Abcam
	β-catenin	1:1k	Rabbit	PA5-16429	Pierce
	P-β-catenin	1:1k	Rabbit	PA1-14447	Pierce
	GAPDH	1:10k	Rabbit	#2118	Cell Signal
	ADAM10	1:1k	Goat	AB946	R&D Systems
	TACE (ADAM17)	1:1k	Rabbit	sc-13973	Santa Cruz Biotechnology
	Fibrinogen	1:1k	Rabbit	sc-33580	Santa Cruz Biotechnology

	Fibrinogen Aα-E	1:1k	Rabbit	polyclonal	Produced by GenScript
	β-Actin	1:10k	Mouse	sc-69879	Santa Cruz Biotechnology
	Axin-2	1:10k	Rabbit	ab32197	Abcam
	Ctnnb	1:1k	Rabbit	PA5-16429	Thermo Fisher
	p33 Ctnnb	1:1k	Rabbit	PA1-14447	Thermo Fisher
	Tubulin	1:10k	Mouse	ab7291	Cell Signal
2˚	Rabbit IgG	1:10k	Donkey	#7074	Cell Signal
	Rabbit IgG	1:10k	Donkey	NIF824	GE Healthcare
	Mouse IgG	1:10k	Goat	NIF825	GE Healthcare
	Goat IgG	1:10k	Donkey	Sc-2020	Santa Cruz Biotechnology

Table 14 – Antibodies used to probe for specific antigens by Western analysis.

We also used our custom Fibrinogen Aα-E serum as described. Bands were visualized with ECL substrate and quantified using ImageJ®. For repeated probing

of blots, the following stripping buffer was used for 7 minutes shaking at room temperature:

15 g glycine

1 g SDS

10 ml Tween20

Bring volume up to 1 L with ultrapure water, adjust pH to 2.

CHAPTER FOUR

LOSS OF AXL SIGNALING IMPAIRS ANTI-INFLAMMATORY SIGNALING IN SLE

INTRODUCTION

Axl is a TAM family receptor tyrosine kinase originally identified as a marker of malignant cells as previously discussed (see p51). This receptor further enhances survival, proliferation, and migration in many different cell types by recognizing the soluble ligand Gas6.[281]

Axl has also been implicated in SLE. Mesangial cell proliferation, which is essential to the development of nephritis in mouse models, requires Axl-mediated Gas6 signaling.[306,330-332] Gas6-null rats are protected in experimental nephritis, reflecting the role of Axl in mesangial cell proliferation.[330] We discovered in our screens that cleaved serum Axl is elevated in SLE. This has since been confirmed by others.[301-303] While others have suggested that serum Axl in SLE may come from mesangial cells, this explanation falls short in two important ways. First, mesangial cell Axl signaling is intact in nephritis. Second, ADAM10, which is the protease implicated in the literature as a cleaver of Axl, is not elevated in mesangial cells during nephritis.[359]

Axl has an under-studied role in immune cells. It is expressed on the surface of macrophages and Axl mRNA is reported in dendritic cells, γδ T cells, CD25+ T cells, and B1a B cells.[360] Axl signaling in macrophages induces transcription factor Twist through an unknown pathway.[309] Twist, in turn, inhibits NFκB-mediated transcription of inflammatory cytokines by occupying NFκB DNA binding sites. Axl-deficient mice experience worse disease than wild-type controls in the multiple sclerosis (MS)-like experimental autoimmune encephalomyelitis (EAE) model.[305] Complete TAM family knockout mice develop severe, spontaneous systemic autoimmunity.[361]

The role of leukocyte Axl is poorly studied in SLE. Gas6—the native ligand for Axl—and soluble Axl are both upregulated in SLE serum.[303] The source of serum Axl in SLE and the status of leukocyte Axl are unknown. Axl is regulated posttranslationally by proteolytic cleavage of the ectodomain to produce non-signaling soluble Axl (sAxl) and a surface stump receptor.[336] ADAM10 mediates this cleavage in healthy cells *in vitro*.[295] TACE (ADAM17), a closely-related protease, is upregulated on SLE PBMCs.

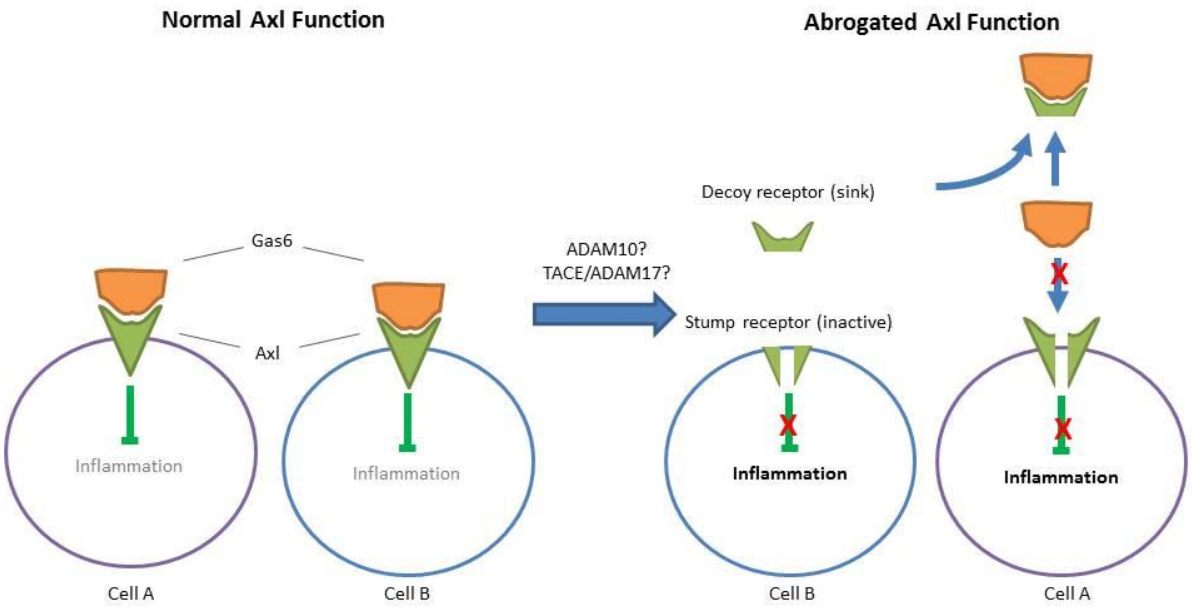


Figure 8 – A model of Axl cleavage and its potential to dampen Axl anti-inflammatory signaling in lupus.

I hypothesized that ADAM10 and/or TACE cleave surface Axl on human PBMCs in SLE, abrogating the Axl anti-inflammatory pathway and worsening disease as modeled in Figure 8. My specific aims and summarized results are:

1. **Aim 1: Determine the source and extent of cleaved Axl in SLE serum.** Soluble Axl (sAxl) is elevated in SLE serum and correlates with disease severity. I found that peripheral blood mononuclear cells (PBMCs) from patients with high serum Axl lose Axl ectodomain proportionately by Western analysis. Lupus-prone mouse strains also exhibit elevated serum Axl and decreased splenocyte Axl.

2. **Aim 2: Determine the cause of Axl cleavage in SLE leukocytes.** I found that the loss of leukocyte Axl in SLE is not due to underexpression

as measured by RT-PCR. Cell surface metalloproteases ADAM10 and ADAM17 (TACE) are elevated on SLE PBMCs and lupus-prone mouse splenocytes by Western analysis. I further showed that ADAM10- and TACE-specific inhibitors synergistically block Axl cleavage in lupus-prone splenocytes.

3. **Aim 3: Determine the physiological significance of Axl cleavage in SLE.** I found that SLE PBMC and lupus-prone mouse splenocytes exhibit decreased Axl activation (*i.e.* phosphorylation) versus healthy control PBMC and splenocytes by Western analysis. I further found that lupus-prone splenocytes fail to induce Twist-mediated NFκB suppression on Gas6 stimulation *in vitro*. B cells only marginally upregulate *Twist* in response to Gas6 stimulation. Treatment with combined ADAM10 and TACE inhibitors rescues this phenotype. Axl knockouts and heterozygotes exhibit worsened anti-GBM disease than wild-type controls. Transfer of Axl-/- macrophages worsens anti-GBM disease, whereas reconstitution of Axl-/- macrophages with Axl may lessen disease. Cleaved soluble Axl ectodomain further inhibits Gas6-mediated Axl signaling.

AIM 1: TO DETERMINE THE SOURCE AND EXTENT OF INCREASED AXL CLEAVAGE IN SLE.

1.1 Soluble Axl (sAxl) is elevated in the serum of active SLE patients

Microarray proteomic analysis suggested that sAxl is elevated in the sera and urine of lupus patients (data not shown). We isolated serum from healthy controls and patients with inactive and active SLE as determined by clinical scores and analyzed the concentration of sAxl by ELISA (R&D Systems, Minneapolis). Patients with active SLE exhibit significantly higher levels of serum sAxl than healthy controls and patients with inactive SLE by ELISA (Figure 9). We then measured sAxl concentration in a random sample of serum from SLE patients and subsequently matched results to SLEDAI scores. SLEDAI and sAxl levels trended together in these samples. Previous work by Ekman *et al* has found significant correlation between serum Axl and SLE disease activity in a large group of patients.[303]

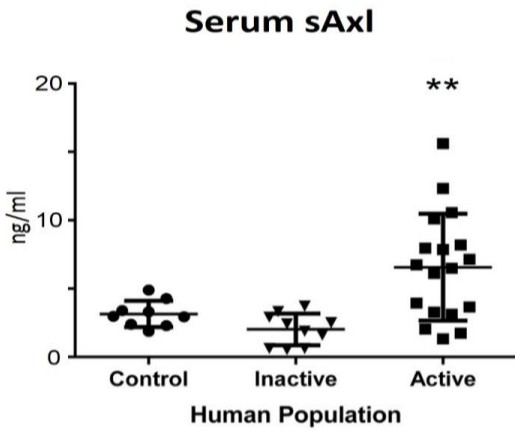


Figure 9 – A) Soluble Axl was measured in the serum of healthy controls (n=9), patients with inactive SLE by clinical score (n=10), and patients with active SLE by clinical score (n=18). Patients with active SLE have significantly higher levels of sAxl than healthy controls (p=0.0084) or patients with inactive SLE (p=0.0007).

1.2 Axl and Axl signaling are elevated in kidney mesangial cells of lupus-prone mice

Kidney mesangial cell proliferation drives lupus nephritis. This proliferation is Axl-dependent in Wistar rat glomerulonephritis.[330] I took kidneys from pre-disease (6 month) and diseased (13 month) B6.Sle1.Sle3 mice and age-matched healthy B6 controls to show that Axl expression is elevated in lupus-prone mice as in nephritic Wistar rats by RT-PCR (Figure 10). Macrophages infiltrate the kidney in nephritis. To determine (a) whether macrophages or a stromal population (*i.e.* mesangial cells) express Axl in lupus nephritis and (b) whether elevated Axl transcription affects Axl activation (*i.e.* phosphorylation), I took frozen kidney samples from 6-month nephritic BWF1 and healthy B6 control kidneys. I performed immunofluorescence staining for Axl ectodomain, activated (phosphorylated) pAxl, and mouse macrophage marker F4/80. As shown in Figure 12, sparse kidney macrophages are the only cells found in healthy B6 kidneys expressing active pAxl or total Axl ectodomain. In contrast, mesangial cells but not macrophages express Axl and pAxl in nephritis as determined by a blinded pathologist (see also Figure 12). As mesangial cell Axl is intact and mesangial cells do not express Axl-cleaving proteases, we considered whether leukocytes may be a systemic source for soluble Axl in SLE.

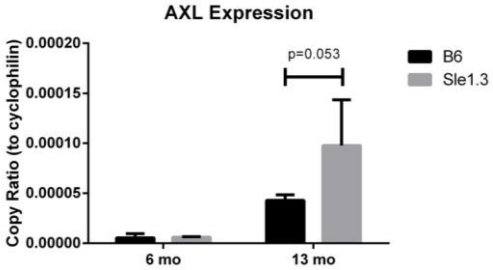


Figure 10 – Axl transcription is increased in mouse lupus nephritic kidneys (n=3).

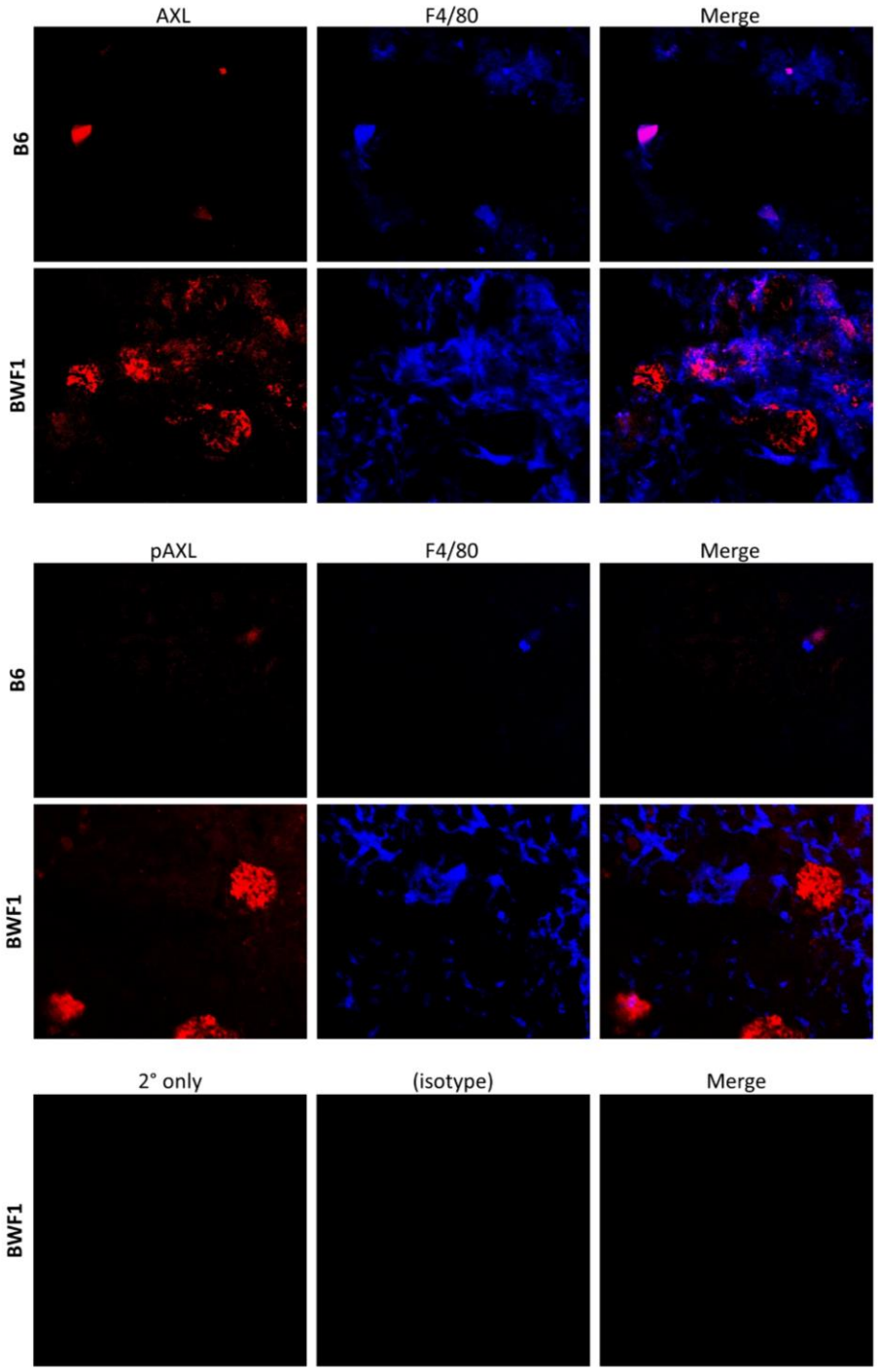


Figure 11 – Immunofluorescence co-staining of 6-month B6 and BWF1 kidneys for Axl (Axl, red, left panel), phosphorylated Axl (pAxl, red, right panel), and mouse macrophage surface marker (F4/80, blue). Kidney macrophages express Axl and activated pAxl in healthy control B6 but not diseased BWF1 kidney. A stromal population expresses Axl and activated pAxl on diseased BWF1 but not healthy control B6 kidney (representative, n=3).

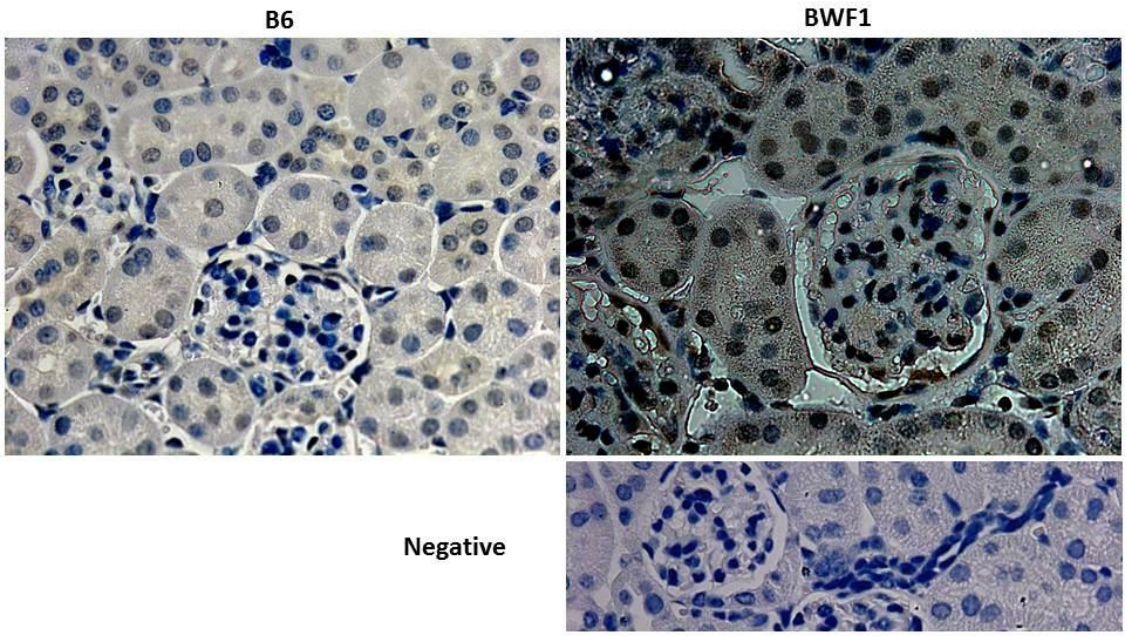


Figure 12 – Immunohistochemical pAxl staining of 8-month kidneys from healthy B6 (left) and diseased BWF1 (right) mice. A blinded pathologist noted from the figure that mesangial cells of the nephritic kidneys express the most elevated pAxl levels.

1.3 Extracellular Axl is missing from PBMCs in SLE patients with elevated serum sAxl

I hypothesized that leukocytes may be a source of serum sAxl in SLE. I isolated peripheral blood monocytes (PBMCs) and serum from healthy controls and SLE patients. I analyzed two sets of healthy and active SLE PBMC lysates by Western blot as shown in Figure 13A using antibodies against the extracellular domain of Axl (Axl) and the internally-phosphorylated (*i.e.* Gas6-activated) Axl (pAxl). It appeared that SLE PBMCs lost Axl ectodomain and internally-phosphorylated Axl, which I quantified by densitometry versus loading control as shown in Figure 13B

($p<0.0001$). This suggested either (a) that Axl mRNA levels are decreased or (b) that the Axl ectodomain is cleaved from SLE PBMCs.

I next tested sera from the same subjects by ELISA for the presence of cleaved Axl. Soluble serum Axl—in contrast to PBMC-associated Axl ectodomain—was significantly *increased* in the serum of these same SLE patients versus healthy controls (see Figure 13C). As shown in Figure 13D, the negative correlation between PBMC extracellular domain Axl and serum sAxl suggests that PBMCs may be a source of sheared sAxl in the blood.

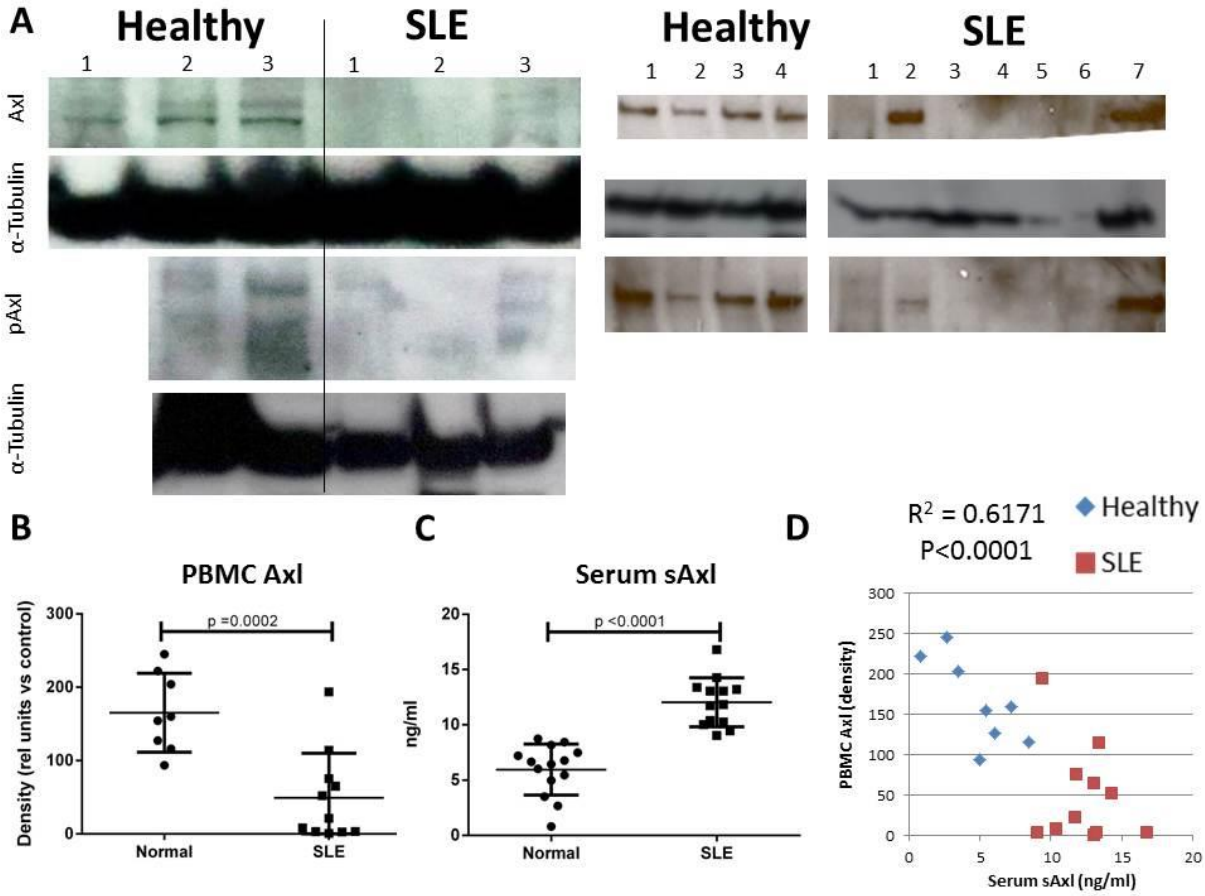


Figure 13 – (A) PBMCs from two separate sets of healthy (n=3, 4) and active SLE (n=3, 7) subjects were isolated and analyzed for Axl by Western blot. SLE PBMCs showed decreased total PBMC extracellular domain Axl and pAxl. (B) Densitometry of Axl versus α-Tubulin loading control quantifies this loss of Axl extracellular domain (p=0.0002). (C) ELISA shows a significant increase in serum Axl (sAxl) in the same patients (p<0.0001). (D) Data from B and C correlate inversely in healthy and SLE patients (R^2=0.6171, p<0.0001 by Pearson correlation). These data suggest that PBMCs may be an important source of sheared sAxl in the blood of lupus patients.

1.4 Soluble Axl (sAxl) is elevated in the serum of lupus-prone mice versus healthy controls

The previous correlation suggests that leukocytes may be a source of serum Axl in SLE. Lupus-prone mouse models mimic SLE and are an important tool for testing hypotheses relating to lupus pathogenesis. To test whether lupus-prone mice also shed Axl ectodomain, I collected sera from healthy C57BL/6 (B6) mice and several

strains of lupus-prone mice and measured serum Axl by ELSA. As shown in Figure 14, most lupus-prone strains also exhibited significantly higher concentrations of sAxl than healthy controls.

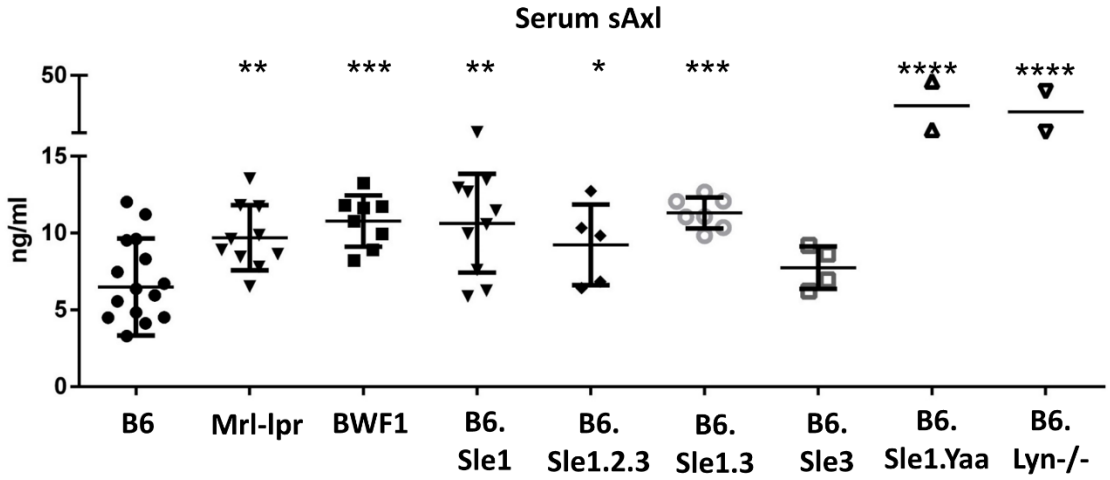


Figure 14 – Increased serum Axl in lupus-prone strains versus ≈8-month-old B6 background. sAxl is significantly higher in Mrl-lpr (≈5 months, p=0.0047), BWF1 (≈8 months, p=0.008), B6.Sle1 (12 months, p=0.0018), B6.Sle1.Sle2.Sle3 (≈8 months, p=0.0474), B6.Sle1.Sle3 (10 months, p=0.0004), Sle1.Yaa (8 months, p<0.0001), and Lyn-/- (8 months, p<0.0001) but not B6.Sle3 (8 months, p=0.2264) mice. Error bars represent standard deviation.

1.5 Splenocytes in lupus-prone mice lose the extracellular domain of Axl

I next looked to determine whether lupus-prone mouse splenocytes mimic human samples in the shedding of serum soluble Axl in SLE. I isolated spleens from 4 month-old healthy B6 and lupus-prone Mrl-lpr mice, cryosectioned, and stained for Axl and DAPI as shown in Figure 15 (green and blue, respectively). Axl is on the surfaces of some B6 splenocytes but is absent from Mrl-lpr splenocyte surfaces.

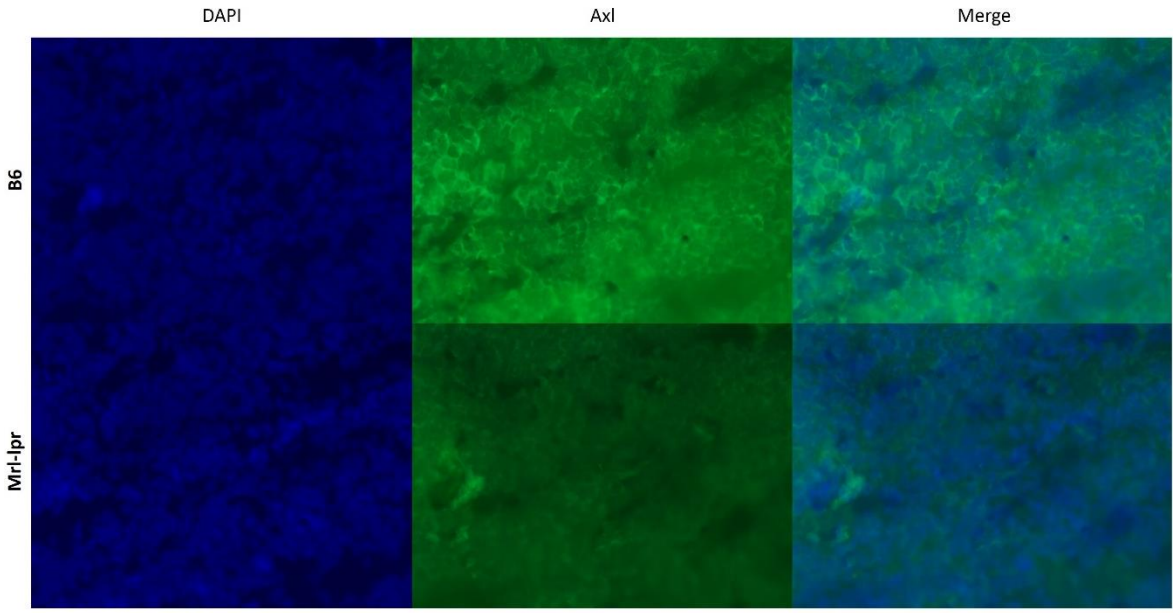


Figure 15 – Immunofluorescent stain of Axl (green) and DAPI (blue) in the spleens of healthy B6 (top) and lupus-prone Mrl-lpr (bottom) mice. Axl appears to be patent on the surfaces of some B6 splenocytes located in putative germinal centers but absent from Mrl-lpr splenocyte surfaces.

To determine which cells lose Axl expression in lupus-prone mice, I isolated splenocytes from four-month-old healthy male B6 and diseased B6.Sle1.Yaa mice for analysis by flow cytometry. Splenocytes were stained using a simple set of markers to identify CD11b+, CD19+, CD4+, and CD8+ cells. Cells were further stained with either rabbit isotype control (BD #55087) or rabbit anti-Axl (LSBio B7213) antibody previously conjugated to Alexa555® (Invitrogen #A10470). As shown in Figure 16, healthy B6 CD11b+ and CD19+ splenocytes express surface Axl. To our knowledge, this is the first report of CD19+ splenocytes expressing surface Axl. Interestingly, B6.Sle1.Yaa CD11b+ splenocytes do not stain positively for Axl versus isotype control (representative diagram of three experiments shown).

Given the transcriptional parity between healthy and diseased mice (see Aim 2.1, p99, Figure 17), macrophages and B cells appear to be a source of serum soluble Axl in lupus-prone mice.

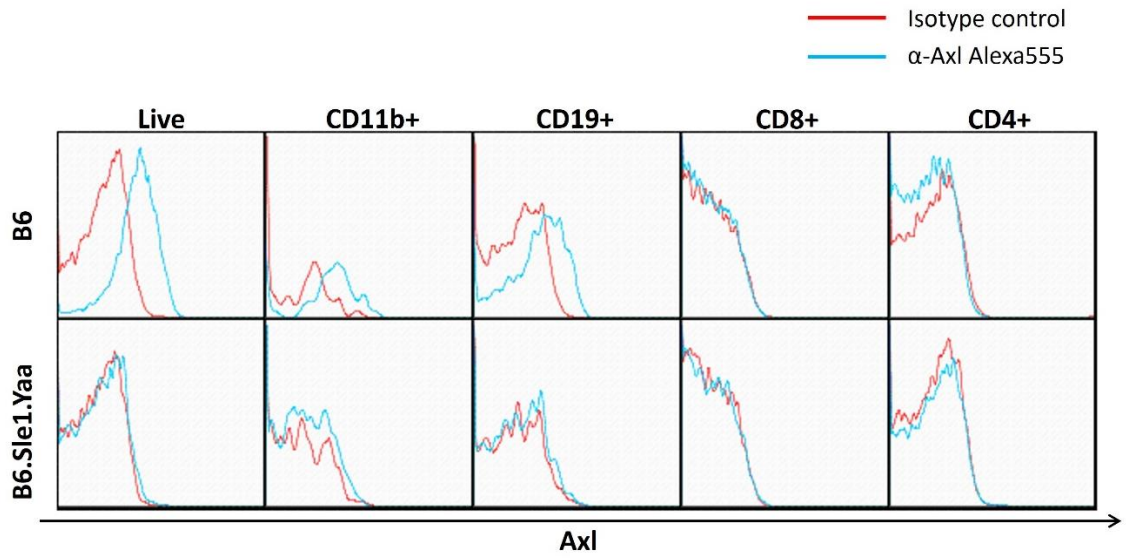


Figure 16 – Axl is lost from the surface of CD11b+ and CD19+ Sle1.Yaa splenocytes. B6 and B6.Sle1.Yaa spleens (n=3, 3) were harvested and splenocytes were stained for CD11b, CD19, CD8, and CD4. Each sample was further stained with A555-conjugated isotype control (red) or α-Axl antibody (blue). As expected and depicted in representative plots, B6 CD11b+ splenocytes stained positively for Axl. Unexpectedly, B6 CD19+ splenocytes also stained positively for Axl. As further expected, B6.Sle1.Yaa splenocytes did not stain positively for Axl. Representative plots shown.

AIM 2: TO DETERMINE THE CAUSE OF INCREASED AXL LOSS IN SLE LEUKOCYTES

2.1 The loss of Axl in mouse splenocytes is not due to decreased mRNA levels

Cleavage of immune cell surface Axl explains both the existence of serum sAxl and the loss of immune cell Axl signaling. However, this does not exclude potential changes in Axl mRNA transcript levels in these cells. To this end I performed quantitative PCR (RT-PCR) on lupus-prone splenocytes. As shown in Figure 17, lupus-prone splenocytes did not exhibit a decrease in Axl mRNA levels.

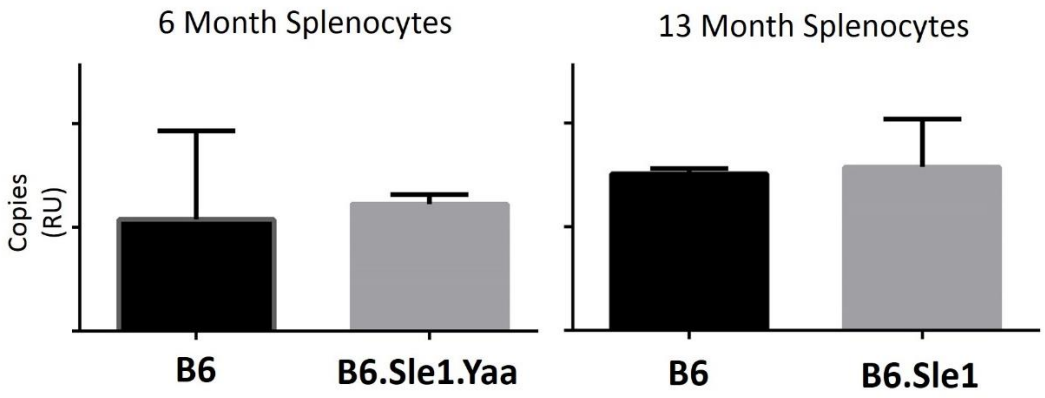


Figure 17 – The level of Axl mRNA in lupus-prone mouse splenocytes is not significantly different from those of healthy controls. Representative of 3 experiments of three mice per group in each experiment.

2.2 ADAM10 and TACE are elevated in human SLE PBMCs and lupus-prone mouse splenocytes

It is known that Axl is cleaved by matrix metalloprotease ADAM10 (A Disintegrin and Metalloproteinase domain-containing protein 10) in healthy cells, leaving a signaling-incompetent Axl receptor stump.[295,336,337] This is an important post-translational regulation checkpoint. ADAM10 had not been previously implicated directly in SLE but is elevated in macrophages in inflammatory conditions. These include

macrophages in the synovium of rheumatoid arthritis patients, in the cerebrospinal fluid (CSF) of multiple sclerosis patients, and in the alveoli of emphysema patients.[362-364] Closely-related matrix metalloprotease TACE (ADAM17, Tumor Necrosis Factor Alpha-Converting Enzyme) is elevated in SLE leukocytes, and our group and others have shown a number of ADAM10- and TACE-processed proteins to be elevated in SLE, notably CXCL16 and TNFα.[277,365,366] I hypothesized that one or both of these related proteases cleave Axl ectodomain from the surface of SLE leukocytes, as there is evidence for substrate overlap in the published literature as shown in Table 15.[367]

Shared ADAM10 and TACE Substrates

Inflammatory Mediators	
TNF-α	(Black et al., 1997)[368]
IL-6	(Müllberg et al., 1994)[371]
IL-15R	(Budagian et al., 2004; Bulanova et al., 2007)[373,374]
CX3CL1	(Hundhausen et al., 2003)[376]
CXCL16	(Abel et al., 2004)[378]

Immune Modulators	
Notch-1	(Brou et al., 2000; Mumm et al., 2000)[369,370]
Ephrins	(Janes et al., 2005)[372]
L-selectin	(Hafezi-Moghadam et al., 2001)[375]
Amyloid protein	(Esch et al., 1990)[377]
VCAM-1	(Garton et al., 2003)[379]
FasL	(Schulte et al., 2007)[380]

Table 15 – A number of inflammatory mediators and immune modulators are cleaved by both ADAM10 and TACE (ADAM17).

In order to confirm the elevation of these proteases in SLE leukocytes, I isolated PBMCs from human SLE patients and controls as well as splenocytes from B6 background control and lupus-prone mice. I isolated protein from these samples and performed Western analysis for the detection of ADAM10 and TACE versus loading

controls. Both ADAM10 and TACE are expressed in lupus-prone and SLE leukocytes versus healthy controls (Figure 18A, B).

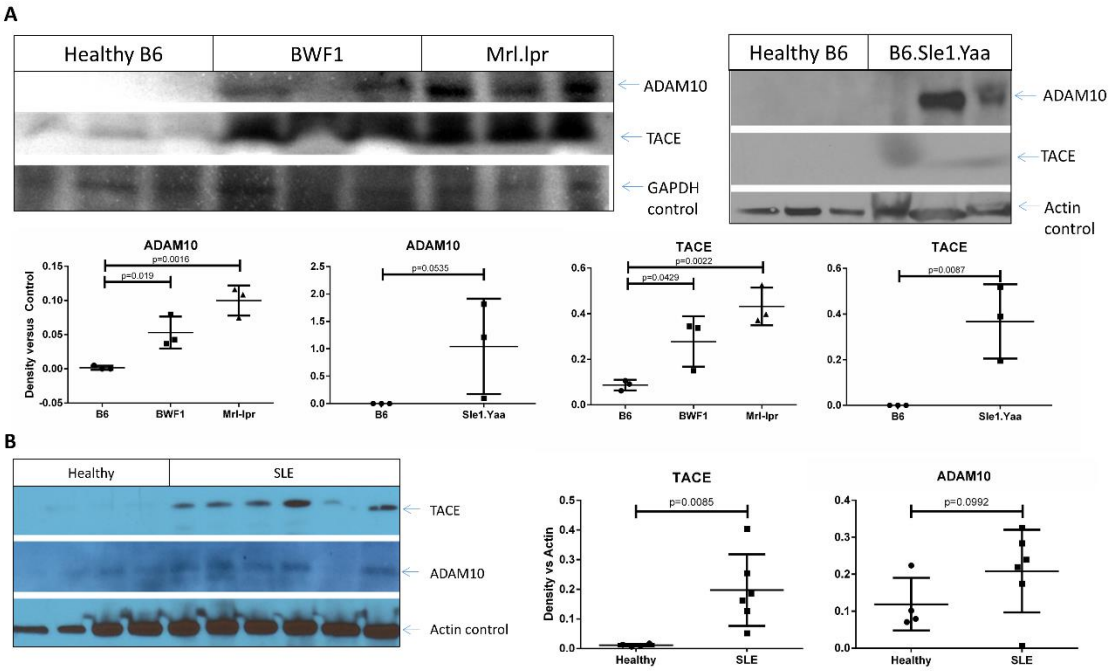


Figure 18 – ADAM10 and TACE are elevated in lupus-prone splenocytes and SLE PBMCs versus healthy controls. (A) Splenocytes were isolated from 6-month-old healthy B6, lupus-prone BWF1, and lupus-prone Mrl-lpr mice as well as from 4-month-old healthy B6 and lupus-prone B6.Sle1.Yaa mice. Protein was extracted and run on a gel in denaturing conditions and stained for ADAM10, TACE, or loading control. Both ADAM10 and TACE are elevated in lupus-prone mouse splenocytes. (B) PBMCs were isolated from healthy controls and SLE patients. Protein was extracted and run on a gel in denaturing conditions and stained for ADAM10, TACE, or actin loading control. Bands were quantified and plotted as density ratio versus loading control. SLE patients had significantly more TACE than healthy controls (p=0.0085).

2.3 ADAM10 and TACE inhibitors synergistically block Axl cleavage in CD11b+, CD11c+, and B220+ splenocytes in lupus-prone mice and in CD19+ and CD14+ human SLE PBMCs

To determine whether ADAM10, TACE, or both proteases are responsible for the cleavage of Axl ectodomain from the surface of splenocytes in lupus-prone mice, I

harvested splenocytes from age-matched healthy B6 and lupus-prone B6.Sle1.Yaa mice and treated them with vehicle control, ADAM10 inhibitor, TACE inhibitor, or combined inhibitors for 18 hours. As shown in Figure 19, combined ADAM10/TACE inhibition profoundly blocked Axl cleavage in CD11b+, B220+, and CD11c+ cells. This confirms that both proteases actively cleave Axl in lupus-prone splenocytes. CD4+ cells did not exhibit Axl expression with or without inhibitor treatment. This evidence suggests that TACE and ADAM10 both contribute to cleavage of Axl in lupus-prone splenocytes.

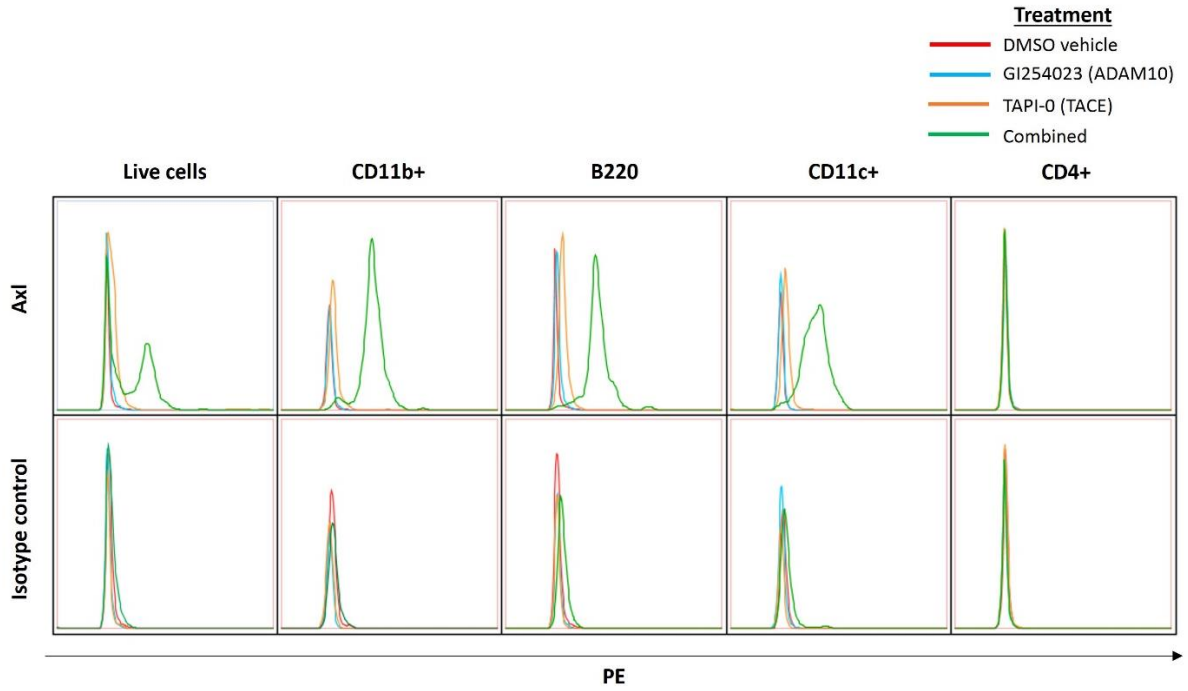


Figure 19 – Sle1.Yaa splenocytes were treated with DMSO vehicle control, a selective ADAM10 inhibitor (GI254023, 50μM), a selective TACE inhibitor (TAPI-0, 50μM), or combined inhibitors. Cells were isolated and stained with anti-Axl PE (top) or isotype control (bottom) in addition to anti-CD11b, B220, CD11c, and CD4 antibodies. Both inhibitors together resulted in greater expression. Note that CD4+ cells—which did not exhibit Axl staining in normal B6 mice—do not express Axl in the presence of inhibitor. Representative of four independent experiments.

To confirm that these proteases are also at work in SLE PBMCs, I isolated PBMCs from SLE patients and treated with vehicle control, ADAM10 inhibitor, TACE inhibitor, or both inhibitors together and stained for surface Axl. As shown in Figure 20A, individual protease inhibitor treatment elicited only marginal increases in surface Axl in each patient PBMC sample. Combined protease inhibitor treatment elicited greater increases in Axl surface expression. This rescue occurred variably on CD14+ or CD19+ SLE PBMCs or both. CD3+ human PBMCs did not express Axl. This variability between patient samples is consistent with observations summarized previously in Figure 13 (see p95). Taken together with diminished Axl phosphorylation in SLE PBMCs, these data suggest that ADAM10 and TACE cleave Axl and abrogate Axl signaling in human SLE PBMCs. The change in mean fluorescence intensity (MFI) was measured according to the following equation:

$$MFI\ ratio = \frac{((MFI_{Axl\ treated}) - (IMFI_{Isotype\ treated}))}{((MFI_{Axl\ untreated}) - (IMFI_{Isotype\ intreated}))}$$

and plotted in Figure 20B. Thus a ratio of 1 means there is no difference in Axl staining intensity in a given sample versus vehicle-treated control. These plots highlight the cooperative effects of ADAM10- and TACE-specific inhibition.

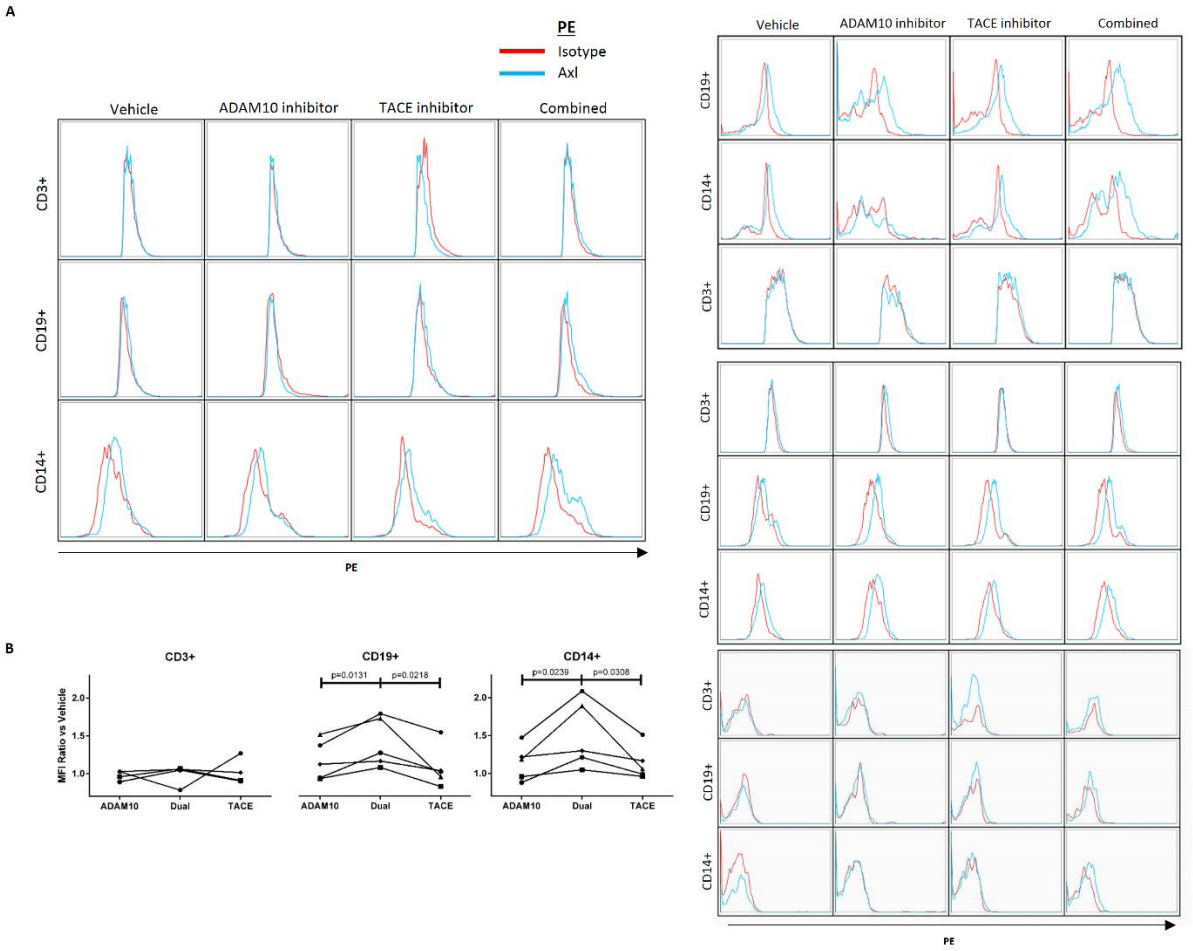


Figure 20 – Human SLE PBMCs upregulate Axl in response to combined protease inhibition. (A) Human SLE PBMCs were treated for 18 hours with DMSO vehicle control (column 1), 50μM ADAM10-specific inhibitor GI254023 (column 2), 50μM TACE-specific inhibitor TAPI-0 (column 3), or both inhibitors together (column 4). Cells were then isolated and stained with PE-conjugated anti-Axl antibody (blue) or isotype control (red) and analyzed by flow cytometry. CD3+ cells did not stain for Axl. CD19+ and/or CD14+ SLE PBMCs showed increased Axl staining in samples treated with both protease inhibitors, which varied from patient to patient. Each block represents one patient. (B) Changes in Axl surface staining in response to protease inhibitor treatment were compared to isotype staining mean fluorescence intensity (MFI) and graphed as a ratio to vehicle control in CD3+, CD19+, and CD14+ subsets (p=0.0131, p=0.0218, p=0.0239, p=0.0308). MFI ratio = ((Axl-stained protease inhibitor-treated sample MFI) - (isotype control-stained protease inhibitor-treated sample MFI)) ÷ ((Axl-stained vehicle-treated sample MFI) - (isotype control-stained vehicle-treated sample MFI)). Thus a ratio of 1 means there is no difference in Axl staining intensity in a given sample versus vehicle-treated control.

AIM 3: TO DETERMINE THE PHYSIOLOGICAL SIGNIFICANCE OF INCREASED AXL CLEAVAGE IN SLE.

3.1 Splenocyte surface Axl fails to signal in lupus-prone mice

The foregoing evidence suggests that Axl is cleaved from immune cells in SLE and lupus-prone mice. I next looked to determine whether this cleavage has phenotypic effects *in vitro* and *in vivo*.

To determine whether Axl signaling is lost in lupus-prone mouse splenocytes as it is in human SLE PBMCs (see Figure 13, p95), I isolated spleens from six-month-old healthy control (B6) and lupus-prone Mrl-lpr mice and extracted protein for analysis of by Western blot. I probed with antibodies against Axl (LSBio #B7213, extracellular epitope/ectodomain) and actively-signaling Y779-phospho-Axl (R&D Systems #AF2228). As shown in Figure 21, Mrl-lpr splenocytes showed significantly lower levels of total Axl and active phospho-Axl than B6 splenocytes. This mimics human PBMC pAxl loss (see Figure 13, p95). As previously mentioned, RT-PCR did not detect any difference in Axl mRNA levels in the spleens of lupus-prone mice versus healthy controls (see Figure 17, p99).

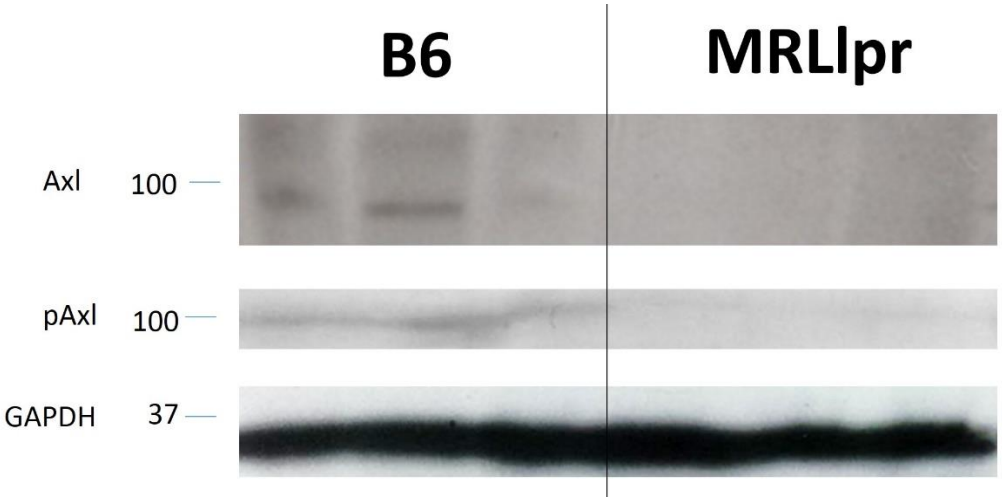


Figure 21 – Splenic Axl and (activated) phospho-Axl are significantly reduced in spleens of 6-month-old lupus-prone Mrl-lpr mice versus age-matched B6 controls as measured by Western analysis and quantified by densitometry (p=0.0011, 0.0015).

3.2 Lupus-prone splenocytes fail to induce Twist in response to Gas6 in vitro *and treatment with ADAM10 and TACE inhibitors rescues* Twist *mRNA levels as well as* Il6 *and* Tnfa *suppression.*

It has previously been reported in the literature that Gas6 induces Twist activation in macrophages through Axl and that Twist blocks NFκB transcriptional activity *in vitro*.[309] To confirm the link between Axl and Twist, I isolated splenocytes from normal B6 and B6.Axl-/- mice, treated them with low-dose LPS (1ng/ml) and vehicle control or Gas6 (400ng/ml), and measured expression of Twist by RT-PCR after 24 hours. I confirmed that Gas6 treatment induces Twist expression only in Axl+/+ splenocytes. The lack of Axl receptor also eliminates Gas6-dependent IL-6

downregulation. Gas6 alone was insufficient to induce Twist expression (data not shown).

I next isolated splenocytes from B6, B6.Sle1.Yaa, and B6.Axl-/- mice and treated with low-dose LPS (1ng/ml) and PBS vehicle control or low-dose LPS plus Gas6 (400ng/ml) for 24 hours. As shown in Figure 22A, B6.Sle1.Yaa splenocytes, like B6.Axl-/- splenocytes, do not upregulate *Twist* mRNA in response to Gas6 stimulation ($p=0.0113$). I previously showed that combined ADAM10 and TACE inhibition rescued surface Axl expression in Sle1.Yaa leukocytes. I next treated mouse splenocytes with these same inhibitors for 24 hours prior to LPS and Gas6 treatment. As shown in Figure 22B, Sle1.Yaa splenocytes do not increase Twist expression in response to Gas6 ($p=0.0251$) but Gas6-dependent B6.Sle1.Yaa splenocyte Twist expression was rescued by ADAM10 and TACE metalloprotease inhibition ($p=0.0058$). ADAM10 and TACE inhibition did not rescue Axl-/- splenocyte Twist expression. Twist functions normally to block the transcription of NFκB targets like the genes encoding tumor necrosis factor alpha (TNFα, *Tnfa*) and interleukin 6 (IL-6, *Il6*). As shown in Figure 22C and D, B6.Sle1.Yaa splenocytes do not downregulate *Il6* and *Tnfa* mRNA levels response to Gas6 ($p<0.0001$, $p=0.0081$). However, the suppression of these inflammatory mRNAs is rescued by ADAM10 and TACE inhibition ($p<0.0001$, $p=0.0093$).

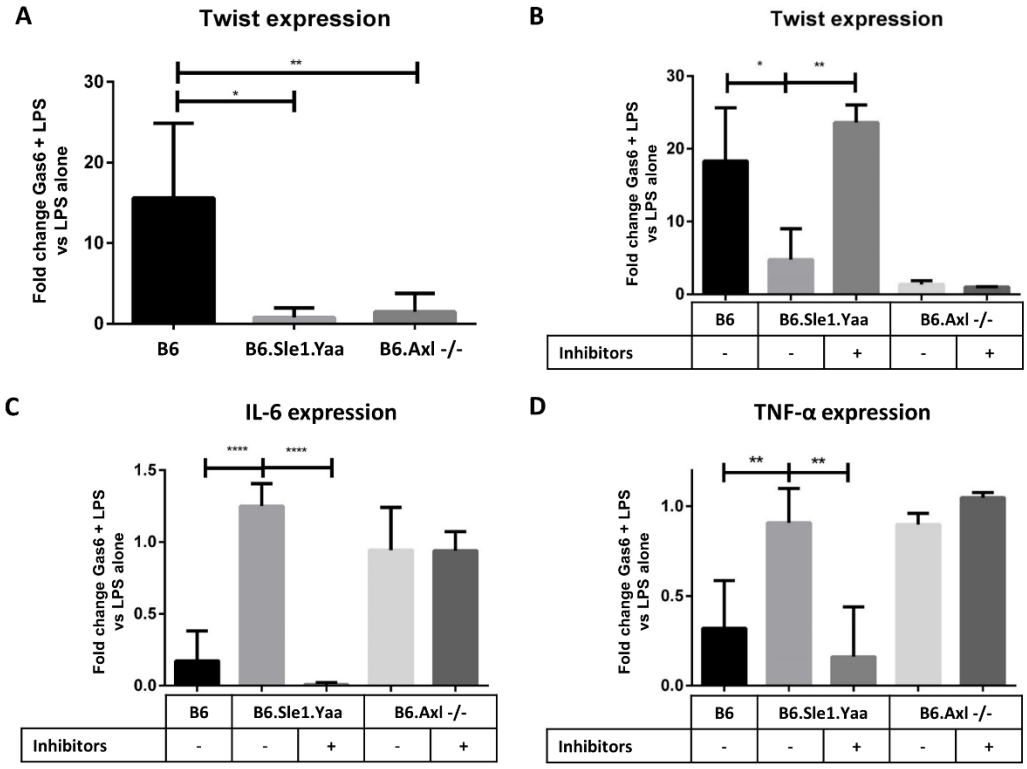


Figure 22 – A) B6.Sle1.Yaa and B6.Axl-/- splenocytes do not express *Twist* in response to Gas6 stimulation (p=0.0113). B) B6.Sle1.Yaa splenocytes induce *Twist* in response to Gas6 stimulation when rescued by inhibition of ADAM10 and TACE (p=0.0251, p=0.0058). C and D) *Il6* and *Tnfa* expression are reduced in response to Gas6 in B6.Sle1.Yaa splenocytes treated with ADAM10 and TACE inhibitors (p<0.0001, p<0.0001 and p=0.0081, p=0.0093 respectively).

3.3 Axl vectors may be used to reconstitute Axl expression in Axl-deficient leukocytes

To further study Axl physiology I produced two Axl vectors from a depository vector from Dana Farber. The first, wild-type construct and the second, uncleavable construct differed only by the deletion of the ADAM10 proteolytic cleavage site sequence identified by Budigan *et al* (^{432}QPLHHLVSEPPPRA^{446}, see APPENDIX B,

p178).[295] As described in methods, I transformed B6.Axl-/- BMDM using the Nucleofector™ system (Lonza, Basel, Switzerland) and analyzed by flow after 24 hours. Both vectors expressed in approximately 40% of recipient BMDM (Figure 23). I used these vectors in subsequent experiments.

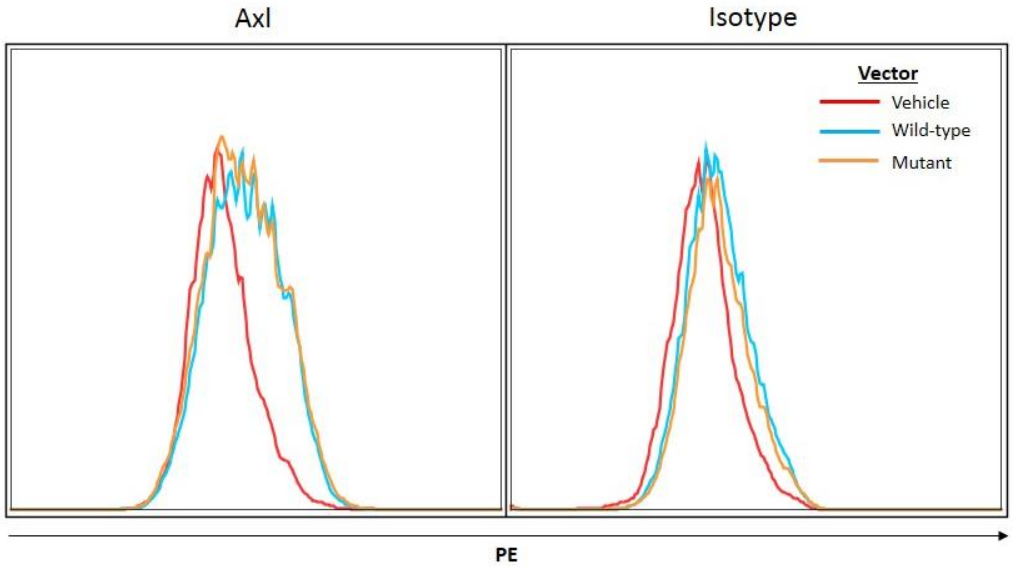


Figure 23 – B6.Axl-/- BMDM transfected with wild-type (blue) or mutant uncleavable (orange) Axl stained for surface Axl in about 40% of cells versus vehicle-transfected controls (red).

3.4 B cells do not upregulate Twist *significantly in response to Gas6 stimulation*

The finding of peripheral B cells expressing Axl is, to our knowledge, novel. It is unknown whether B cells, like macrophages, induce Twist in response to Gas6. The previous results in 3.2 were obtained using low-dose (1ng/ml) LPS stimulation (Figure 22). This low dose does not stimulate B cells.[381] However, I found previously that lupus-prone B splenocytes lose surface Axl staining (Figure 16, p98). To

determine whether B cells normally upregulate *Twist* in response to Gas6 stimulation, I isolated B6 splenocytes from B6, B6.Sle1.Yaa, and B6.Axl-/- mice and treated for 24 hours with LPS (1µg/ml). I then transfected cells with blank, wild-type Axl, or mutant uncleavable Axl vector constructs (see APPENDIX B, p178). After an additional 24 hours, I treated with Gas6 (400ng/ml) for 24 hours and harvested cells for RT-PCR analysis (Figure 24). B6 splenocytes do not markedly upregulate *Twist* mRNA levels in response to Gas6. Reconstitution with Axl vectors rescues this minimal upregulation. Uncleavable Axl vector rescue trends slightly higher than wild-type Axl vector rescue, although neither is likely physiologically relevant (data not shown). In future studies we will consider other mechanisms by which B cell Axl loss in SLE may contribute to disease.

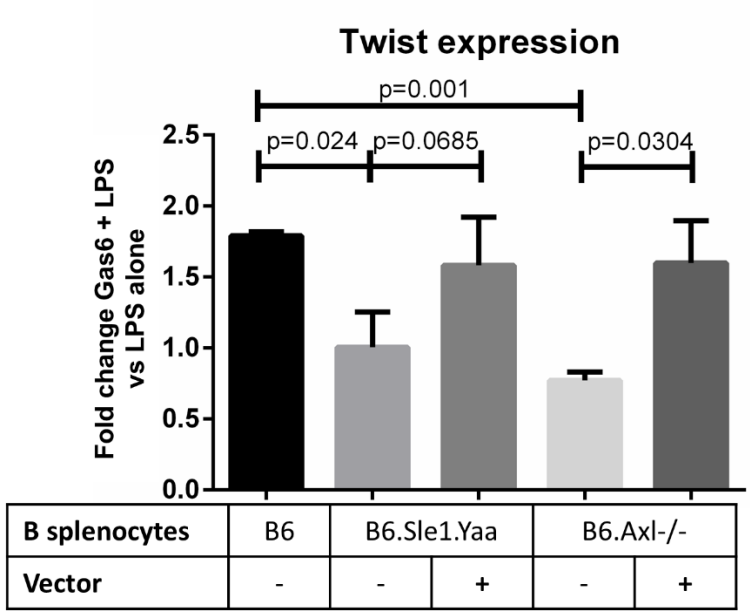


Figure 24 – B splenocytes were treated 24 hours with 1µg/ml LPS and transfected with Axl expression vectors described in Appendix B and incubated in LPS for an additional 24 hours. Subsequently, Gas6 (400ng/ml) was added to half of the cells and Twist mRNA levels were measured by RT-PCR. *Twist* levels are plotted as the ratio between Gas6-treated and vehicle control-treated cells. While B6.Sle1.Yaa and B6.Axl-/- B splenocytes do not upregulate *Twist* in response to Gas6 stimulation and a normal response is rescued by vector, these levels are very low, suggesting that B splenocyte Axl does not significantly alter cell function through Twist.

3.5 B6.Sle1.Yaa bone marrow-derived macrophages retain basal Axl expression similar to B6 bone marrow-derived macrophages

To determine whether the loss of Axl from the surface of lupus-prone macrophages is intrinsic to the macrophages or induced in the periphery, I isolated bone marrow from B6, B6.Sle1.Yaa, and B6.Axl-/- mice and induced them *in vitro* to produce bone marrow-derived macrophages (BMDM). I measured surface Axl versus isotype control staining by flow cytometry and found that B6.Sle1.Yaa BMDM retain a basal level of surface Axl similar to that of healthy control B6 BMDM (Figure 25). Unsurprisingly, Western analysis did not detect ADAM10 or TACE in either healthy B6 or lupus-prone Sle1.Yaa BMDM, even after long (>2hr) exposures (Figure 26). This aliquot of antibody was subsequently re-used on a positive sample as a control. This suggests that macrophage Axl loss is not intrinsic to the Sle1.Yaa macrophage but rather induced by inflammatory environmental cues, which is consistent with the finding of macrophage protease upregulation in a number of inflammatory conditions.[362-364]

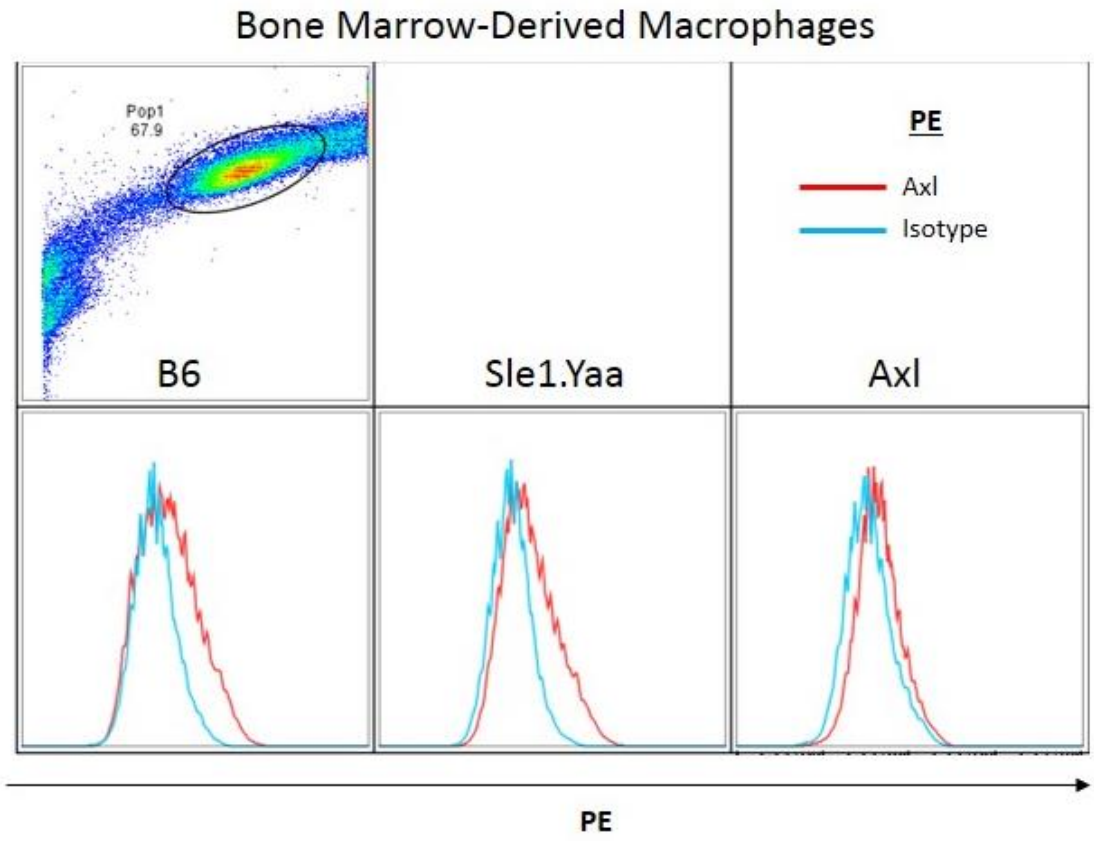


Figure 25 – Bone marrow-derived macrophages from Sle1.Yaa retain basal Axl expression as measured by flow cytometry (red) versus isotype control (blue).

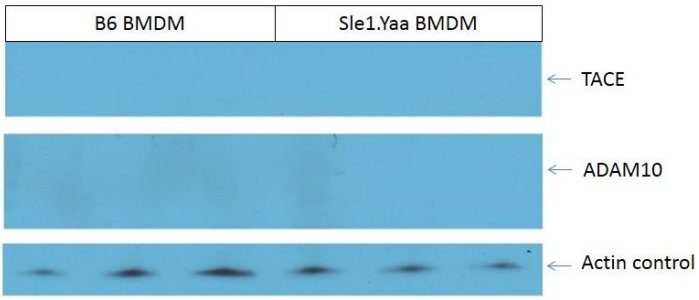


Figure 26 – Even after long-term (>2 hr) exposure, Western analysis did not detect any TACE or ADAM10 expression in B6 or lupus-prone Sle1.Yaa splenocytes

Given that B6.Sle1.Yaa bone marrow-derived macrophages (BMDM) do not intrinsically cleave Axl, it seemed likely that this change is induced in an inflammatory milieu. To show that B6.Sle1.Yaa BMDM are not intrinsically different

in their Axl expression response to common stimuli, I treated BMDM over 18 hours with vehicle control, CpG DNA, LPS (TLR4), R848 (TLR7/8), Poly I:C (TLR3), or type I interferon. Axl staining increased on treatment with LPS, Poly I:C, and Type I interferon, as expected. Interferon, which is relevant in SLE, may increase Axl expression on B6.Sle1.Yaa more than on B6 BMDM. No significant difference was noted between B6 and B6.Sle1.Yaa BMDM Axl staining in response to other stimuli (Figure 27). It appears that B6.Sle1.Yaa BMDM are not intrinsically different in their regulation of Axl expression than healthy B6 control BMDM, although this experiment was only performed once and will need to be repeated.

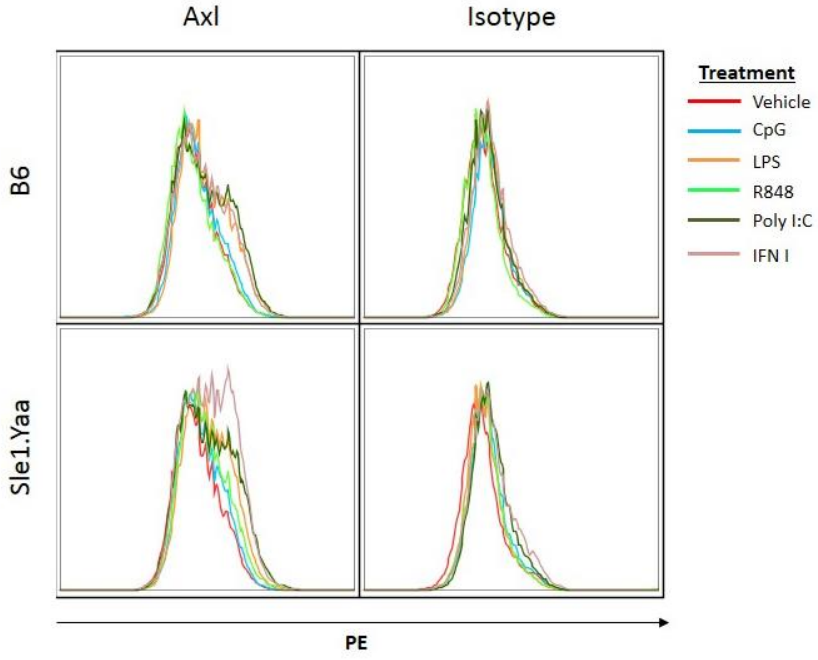


Figure 27 – Bone marrow-derived macrophages (BMDM) from healthy B6 and lupus-prone B6.Sle1.Yaa mice were treated for 18 hours with vehicle control (red), CpG (blue), LPS (orange), R848 (light green). Poly I:C (dark green), and IFN I (salmon).

3.6 Axl-knockout and Axl-heterozygote mice develop more severe anti-GBM nephritis than normal B6 controls

End-organ damage is the third and final checkpoint failure in SLE pathogenesis. Inflammation in the kidneys (*i.e.* nephritis) is a leading cause of morbidity and mortality in SLE.[258] Anti-glomerular basement membrane disease (anti-GBM) is a valuable mouse model of nephritis in which rabbit IgG and complete Freund's adjuvant (CFA) are injected into healthy young mice five days prior to tail vein injection of rabbit anti-glomerular basement protein antiserum.[358] Mice develop nephritis over the course of three weeks as measured by serum creatinine (SCr). This model is ideal for determining the effects of genetic mutations on end-organ damage over a short period of time.

As previously explained, Axl is likely to participate in lupus nephritis in two contradicting ways as summarized in Figure 28. First, Axl signaling inhibits inflammatory activity in leukocytes through Twist as we have shown previously. Second, Gas6 in the kidney contributes to nephritis and inflammation through Axl-mediated mesangial cell proliferation.[330] In short, Axl signaling has opposing effects in kidney mesangial cells (pro-disease) versus peripheral leukocytes (anti-inflammatory).

The loss of both of these signals in anti-GBM disease would be expected to have opposing consequences: A loss of Axl in leukocytes may make them more prone to inflammation, whereas a loss in glomeruli may attenuate nephritis. I hypothesized that anti-GBM nephritis will be worse in Axl heterozygotes than in B6 or B6.Axl-/- controls. This seems likely because residual mesangial cell Axl would mediate nephritic proliferation while attenuated leukocyte Axl expression would fail to block inflammatory cytokine secretion.

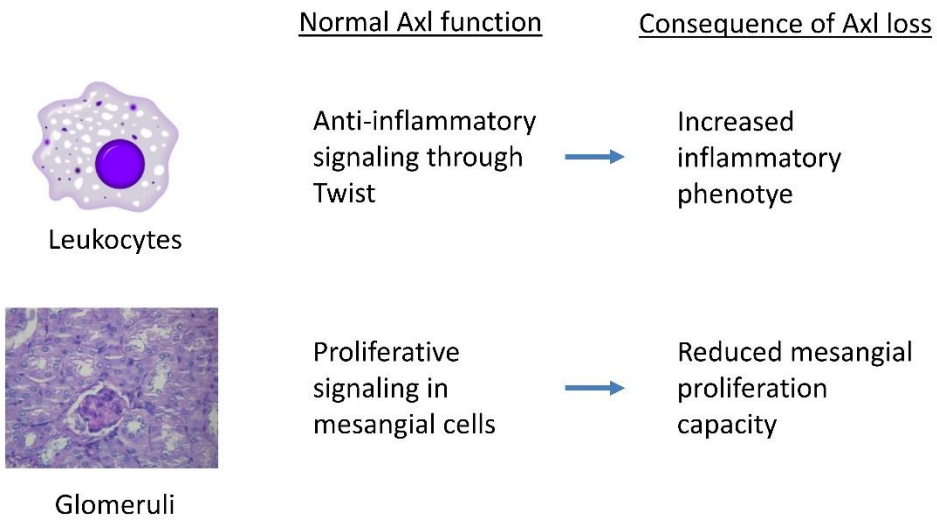


Figure 28 – Normal Axl function in leukocytes and glomeruli. In leukocytes, Axl acts as an anti-inflammatory mediator through twist. In glomeruli, Axl acts in mesangial cells to mediate proliferative signaling. The loss of both of these signals in anti-GBM disease would be expected to have opposing consequences: A loss of Axl in leukocytes may make them more prone to inflammation, whereas a loss in glomeruli may attenuate nephritis.

To test this hypothesis, I induced nephritis with the anti-GBM model described in Methods (Chapter 3, p65). In brief as depicted in Figure 29A, mice were injected *i.p.* at day zero (D0) with rabbit IgG and *i.v.* at day five (D5) with rabbit anti-glomerular

basement membrane protein. I subjected sibling wild-type B6 (B6 background control, n=5), B6.Axl^{het} (B6 background Axl heterozygotes, n=4), and B6.Axl$^{-/-}$ (B6 background Axl knockouts, n=7) to anti-GBM disease and followed them for three weeks, sacrificed, and analyzed for creatinine, anti-dsDNA autoantibodies, and urine protein.

As I expected, Axl heterozygotes exhibited the most pronounced nephritis as measured by serum creatinine (Figure 29B). Axl heterozygotes also exhibited elevated levels of urine protein versus B6 controls (Figure 29C).

Interestingly, complete Axl knockout mice exhibited significantly elevated anti-dsDNA IgG, a marker of systemic inflammation (Figure 29C). These levels are not as high as those in spontaneous lupus models with developed systemic pathology, likely because anti-GBM mice do not develop frank systemic disease. This further suggests that immune cell Axl may be directly responsible for this change.

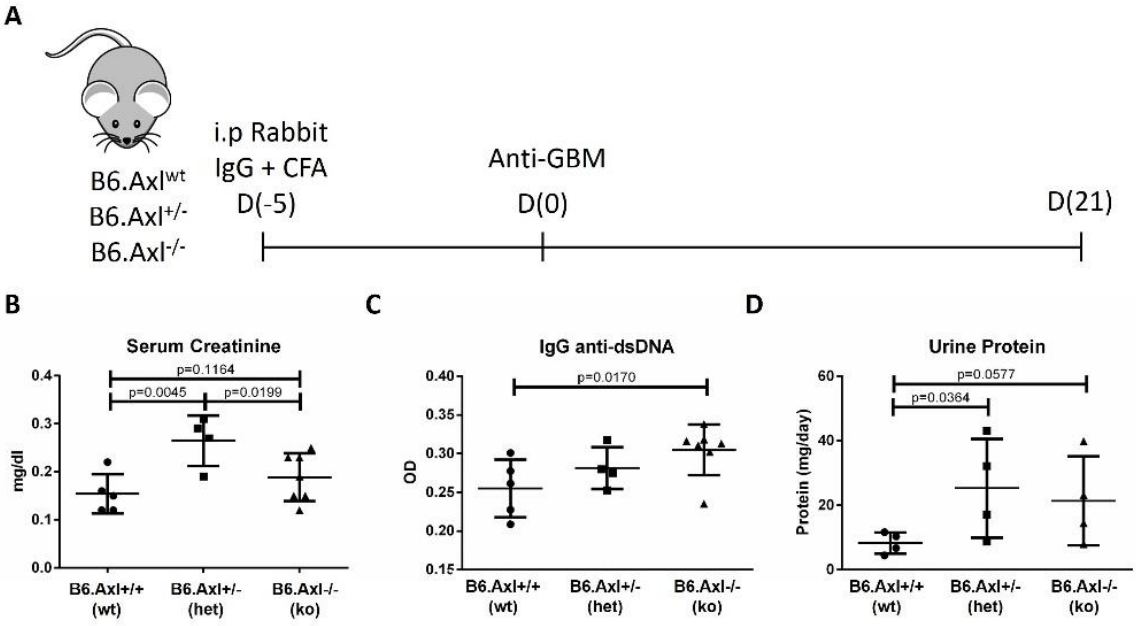


Figure 29 – In an anti-GBM trial, Axl heterozygotes exhibited worsened kidney pathology versus B6.Axl+/+ and B6.Axl-/- controls. Anti-glomerular basement membrane (GBM) disease is induced in mice by D(-5) intraperitoneal injection of a 225µl mixture of 100µl Complete Freund's Adjuvant (CFA), 100µl PBS, and 25µl rabbit IgG, D0 intravenous injection of mouse anti-rabbit glomerular basement membrane serum at 150µl per 20g (A). Axl heterozygotes (het, n=4) fared more poorly than Axl knockouts (ko, n=7) or wild-type mice (wt, n=5) as measured by serum creatinine (B) and urine protein (D) and Axl knockouts showed increased serum anti-dsDNA IgG, indicative of systemic autoimmunity (C).

3.7 Transfer of Axl-/- macrophages worsens anti-GBM disease

A variation of the anti-GBM model directly tests the phenotypic effects of macrophages *in vivo* by cell transfer.[358] To determine whether macrophage Axl deficiency alone contributes to nephritis, I transferred B6.Axl+/+, B6.Axl-/-, and Axl vector-reconstituted B6.Axl-/- macrophages into anti-GBM-treated recipient mice that develop nephritis over three weeks. In brief as outlined in Figure 30A, young healthy sibling B6 mice were challenged with intraperitoneal rabbit IgG plus CFA six days prior (D-5) to anti-GBM antibody tail vein injection (D1). On D0, these mice randomly

received adoptively-transferred bone marrow-derived macrophages (BMDM) from B6.Axl+/+, B6.Axl-/-, or wild-type Axl- or uncleavable Axl-reconstituted B6.Axl-/- donor littermates. After three weeks, mice receiving Axl-deficient donor BMDM experienced significantly worse end organ damage as measured by serum creatinine than those receiving B6.Axl+/+ donor BMDM (Figure 30B). Interestingly, this increase in serum creatinine was normalized by reconstitution of B6.Axl-/- donor macrophages with vectors expressing wild-type or uncleavable Axl. B6 recipients of Axl-deficient donor BMDM also exhibited significantly-elevated autoantibody levels, albeit lower than matched spontaneous lupus controls (Figure 30C). B6.Axl-/- donor BMDM recipients further trended toward elevated 24-hour urine protein (Figure 30D). We hypothesized that the lack of Axl in BMDM may affect disease through by secreting increased inflammatory mediators such as IL-6. Axl-deficient macrophages significantly increase serum IL-6 levels in anti-GBM disease (Figure 30E). Such high levels suggest that recipients of Axl-deficient BMDM may also exhibit systemic effects. Indeed, a blinded observer was able to identify recipients of Axl-deficient donor BMDM by their appearance, which included hair fraying, hair loss, and crouched habitus (Figure 30F). B6 recipients of B6.Axl+/+ donor BMDM did not exhibit these features. B6.Axl-/- BMDM grew identically to B6.Axl+/+ BMDM *in vitro*, suggesting that these differences were not altered by cell survival. Rescue was not significantly different between uncleavable and Axl vectors and this part of the experiment will need to be repeated using appropriate controls.

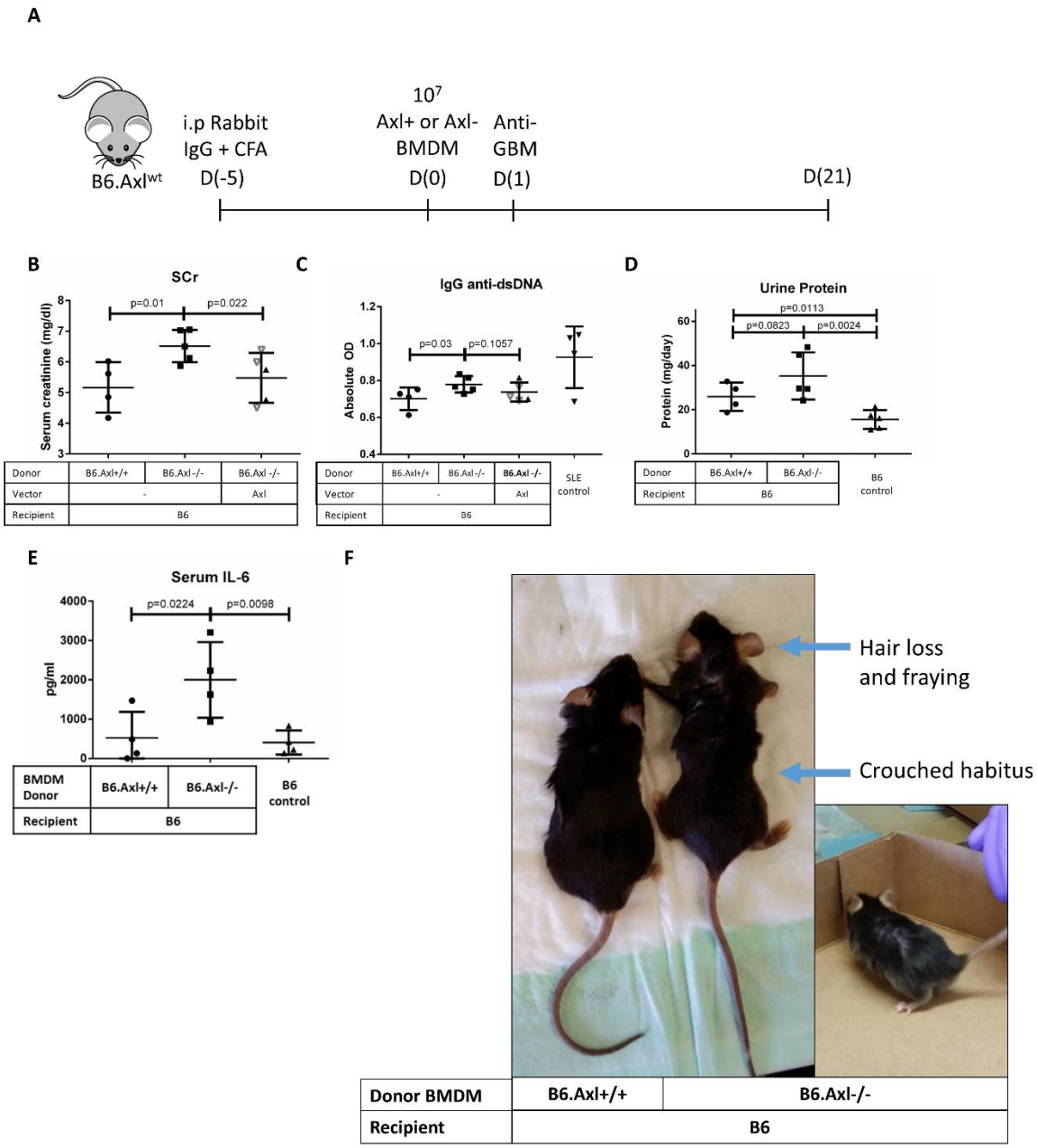


Figure 30 – Anti-GBM-treated mice received 10^7 bone marrow-derived macrophages (BMDM) from sibling B6, B6.Axl-/-, or B6.Axl-/- Axl vector-reconstituted donors (A). Axl-deficient donor macrophage recipients exhibit elevated serum creatinine (B, p=0.01), elevated anti-dsDNA IgG autoantibodies (C, p=0.03), elevated urine protein (D, p=0.0823), and elevated serum IL-6 (E, p=0.0224) as well as hair loss, hair fraying, and crouched habitus noted by a blinded observer (F). Mice receiving Axl-reconstituted macrophages (open triangles represent uncleavable-reconstituted and closed triangles represent wild type-reconstituted BMDM recipients) had significantly lower serum creatinine than those receiving Axl-deficient macrophages, but appropriate controls will be necessary to determine the significance of this observation.

3.8 Exogenous sAxl may abrogate Gas6 induction of Twist in vitro

Gas6 is usually complexed with sAxl *in vivo* in the bloodstream.[382] Further, Gas6 has a higher binding affinity to Axl than to other receptors.[286] I sought to determine whether soluble Axl cleaved from leukocytes may act as a "sink" to sequester the ligand Gas6 to prevent engagement of intact Axl receptors.

To determine whether a soluble factor from B6.Sle1.Yaa splenocytes inhibits splenocyte Twist upregulation, I cultured B6.Axl-/- and B6.Sle1.Yaa splenocytes in 1μg/ml LPS and growth media for 48 hours, isolated cell supernatants, and cultured normal B6 splenocytes in these supernatants with or without 400ng/ml Gas6 for 24 hours. Twist mRNA levels were reported as a ratio between Gas6-treated and untreated splenocytes (Figure 31A). B6.Sle1.Yaa supernatants inhibit Gas6-mediated Twist induction in normal B6 splenocytes.

To determine whether the soluble factor inhibiting Gas6-mediated Twist upregulation is cleaved by proteases ADAM10 and/or TACE, I isolated B6 and B6.Sle1.Yaa splenocytes and treated for 48 hours with 1μg/ml LPS with or without ADAM10- and TACE-specific protease inhibitors. Protease inhibitor-treated cells should not cleave Axl (among other substrates) into their supernatant, which I hypothesized would alleviate the inhibitory effect of the supernatant. I treated newly-isolated healthy B6 splenocytes with these supernatants and 400ng/ml Gas6 or vehicle control for 24

hours. I also simultaneously treated healthy B6 splenocytes with only LPS or LPS plus 400ng/ml Gas6 as a control. Twist mRNA levels were reported as a ratio between LPS plus Gas6-treated and LPS only-treated splenocytes (Figure 31B). Pre-treatment of B6.Sle1.Yaa splenocytes with inhibitor produced supernatants that rescued *Twist* expression to the same level as B6 supernatants and no supernatant (see dotted line). This suggests that a substrate or substrates of ADAM10 and/or TACE from lupus-prone splenocytes inhibits Gas6-induced *Twist* expression.

While I hypothesized that this substrate is the Axl ectodomain, these experiments did not exclude two other possible explanations as outlined in Figure 32. First, these experiments do not exclude the possibility of some other ADAM10 and/or TACE substrate acting as a decoy receptor to prevent Gas6-mediated *Twist* response in the healthy cells (Figure 32, second column). Second, these results could be explained by a soluble factor from B6.Sle1.Yaa splenocytes acting to upregulate ADAM10 and/or TACE in the healthy B6 cells to cleave Axl and prevent signaling. This possibility is not excluded because protease inhibitors are carried over in the treated supernatants in the experiment in Figure 31B. ADAM10 and TACE each cleave many substrates, many of which modulate immunity and some of which may potentially bind Gas6.

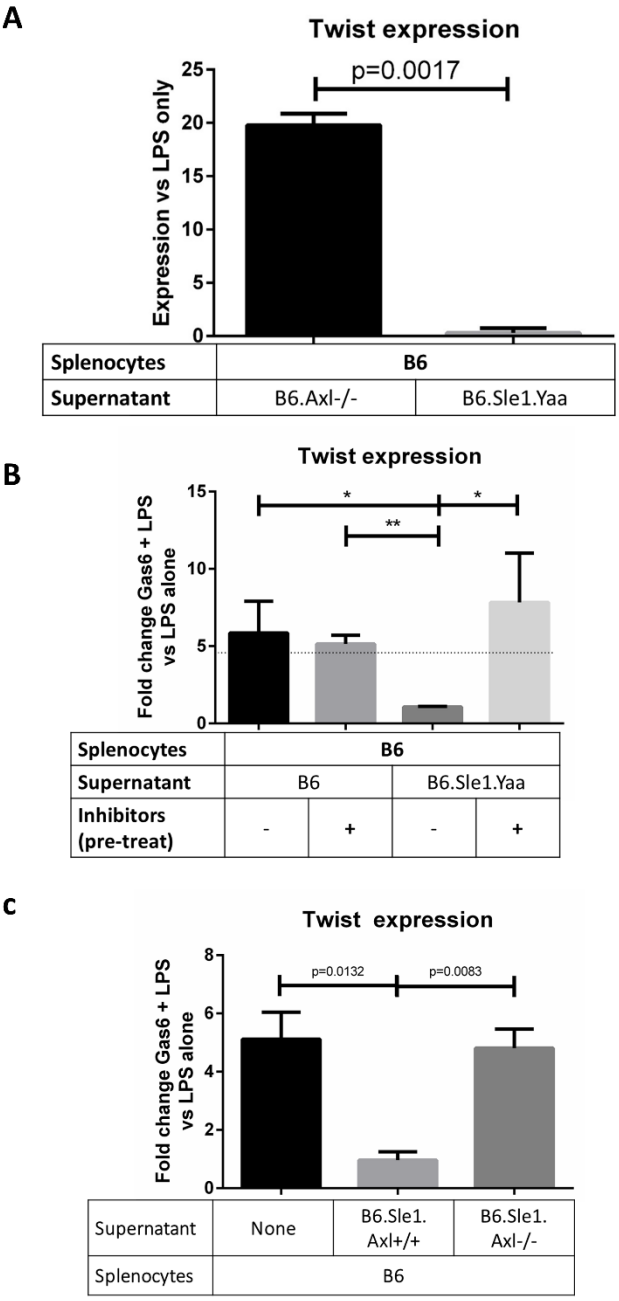


Figure 31 – "Trans" sAxl inhibits Gas6-mediated *Twist* mRNA induction. (A) B6.Axl-/- and B6.Sle1.Yaa splenocytes were cultured for 48 hours in 1µg/ml LPS. Supernatants were isolated and used to treat fresh B6 splenocytes for 24 hours with or without 400ng/ml Gas6. The ratio of Gas6-treated to control is reported. B6.Sle1.Yaa splenocyte supernatant inhibits *Twist* expression in healthy B6 splenocytes (p=0.0017). (B) B6 and B6.Sle1.Yaa splenocytes were cultured in the presence or absence of ADAM10/TACE inhibitors in 1µg/ml LPS and supernatants for 48 hours, after which supernatants were isolated and used to treat healthy B6 splenocytes with or without 400ng/ml Gas6 over 24 hours. The ratio of Gas6-treated to LPS-only control is reported. Splenocytes not treated with supernatants were simultaneously treated with LPS with or without Gas6 and measured as a healthy baseline control (dotted line). Treatment of B6.Sle1.Yaa splenocytes with ADAM10/TACE inhibitors rescues supernatant-induced blockage of Gas6-induced *Twist* expression. This suggests that a factor cleaved by ADAM10 and/or TACE from B6.Sle1.Yaa splenocytes blocks Gas6 stimulation of the Axl receptor. (C) B6.Sle1.Axl+/+ and B6.Sle1.Axl-/- splenocytes were cultured in 1µg/ml LPS for 48 hours, after which supernatants were isolated and used to treat fresh healthy B6 splenocytes in the presence or absence of 400ng/ml Gas6 over 24 hours. The ratio of Gas6-treated to LPS-only control is reported. Splenocytes not treated with supernatants were also treated simultaneously. Unlike normal B6.Sle1 supernatants, B6.Sle1.Axl-/- supernatants do not inhibit Gas6-induced *Twist* expression.

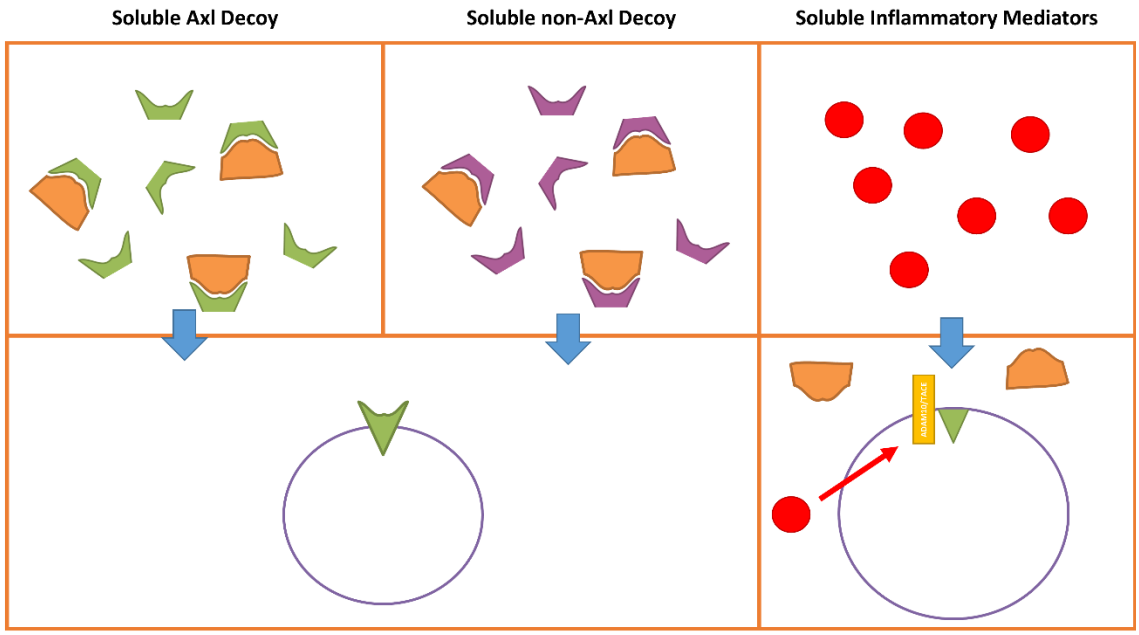


Figure 32 – The experiments in Figure 31A and B pose three plausible explanations for the inhibitory capacity of B6.Sle1.Yaa splenocyte supernatants on Gas6-mediated *Twist* upregulation. First, as I hypothesize, soluble Axl from B6.Sle1.Yaa splenocytes may act as a decoy to prevent Gas6 from engaging intact Axl receptor in the healthy B6 cells (first column). Second, some other ADAM10- and/or TACE-cleaved product may also act as a decoy receptor (second column). Lastly, an unrelated soluble inflammatory mediator from B6.Sle1.Yaa splenocytes may upregulate ADAM10 and/or TACE to cause shedding of Axl from healthy B6 splenocytes (third column). This possibility is not excluded by the experiment in Figure 31B because ADAM10/TACE inhibitors are carried over in the treated supernatants. These alternative hypotheses are addressed in the experiment in Figure 31C.

To exclude both of the alternative hypotheses diagrammed in Figure 32, I isolated B6.Sle1. and B6.Sle1.Axl-/- splenocytes and treated them with 1µg/ml LPS for 48 hours. I then isolated the supernatants from these cells to treat healthy B6 splenocytes with 400ng/ml Gas6 or vehicle control. I also treated healthy B6 splenocytes with LPS or Gas6 plus LPS in the absence of supernatants as a control. Twist mRNA levels were reported as a ratio between LPS plus Gas6-treated and LPS only-treated splenocytes (Figure 31C). B6.Sle1.Axl-/- supernatants did not exhibit any suppression versus supernatant-free treated B6 splenocytes. In contrast, B6.Sle1 supernatants containing Axl suppressed Gas6-mediated *Twist* induction entirely. This excluded the alternative hypothesis in column 2 of Figure 32. Further treatment of the B6 splenocytes with protease inhibitors did not significantly improve *Twist* induction in these experiments (data not shown). This excluded the alternative hypothesis in column 3 of Figure 32.

Taken together, these data suggest that soluble Axl ectodomain acts to block Gas6-mediated signaling. These experiments will need to be repeated for future publication.

SUMMARY

In summary, I found that macrophages and B cells in human SLE and lupus-prone mice express proteases ADAM10 and TACE (ADAM17). These proteases cleave Axl ectodomain from the surface of these cells. In macrophages, this cleavage abrogates Gas6-induced Twist expression and Gas6-induced IL-6 and TNF-α suppression. Soluble Axl also acts as a sink receptor to block this anti-inflammatory pathway. *In vivo*, Axl-deficient macrophages worsen end-organ damage in anti-GBM nephritis. A summary of this model is found in Figure 8 (p88).

CHAPTER FIVE

LOSS OF B-CATENIN SIGNALING MAY LIMIT ANTIINFLAMMATORY RESPONSE IN SLE

INTRODUCTION

As previously explained, I used Ingenuity™ canonical pathways with results from a targeted proteomic screen to identify the Wnt/β-catenin pathway as potentially dysregulated in immune cells in SLE. Wnt/β-catenin activity has previously been implicated in immunity as an important determinant of dendritic cell function.[347]

The Wnt/β-catenin pathway is central to embryogenesis and tumor development and was first characterized in *Xenopus, Drosophila*, and mice.[383,384] Wnt is a chemotactic factor that signals through seven-membrane-spanning protein Frizzled that responds to signal by releasing Disheveled (Dsh). Dsh

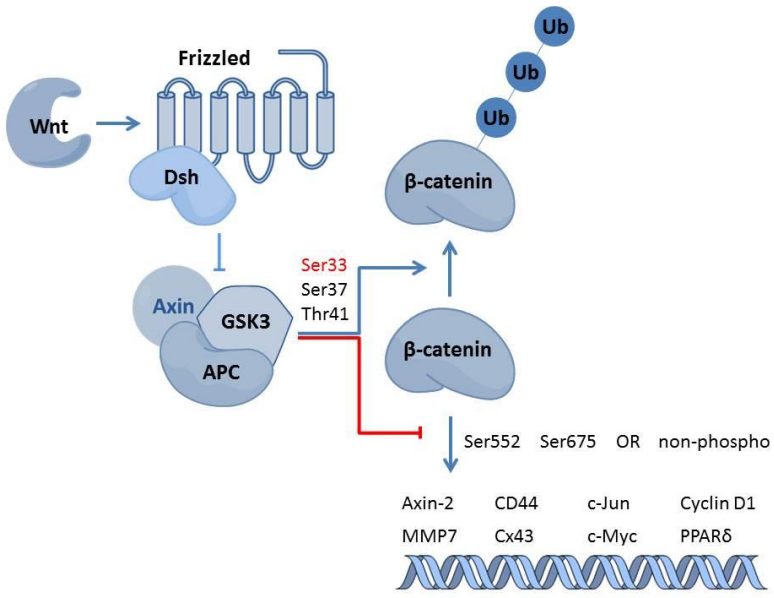


Figure 33 – The canonical Wnt/β-catenin pathway is regulated by β-catenin phosphorylation.

inhibits the formation of the catenin destruction complex that comprises Axin-2, GSK3 (Glycogen synthase kinase 3), and APC (Adenomatous polyposis coli) (Figure 33).[339,340] When Wnt signaling is preventing the activity of the destruction complex, unphosphorylated or Ser552- or Ser675-phospho β-catenin translocates to the nucleus and leads to transcription of downstream TCF/LEF promoter targets, including Axin-2 in a negative feedback loop.[338] In the absence of Wnt signaling, however, the destruction complex phosphorylates β-catenin at Ser33, Ser37, and Thr41.[341] The phosphorylated β-catenin is recognized by ubiquitin ligases and degraded in the cytosol and thus does not translocate to the nucleus to initiate transcription.

Interestingly, the Wnt/β-catenin signaling pathway has previously been implicated in kidney disorders. Further, DKK-1—an inhibitor of Wnt/β-catenin signaling—is elevated in lupus-prone mouse and human SLE serum.[348,385]

Separately, β-catenin participates in cell-cell adhesions known as *adherens junctions.* These junctions are important in tubular epithelial cells in the kidney and elsewhere in the body. Individual β-catenin molecules cannot participate in both transcription and cell-cell adhesion due to spatial exclusion.[344]

I hypothesize that the beta-catenin pathway in immune cells and kidney may contribute to inflammation and nephritis in SLE. My specific aims are:

1. **Aim 1: Determine the state of Wnt/β-catenin signaling in lupus-prone leukocytes.** I found that the Wnt/β-catenin pathway is depressed in leukocytes from lupus-prone mice by Western analysis and RT-PCR. This β-catenin loss is most pronounced in CD19+ and CD11b+ cells. Selective deletion of β-catenin in lysosome-containing cells in lupus-prone mice does not affect disease progression.

2. **Aim 2: Characterize the state of Wnt/β-catenin signaling in lupus-prone kidney.** I found that Wnt/β-catenin signaling is elevated in the kidneys of lupus-prone mice by RT-PCR. Endothelial cells appear to be a source of this increased β-catenin transcription and may exhibit decreased adhesion.

AIM 1: TO DETERMINE THE STATE OF WNT/B-CATENIN SIGNALING IN LUPUS-PRONE LEUKOCYTES

1.1 The Wnt/β-catenin pathway is depressed in SLE immune cells versus healthy controls

To determine whether β-catenin and a downstream transcription target protein are expressed in leukocytes in SLE, I isolated human PBMCs and probed for β-catenin and Axin-2—a β-catenin-transcribed negative feedback product that stabilizes the β-catenin destruction complex—by Western analysis. Both β-catenin and Axin-2 are significantly reduced in SLE PBMCs as quantified by densitometry versus GAPDH loading control (Figure 34).

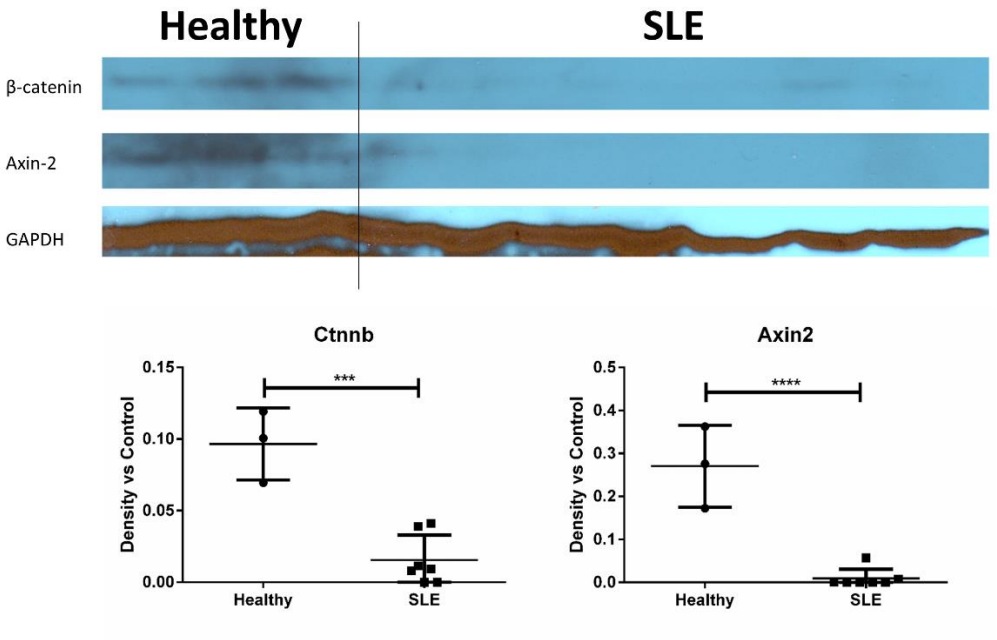


Figure 34 – β-catenin and Axin-2 are lost in SLE PBMCs (p=0.0003, <0.0001). Axin-2 is a transcriptional target of β-catenin and acts as a negative feedback mechanism for the Wnt/β-catenin pathway.

To determine whether this loss of immune β-catenin activity also occurs in lupus-prone mice, I isolated splenocytes from 6-month-old healthy B6 and lupus-prone Mrl-lpr mice and stained for β-catenin, phosphorylation-inactivated p33 β-catenin, and Axin-2. As shown in Figure 35, β-catenin is lost from diseased Mrl-lpr versus healthy B6 controls.

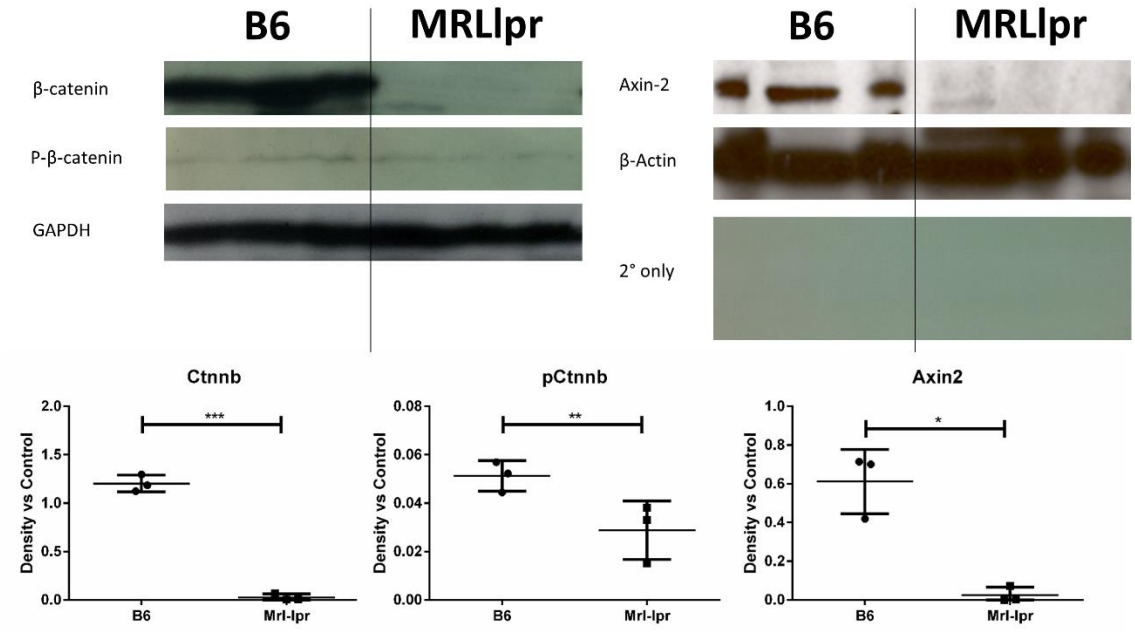


Figure 35 – β-catenin (p<0.001), p33-inactivated β-catenin (p=0.0041), and Axin-2 (p=0.0464) are lost from diseased lupus-prone mouse splenocytes versus healthy controls.

I then looked to determine whether Axin-2 loss in these splenocytes is due to decreased β-catenin transcriptional activity by RT-PCR. As shown in Figure 34, transcription of β-catenin and its product Axin-2 are depressed in diseased lupus-prone mice.

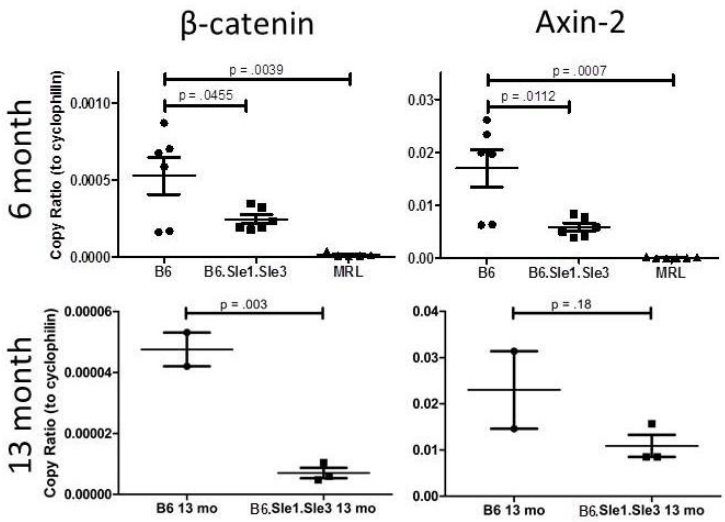


Figure 36 – β-catenin transcriptional activity is lost in lupus-prone splenocytes.

1.2 Many different immune cells lose β-catenin activity in SLE

Recognizing that immune cells broadly lose β-catenin activity, I set out to determine which cellular subsets contribute most to this loss. I first performed immunofluorescence (IF) against phosphorylated (*i.e.* Ser33P, inactive β-catenin) as shown in Figure 37. Splenocytes broadly exhibited an increase in β-catenin inactivation (*i.e.* phosphorylation), some of which was localized to F4/80+ macrophages in the spleen.

It became clear that flow cytometry would offer a better method for determining which cells in the spleen lose β-catenin in SLE. I isolated splenocytes from 8-month-old B6 (healthy control), B6.Sle1 (mildly diseased), and B6.Sle1.Yaa (severely diseased) mice and stained intracellularly for unphosphorylated β-catenin (Figure

36). All major splenocytes populations—B cells, macrophages, and T cells—showed patent β-catenin. This β-catenin is lost in splenocytes in diseased mice. This loss is most pronounced in CD19+ and CD11b+ cells (Figure 38).

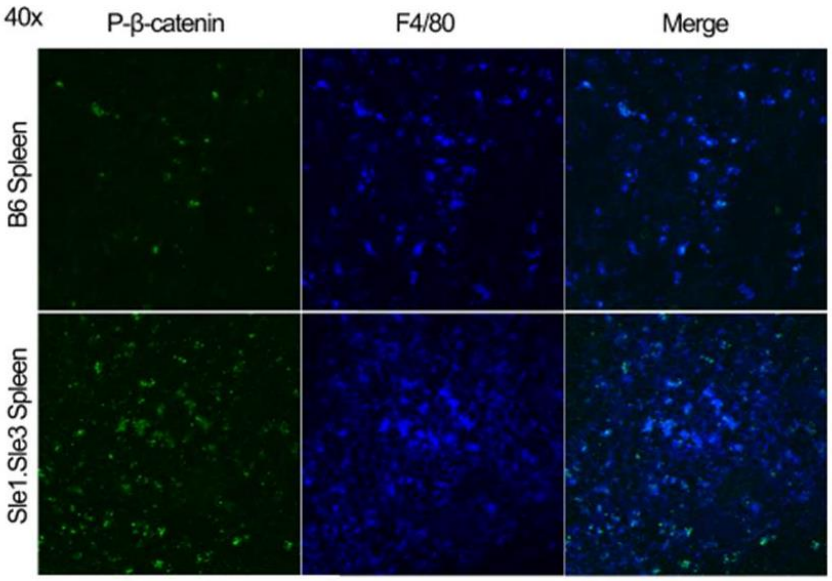


Figure 37 – Broad splenocytes—including F4/80+ macrophages—exhibit an increased rate of β-catenin inactivation.

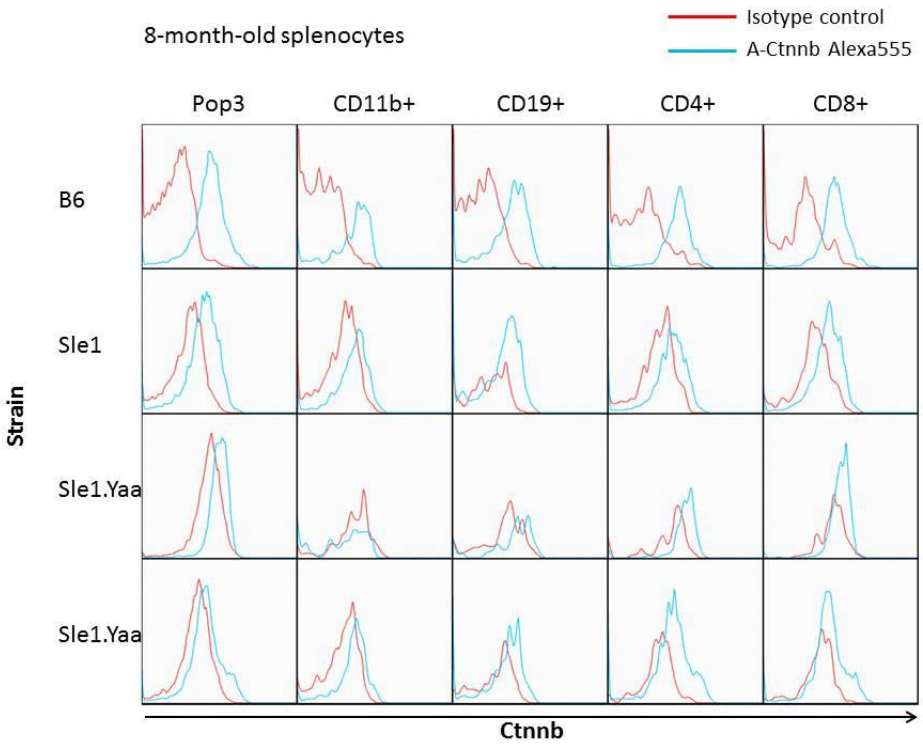


Figure 38 – Flow cytometry with intracellular staining shows that CD11b+, CD19+, and potentially CD4+ and CD8+ cells lose β-catenin in lupus-prone mice. Pop3 refers to all live lymphocytes.

1.3 B6.Sle1.Yaa.LyzM-cre.β-catenin$^{fl/fl}$ mice do not develop more severe lupus

While all leukocytes normally express β-catenin and lose this expression in SLE, I hypothesized that macrophage β-catenin loss may negatively influence disease progression as macrophages are important cytokine producers in SLE. To determine whether macrophage-specific β-catenin loss affects disease progression, I bred Sle1, Yaa, LyzM-cre, and Ctnnb-fl/fl loci onto B6 background mice and followed for disease progression. As shown in Figure 39, mice with myeloid-specific β-catenin loss did not have worse disease as measured by dsDNA autoantibodies, serum creatinine levels, and 24-hour urine protein. They did exhibit significantly depressed levels of anti-dsDNA IgM than sibling controls.

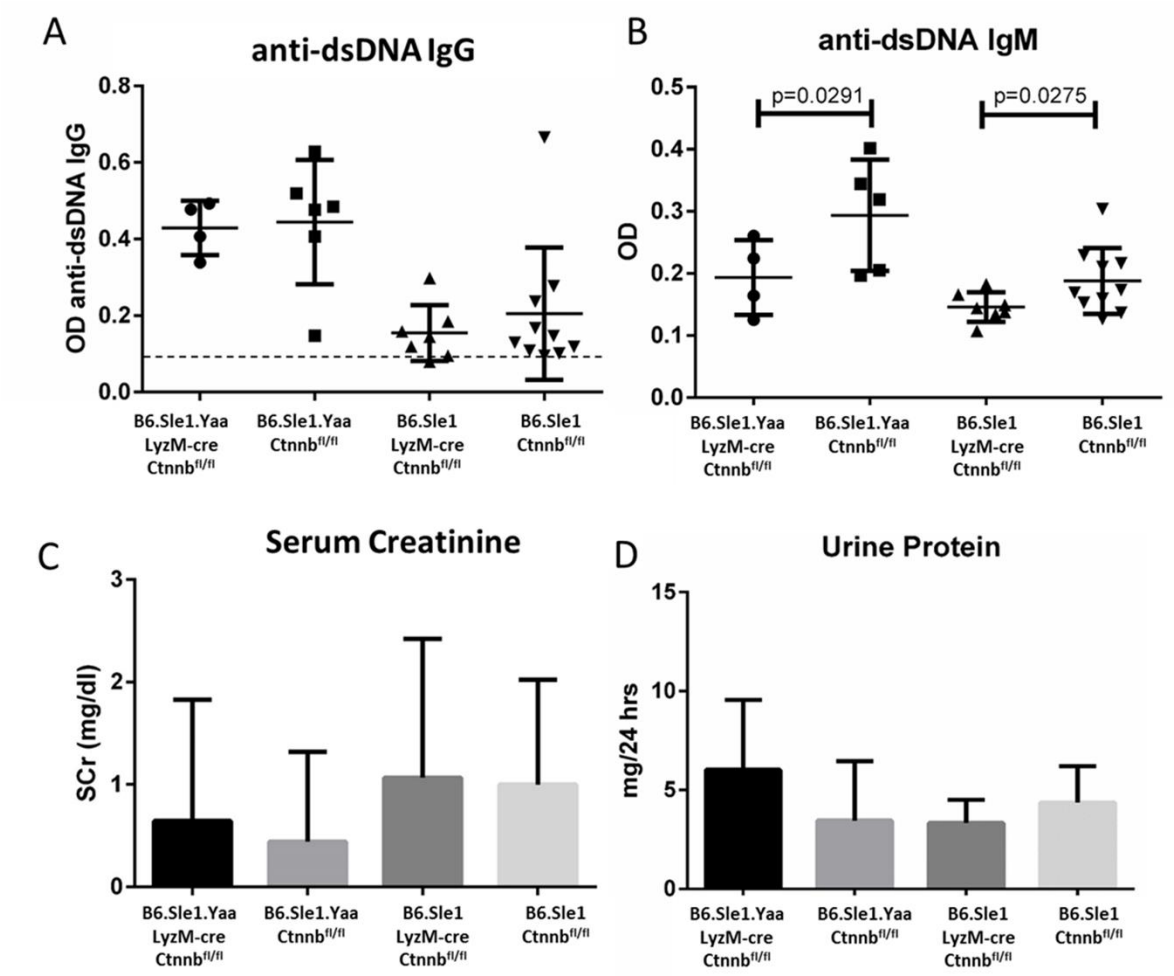


Figure 39 – Lupus-prone mice with myeloid-specific β-catenin loss did not have worse disease as measured by dsDNA autoantibodies (A), serum creatinine levels (C), and 24 hour urine protein (D). These mice did, however, exhibit significant reductions in anti-dsDNA IgM (B).

AIM 2: TO CHARACTERIZE THE STATE OF WNT/B-CATENIN SIGNALING IN LUPUS-PRONE KIDNEY.

2.1 β-catenin is depleted in the kidneys of lupus-prone mice

I further sought to characterize β-catenin signaling in the kidneys of lupus-prone mice. B-catenin is essential to the development of the kidney, and renal vesicles fail to form in the absence of *Wnt* genes.[386] In adherent cells, β-catenin acts not only in signaling but also in intracellular complexes tethering actin filaments to the plasma membrane in adherens junctions between cells.[387,388] This β-catenin is not phosphorylated at Ser33 and is not directly susceptible to degradation by the catenin destruction complex nor does it signal actively.[389]

I first sought to establish the location and relative quantity of β-catenin in lupus-prone kidney. I first isolated whole kidneys from healthy B6 and lupus-prone mice and extracted protein for Western analysis. I found that lupus-prone kidneys express slightly less β-catenin. Neither B6 nor lupus-prone kidneys expressed P-Ser33-β-catenin, suggesting that very little β-catenin is degraded in the kidney (Figure 40).

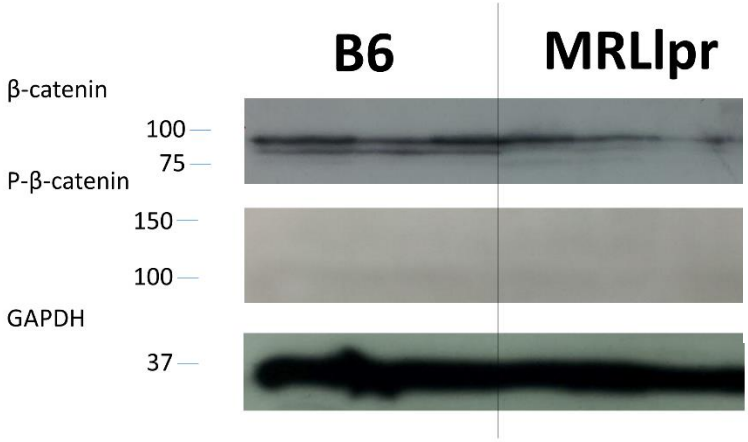


Figure 40 – Kidneys of lupus-prone Mrl-lpr mice may show slightly decreased β-catenin protein levels.

To determine the location of β-catenin in SLE, I isolated kidneys from B6 and Mrl-lpr mice and cryosectioned for immunofluorescent and immunohistochemical staining. B-catenin was localized in tubular epithelial cells in both healthy and lupus-prone kidneys (Figure 41). Lupus-prone mice also express lower amounts of β-catenin in the kidney, confirming the results of the previous Western analysis. It appears that β-catenin in healthy B6 tubular epithelial cells may be localized at the periphery of cells, whereas in Mrl-lpr tubular epithelial cells it appears more centrally. This may be because β-catenin in these cells is active in transcription but not in cell adhesion. Further study will be needed.

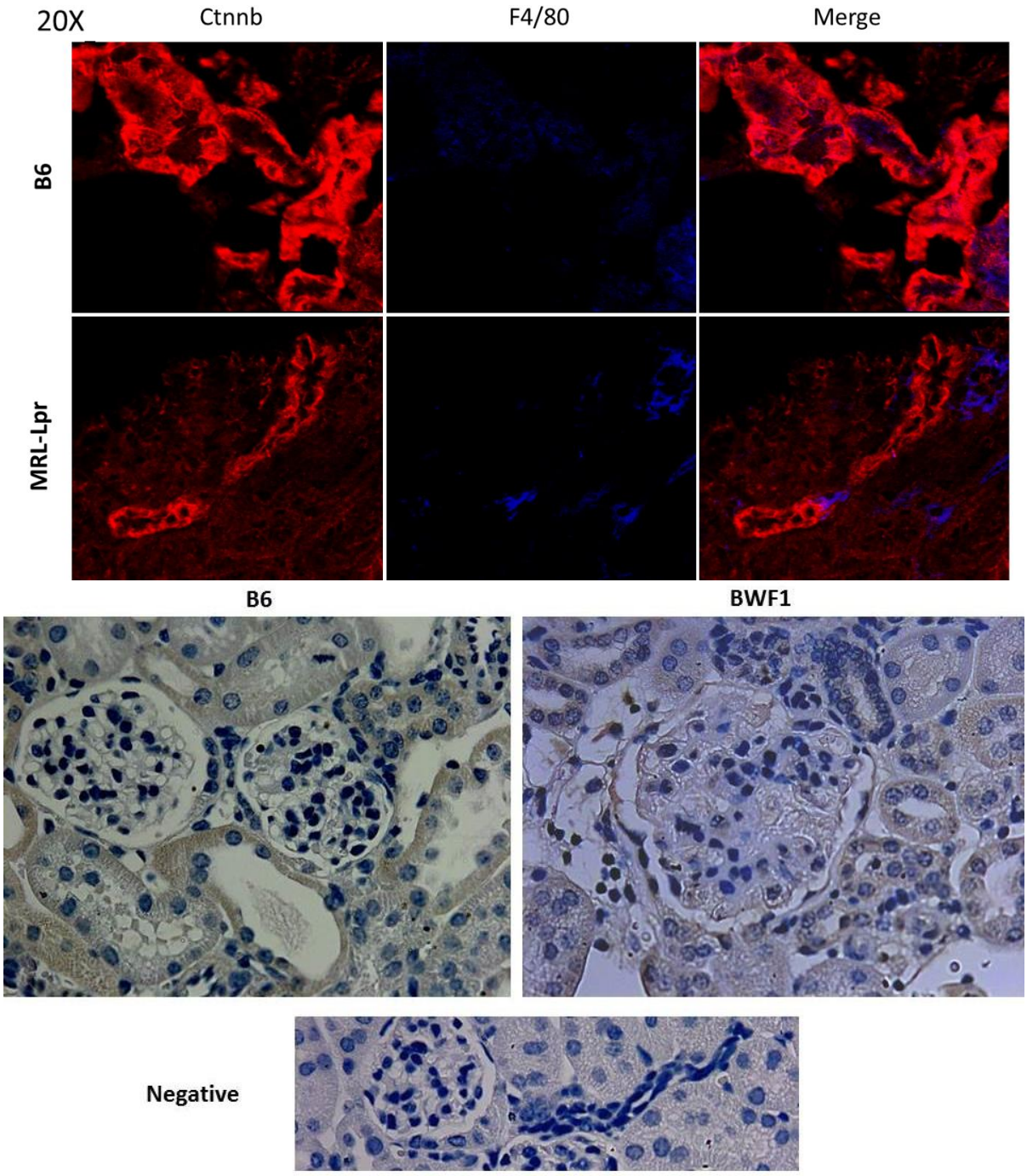


Figure 41 – Immunofluorescence and immunohistochemistry of β-catenin in SLE shows a loss of β-catenin in tubular epithelial cells. Lupus-prone podocytes also express β-catenin.

2.2 β-catenin signaling is elevated in kidneys of lupus-prone mice

The previous results suggest that a smaller pool of β-catenin is available in tubular epithelial cells. B-catenin is known to serve two distinct and mutually exclusive roles: Wnt-induced transcription and participation in adherens junctions. Given this, I sought to establish whether the remaining β-catenin is involved in transcription. Interestingly, β-catenin transcription targets *Ctnnb*. *Axin2*, and *MMP7* mRNA are increased in lupus-prone kidney (Figure 42). This suggests that much less β-catenin is available for participation in adherens junctions.

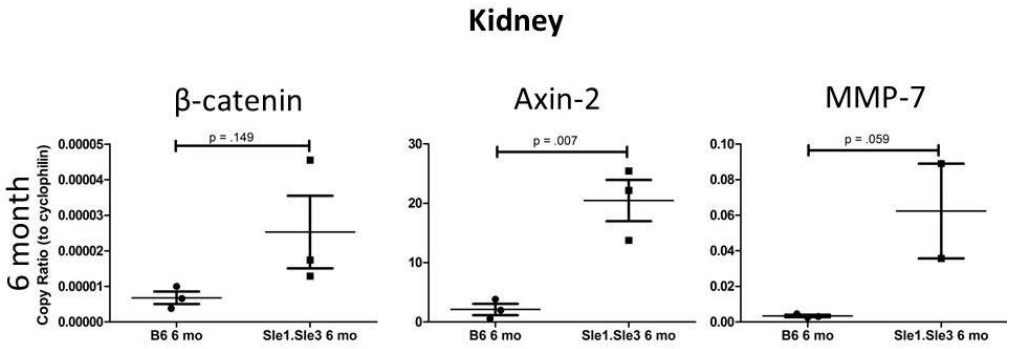


Figure 42 – β-catenin transcription targets Ctnnb. Axin2, and MMP7 mRNA are elevated in lupus-prone kidney.

SUMMARY

In summary, I found that immune cells exhibit decreased β-catenin levels and β-catenin transcription target mRNA levels in SLE. Deletion of β-catenin in macrophages does not alter disease course in B6.Sle1.Yaa mice. In contrast, β-catenin transcription targets are elevated in the kidneys of lupus-prone mice despite

a decrease in total β-catenin protein. This suggests that less β-catenin is less available for adherens junction formation.

CHAPTER SIX

RARE FIBRINOGEN VARIANT AA-E MAY INDUCE THROMBOTIC COMPLICATIONS IN SOME SLE PATIENTS

INTRODUCTION

Systemic Lupus Erythematosus (SLE) progression manifests in several ways. Thrombosis is the most common cause of death in patients with SLE (26.5%).[258] A major marker and cause of thrombosis in SLE is anti-phospholipid antibodies (aPL), which sometimes lead to fulminant anti-phospholipid syndrome (APS) characterized by thrombotic events (*e.g.* deep venous thrombosis, pulmonary embolism, or stroke) and pregnancy complications. However, anti-phospholipid autoantibodies do not explain all SLE thrombotic complications.[390-392]

As previously described, an unbiased proteomic screen revealed that Fibrinogen Aα-E may be elevated in the serum of some SLE patients. Aα-E is the less common of two alternatively spliced fibrinogen alpha chain. It represents a stable 1-2% of fibrinogen alpha in healthy adults.[350] Infant Aα-E levels fluctuate up to 5-6% with unknown significance.[393] The common Aα isoform is 610 amino acids in length, whereas Aα-E includes exon 6 (vi) to reach 846 amino acids (Figure 43). This larger Aα-E is more stable than the common alpha chain and individuals producing

significantly elevated Aα-E may experience thrombotic events.[350,351,394,395] Clots comprising Aα-E contain thinner fibers and erratic branching.[396] Individuals with mutations producing ultra-stable fibrinogen form comparable fibers and unstable clots due to a similar γ-γ dimer motif.[397,398]

Two previous papers suggest that fibrinogen *as a whole* is elevated in SLE, but neither explores whether the rare Aα-E is elevated.[259,399] Thrombotic events (*e.g.* deep vein thrombi (DVT), pulmonary emboli (PE), and cerebrovascular accidents (CVA)) are the most common cause of death in patients with SLE (26.5%).[258] Researchers have previously puzzled over thrombotic complications in lupus that are *not* explained by anti-phospholipid syndrome (APS).[258,264,390-392] Aα-E elevation in SLE may thus offer an explanation as well as a therapeutic target. I hypothesized that Fibrinogen alpha isoform Aα-E is a marker of aPL-negative thrombotic risk in SLE. My specific aim is:

1. **Aim 1: Determine the elevation of Aα-E in SLE patient sera and its correlation with thrombotic complications.** Fibrinogen Aα-E is elevated in patients as confirmed by Western analysis. Furthermore, patients with increased Aα-E are most likely to have a positive history of thrombotic complications. No such variant was detected in lupus-prone mice. A custom polyclonal anti-Aα-E antiserum specifically detects Aα-E but is not suitable for ELISA.

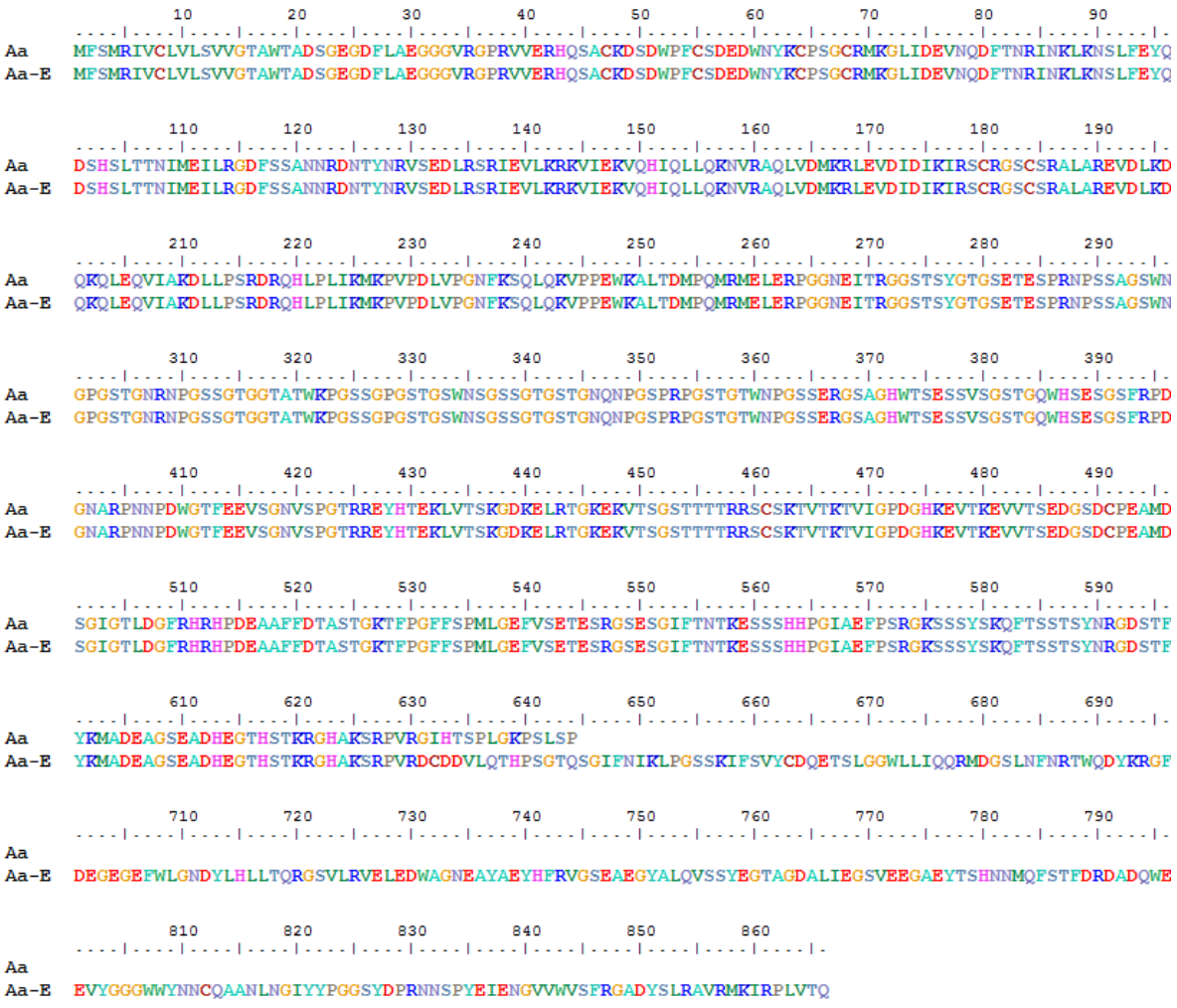


Figure 43 – Alignment of common (Aα) and rare (Aα-E) isoforms of the fibrinogen alpha chain. The Aα isoform is 610AA in length and usually comprises 98-99% of fibrinogen alpha chain in normal serum. The Aα-E isoform includes exon VI, is 846AA in length, and usually comprises only 1-2% of fibrinogen alpha chain in normal human serum.

1.1 Fibrinogen variant Aα-E is elevated in SLE serum

As commercial ELISA kits and antibodies do not distinguish between the rare (Aα-E) and common (Aα) variants of the fibrinogen alpha chain, I first evaluated the relative quantities of the isoforms by Western blot. As shown in Figure 44, the common isoform (Fib Aα) is found at ~69kDa while the rare isoform (Fib Aα-E) is found at ~110kDa.[350] An additional, intermediate Aα-E-specific band is often observed. As expected, healthy controls showed normal, low levels of the common variant and undetectable levels of the Fib Aα-E isoform. In two randomly selected SLE sample sets, however, we observed a significant Aα-E variant band which—though in normal healthy controls it is reported to comprise just 1-2% of total fibrinogen—appears to comprise a substantial proportion of the total, elevated fibrinogen levels in five out of thirteen patients. I was blinded to the clinical histories of these patients until after these blots were compiled.

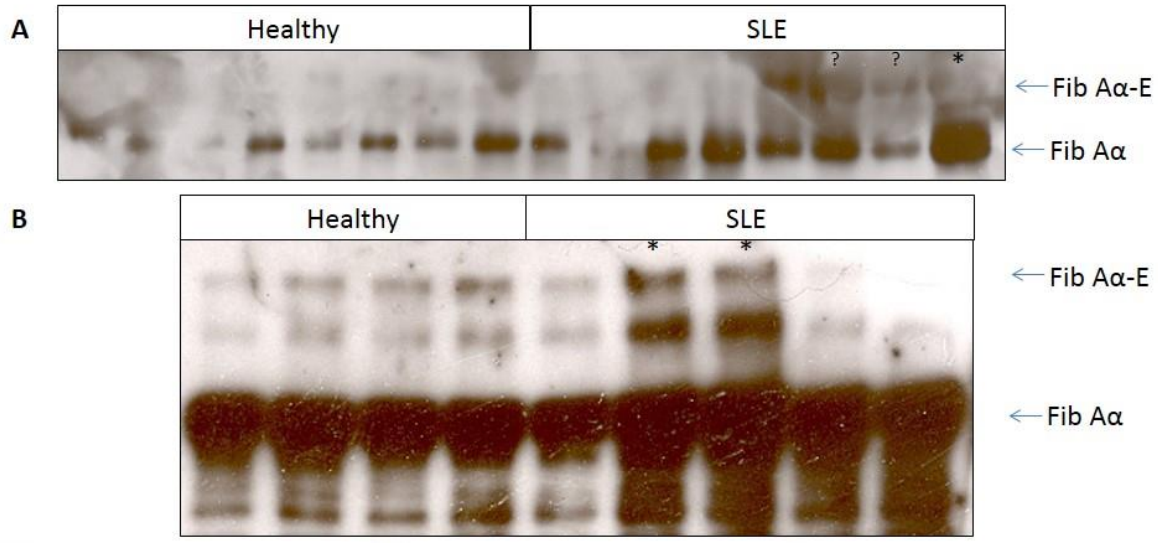


Figure 44 – Fibrinogen Aα-E is enriched in patients with SLE. Patients with histories of thrombotic disease are denoted by asterisk (*) and patients lost to follow-up are marked with a question mark (?).

1.2 Patients with increased Aα-E levels also exhibit a history of thrombotic complications

After performing Western analysis, we consulted deidentified clinical histories of patients in the sample and determined that three patients had a clear history of thrombotic complications such as deep vein thrombosis (DVT), pulmonary embolism (PE), and/or cerebrovascular accident (CVA). We then unblinded and correlated these histories with our Western analysis. Patients with a positive history are marked with asterisks in Figure 44.

I subjected data from these blots to densometric analysis with ImageJ. Results of the analysis are included in Figure 45, where patients with a history of thrombotic

complications are shown in red and patients lost to follow-up are shown in blue. These panels showed respective significant and insignificant overall changes in Aα-E levels between healthy and SLE samples (p=0.0154 and p=0.1165). Between non-thrombotic and thrombotic SLE patients, however, both panels exhibited a significant difference (p=0.029 and p<0.0001). When combined (see Figure 45C) as comparable ratios of Aα-E to Aα densities, SLE patients with thrombotic complications exhibited significant elevation of Aα-E versus SLE patients without thrombosis (p=0.0074, patient lost to follow-up excluded). These data suggest a correlation between Fibrinogen Aα-E and thrombosis.

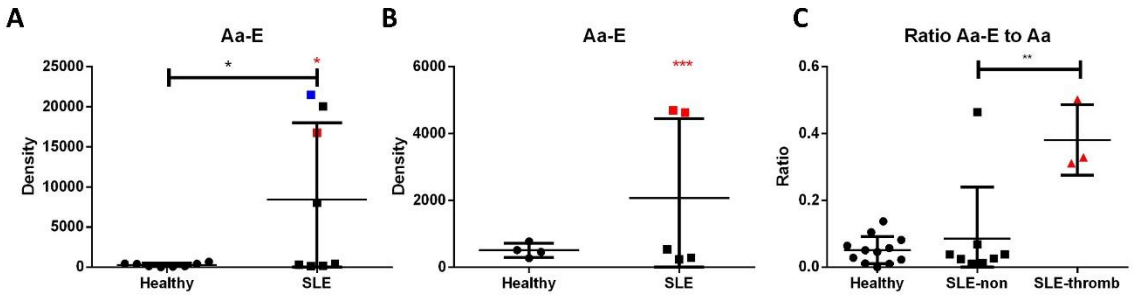


Figure 45 – Results from densometric calculations of Figure 42 using ImageJ. Aα-E density was calculated separately for the first (A) and second (B) blots in Figure 42. The ratio of Aα-E to Aα bands in both blots are also compiled (C). Datapoints from individuals with a history of complications are shown in red. Datapoints from individuals lost to follow-up are shown in blue. The first blot indicated a significant difference between healthy and SLE AαE (see A, p=0.0154), although the second blot did not (see B, p=0.1165). The first blot showed a significant difference between thrombotic and non-thrombotic SLE Aα-E only when a patient lost to follow-up was included (see A, p=0.029). The second blot also showed a significant difference between thrombotic and non-thrombotic SLE Aα-E (see B, p<0.0001). The compiled ratio of Aα-E to Aα, which could be compared across blots, was significantly higher in thrombotic versus non-thrombotic SLE (see C, p=0.0074). Note that a patient lost to follow-up was excluded from the analysis. Red asterisks indicate the inclusion of only those patients not lost to followup.

1.3 Healthy and lupus-prone mice do not exhibit variant Fibrinogen alpha chain bands

While the specific alternative splicing that results in Aα-E in humans is not predicted in mice, we hypothesized that some variant might exist in lupus-prone mice. As shown in Figure 46, no additional bands were found in the sera of lupus-prone mice. Of note, no lupus-prone spontaneous mouse model exhibits thrombotic complications, an important barrier to the study of thrombosis in SLE.[400]

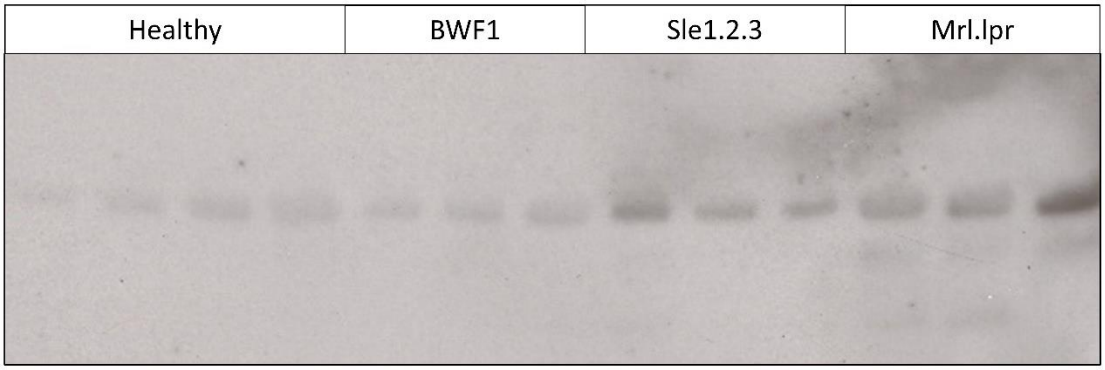


Figure 46 – No Fibrinogen Aα-E isoform is detected in 8-month-old healthy (B6) or lupus-prone (BWF1, Sle1.2.3, Mrl.lpr) mice.

1.4 Custom Fibrinogen Aα-E rabbit antiserum specifically detects Aα-E in human sera

As no commercial antibody specific to the Fibrinogen Aα-E chain existed, we contracted with GenScript™ to produce Aα-E-specific rabbit antiserum. This antiserum would serve both to validate that the higher bands are Aα-E and further to provide specific ELISA or Western analysis antibody for quantifying serum Aα-E content.

Rabbit polyclonal anti-Aα-E serum was generated against specific Aα-E epitopes as described under Methods (see “Production of anti-Fibrinogen Aα-E rabbit antiserum”, p65). Serum from selected high Aα-E and low Aα-E patients in Figure 44 were run on two SDS-PAGE gels and analyzed simultaneously by Western blot using anti-Aα-E serum (Figure 47A) or anti-Fibrinogen Aα antibody (Figure 47B). Patient sera previously identified as having high levels of Aα-E exhibited a specific band versus SLE controls (n=4, 5; p<0.0001 by ImageJ).

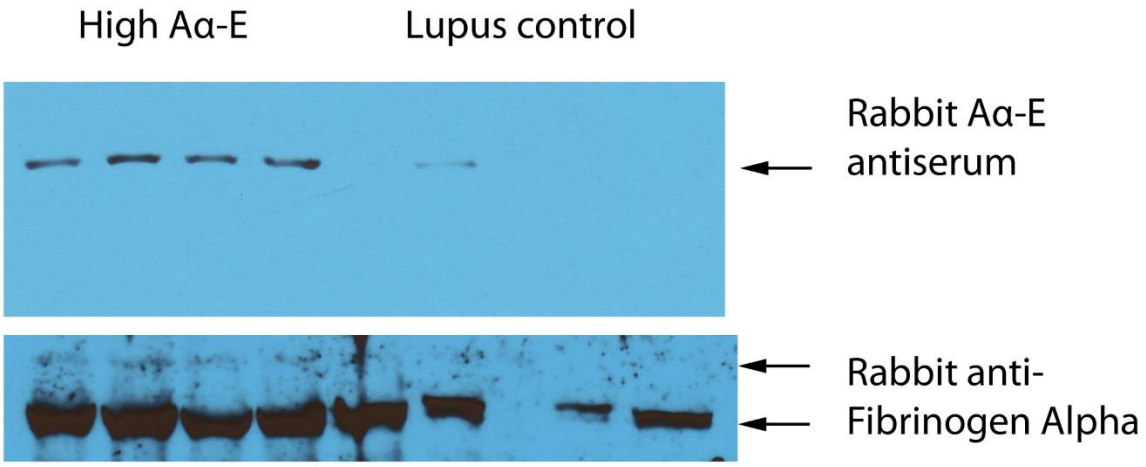


Figure 47 – Specific anti-Aα-E antiserum detects the Aα-E chain (A) and total anti-fibrinogen alpha antibody detects all alpha isoforms (B). Patient sera previously identified as having high levels of A α -E exhibited a specific band versus SLE controls (n=4, 5; p<0.0001). Note: Longer exposures of the top blot indicate, as in previous blots, two Aα-E-specific upper bands.

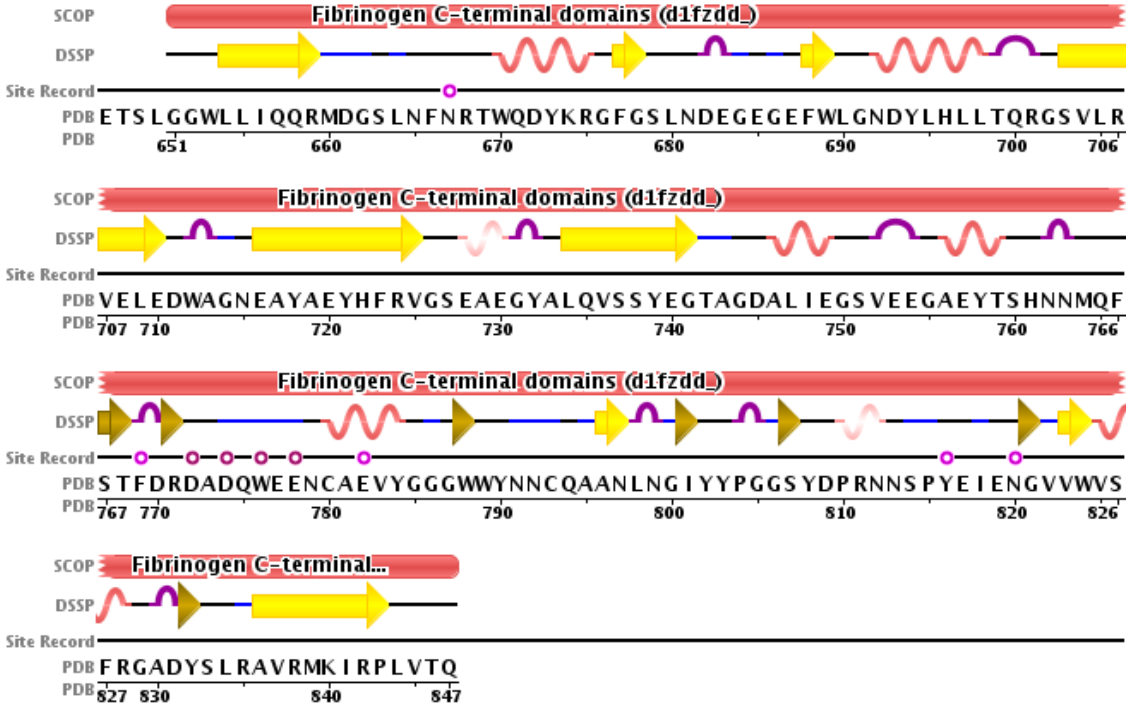
1.5 Custom Fibrinogen Aα-E rabbit antiserum does not successfully detect Aα-E by ELISA

Enzyme-linked immunosorbance assay (ELISA) is a robust method for quantifying analytes in patient serum or urine. This method forms the basis for most clinical tests and allows simple quantification of analyte concentration by reference to a standard curve. It further allows direct comparison of a large number of samples.

I used our custom anti-Aα-E serum and a commercially-available whole Fibrinogen alpha chain ELISA kit (GenWay Bio #40-288-22856) to determine the feasibility of a new Aα-E specific ELISA. The sequence of the extended Aα-E domain is shown in Figure 48 (PDB reference #1FZD).[401] Our polyclonal rabbit anti-Aα-E antiserum was raised against peptide CRGSVLRVELEDWAG (peptides 701 through 714 in the figure) conjugated to carrier protein keyhole limpet hemocyanin (KLH) which forms an accessible β strand as indicated by blue arrows.

ELISA protocols are explained in Materials (see p76). In brief, I coated a 96-well Immulon® H2B plate with the provided coating antibody (GenWay #15-288-22856) or our rabbit antiserum. I analyzed samples known to have high or low Aα-E by Western analysis with the provided detection antibody. While the commercial ELISA kit detected total Aα-E normally, no discernable Aα-E was detected. I purified antibody from the rabbit serum and repeated the coating, but no Aα-E was detected

as shown in Figure 49. Additional attempts to generate a Fibrinogen Aα-E-specific ELISA using bead-purified antibodies and using Aα-E-specific antibodies as HRP-conjugated detection antibodies were also not successful. It is possible that protein G-mediated bead purification, which purifies all antibodies without regard for antigen specificity, may be insufficiently selective for Aα-E antibodies. Additional affinity purification may thus resolve this problem. It is further possible that the peptide used for immunization did not produce antibodies that bind the folded structure of Fibrinogen Aα-E, shown in Figure 48.



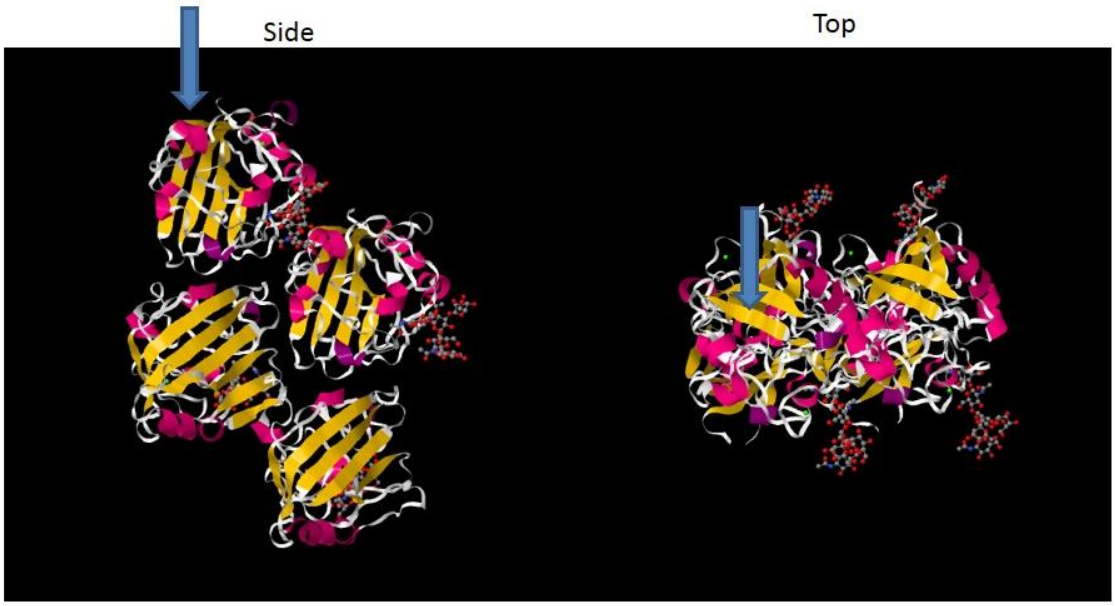


Figure 48 – Four Aα-E chains assemble together to form part of the Fibrinogen-420 complex. Arrows indicate the location of the sequence RGSVLRVELEDWAG (amino acids) targeted by our custom antisera (Images generated at www.pdb.org, PDB reference #1FZD).

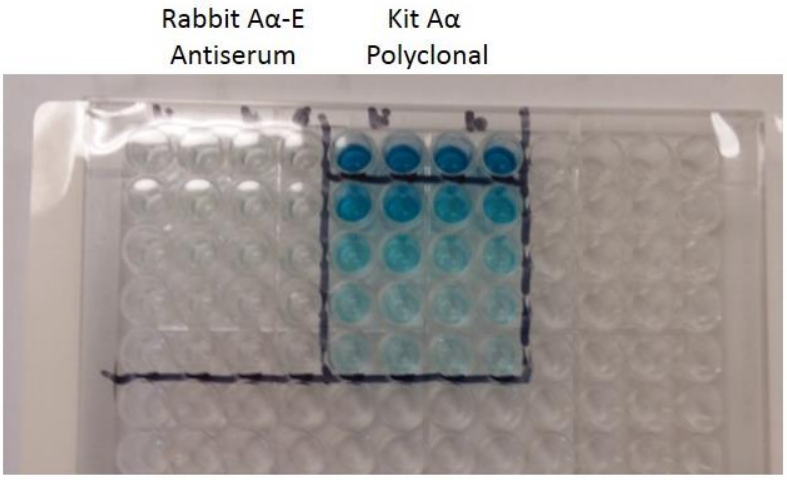


Figure 49 – Rabbit Aα-E antiserum-coated ELISA plates fail to detect Aα-E in known samples despite bead purification of antibodies.

SUMMARY

In summary, rare fibrinogen alpha chain isoform Aα-E appears to be elevated in the serum of SLE patients with thrombotic complications. This isoform may be a useful marker for following and/or predicting such events in SLE patients without other thrombotic indicators such as anti-phospholipid antibody (aPL).

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

INTRODUCTION

As previously discussed, SLE pathogenesis requires the breakdown of three major checkpoints: adaptive immune tolerance, peripheral innate responsiveness, and end-organ inflammation (Figure 1, p14).[3] Adaptive immune dysfunction produces autoantibodies leading to immune complex formation and deposition in the skin, joints, and kidneys. Innate immunity plays an important role in determining disease severity and progression. End-organ damage results in arthritides/arthralgias, photosensitivity, renal disease, and other manifestations that contribute to morbidity and mortality in disease.

Protein and metabolic markers are helpful in understanding, diagnosing, treating, and following SLE. Multiple modalities in proteomic screening aid the discovery of such markers (see Chapter 2, p46). In the present work we identified three factors and related pathways that were discovered using these methods. A brief discussion of findings and future directions is below.

DISCUSSION—AXL TYROSINE KINASE IN SLE

Summary of Axl Tyrosine Kinase in SLE

In this study I have demonstrated that matrix metalloproteases ADAM10 and TACE (ADAM17) cleave surface Axl in CD11b+/CD14+ and CD19+ lupus-prone and SLE leukocytes. This loss abrogates Axl-mediated macrophage anti-inflammatory activity *in vitro* and *in vivo*. To our knowledge these are the first data to show a functional significance of Axl ectodomain shearing in lupus.

Under normal conditions, Axl acts to transduce anti-inflammatory signals in macrophages by Twist-mediated suppression of inflammatory cytokines.[309] This represents a part of the innate immune checkpoint, and dysregulation of this system may contribute to the pathology of autoimmune disease. In the present study we observe that SLE patients and lupus-prone mice exhibit increased levels of sheared soluble Axl ectodomain (sAxl) in the blood and reduced surface Axl and active Y779-phosphorylated Axl on immune cells. This occurs despite increased Axl ligand Gas6 in human SLE and lupus-prone mouse serum.[303,330,332] Both Axl-deficient and lupus-prone mouse macrophages fail to block expression of IL-6 and TNF-alpha in response to Gas6 *in vitro*. Furthermore, B6.Axl-/- macrophages worsen end organ damage *in vivo*.

Young disease-transitioning B6.Sle1 mice show partial loss of leukocyte Axl versus B6 controls (data not shown). Further, bone marrow-derived macrophages (BMDM) from diseased B6.Sle1.Yaa mice do not express detectable levels of ADAM10 and TACE (ADAM17) but do express basal levels of Axl similar to wild type B6-derived BMDM. Thus leukocyte Axl shearing in SLE appears to occur mainly in the periphery in response to an inflammatory milieu. This is consistent with known upregulation of macrophage proteases in inflammatory conditions.[362-364] We speculate that Axl loss in macrophages may contribute to "flares"—periodic, severe worsenings of SLE symptoms with no clear cause—by failing to appropriately rein in inflammatory cytokines. This is supported by multiple reports and our observations correlating serum sAxl levels with disease severity on the SLEDAI scale (Systemic Lupus Erythematosus Disease Activity Index).[301,303]

Axl cleavage has "cis" inflammatory effects

The loss of Axl from blood monocytes could decrease Axl anti-inflammatory signaling in two ways (see model in Figure 50). First, cleavage of cell-surface Axl abrogates Axl signaling through the remaining "stump" receptor. This "cis" suppression of Axl anti-inflammatory signaling on macrophages is supported by our studies outlined here.

Second, this cleavage produces a decoy receptor “sink” that may block Gas6 signaling in other cells. This “trans” suppression would affect cells in which Axl and/or other Gas6 receptors like Mer and Tyro3 remain intact. Because Gas6 has higher binding affinity to Axl than to other receptors,[286] this sink may effectively abrogate TAM receptor signaling pathways in other cells. This is especially important in SLE, as elevated Gas6 may be an important anti-inflammatory feedback mechanism.[303] I have shown that LPS-stimulated 48-hour supernatants from B6.Sle1.Yaa mice inhibit Gas6-mediated upregulation of healthy B6 splenocyte *Twist* (Figure 31A, p122). I hypothesized that this may be due to cleaved Axl ectodomain, which may act as a “sink” for Gas6 as we suggested above and diagram in Figure 50. Subsequent experiments confirmed that soluble Axl in these supernatants is responsible for B6.Sle1.Yaa splenocyte supernatant suppression of Gas6-mediated *Twist* induction in healthy B6 splenocytes (Figure 31B-D). These results will need to be repeated with more reliable reagents.

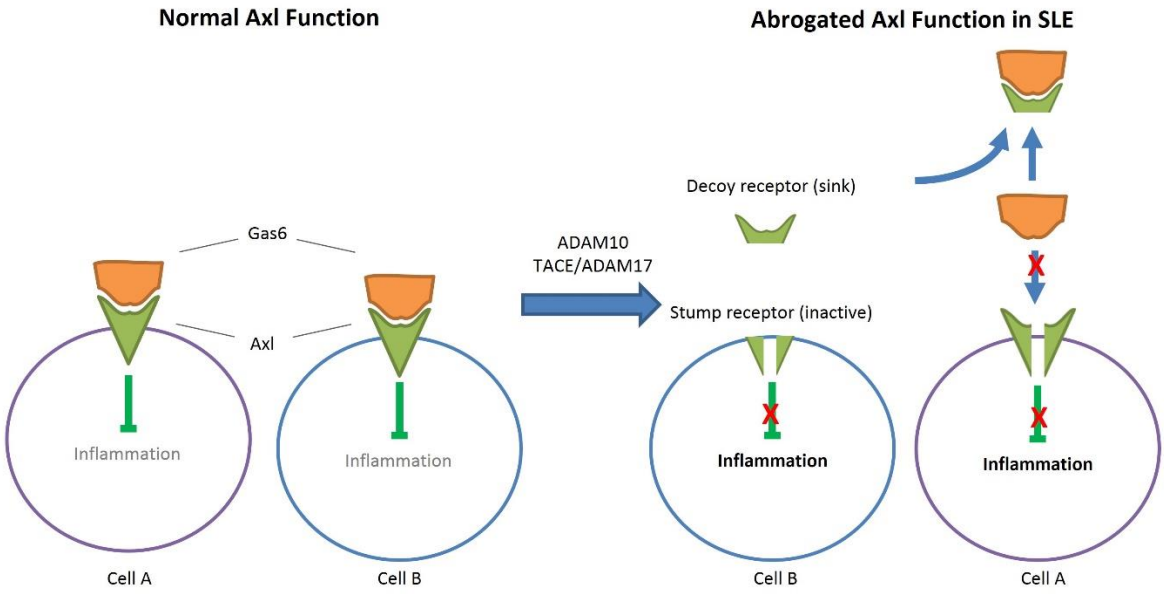


Figure 50 – A model of Axl signaling loss in SLE leukocytes. ADAM10 and TACE (ADAM17) each cleave surface Axl to directly abrogate macrophage Axl anti-inflammatory activity. Soluble Axl ectodomain also appears to act as a "sink" receptor to prevent Gas6-mediated signaling.

Macrophage subtypes in SLE may involve TAM receptors

As previously discussed, macrophage phenotypes are commonly divided into M1 and M2a, b, and c. M1 and M2b appear to be increased in SLE and contribute to disease whereas M2a and M2c are decreased in SLE and would be expected to ameliorate disease. Surface-bound Mer may be a marker of M2c macrophages. As cleaved soluble Mer correlates with disease severity in SLE,[208] cleavage of TAM family tyrosine kinases could be a mechanism by which the inflmamatory milieu influences macrophage phenotypes. Given similar proteolytic patterns, Axl may also be a marker for an anti-inflammatory subset that we previously predicted would be

reduced in SLE.[216] The loss of Axl, as we have shown, alters macrophage phenotype through reduced Gas6 responsiveness. Thus Axl loss in SLE may contribute to skewing toward inflammatory M1 and M2b subtypes. More study will be needed to determine these roles.

Potential contributions of B cell Axl loss

I have shown that B cells isolated from B6.Sle1.Yaa and B6.Axl-/- mice do not upregulate Twist in response to Gas6 stimulation. Interestingly, healthy B6 B cells upregulate Twist only minimally in response to Gas6 (see Figure 24, p110). While this is not likely to be physiologically significant, we hypothesize that the loss of Axl from B cell Axl may have some other phenotypic effects in SLE. Axl activates numerous pathways, including Akt, ERK, and STAT1. These may contribute to B cell functional changes in SLE. It will be interesting to determine how Gas6 stimulation affects healthy B cell activity.

Axl loss as an explanation of interferon signature pathogenicity.

Type I interferons (IFN I) are known to be elevated in SLE, inducing the so-called "interferon signature."[118,119] Type I interferon generally has two effects on immunity: anti-viral and anti-inflammatory. The anti-viral effects of type I interferon were discovered in the 1950s and involve cell resistance to viral infections.[120] IFN I acts both to combat the virus and decrease the viability of infected cells.[121,122] The anti-

inflammatory effects of type I interferon are less well-studied and generally act in a negative feedback mechanism. It limits the effects of proinflammatory cytokines IL-1 and TNF-α and induces IL-10 production.[402]

In SLE, plasmacytoid dendritic cells produce interferon alpha.[123] Other leukocyte cell types each contribute to the resultant spectrum of interferon-induced genes, termed the "interferon signature."[124] IFN-α acts to promote the maturation of dendritic cells (DCs), promote plasma cell development, induce BAFF to maintain mature B cells, and upregulate IRF7 in plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs), and monocytes.[79,127,128]

In some ways, the interferon signature acts as a final common pathway for lupus flares.[138] Notably, IFN I does not exert normally-associated anti-inflammatory effects in SLE.[118,119,402,403] Many studies have provided explanations for this phenomenon, including activation of different subsets of autoreactive cells.[117] The present study may also offer additional explanation of the "interferon signature" in SLE. At least part of the IFN I anti-inflammatory effect is Axl-dependent.[309] Our data suggest that Axl loss in SLE leukocytes may contribute to the pathological effects of type I interferon observed in SLE. Further study is warranted to address this issue.

Serum Axl variation in SLE may be due to demographic differences

Recent interest in soluble Axl in SLE serum has led to conflicting reports of significant versus insignificant correlation between serum sAxl and disease severity.[301-303] This may be due to variation in patient populations studied. Our patient cohorts in Figure 1 and others reporting significant correlation of sAxl and disease have generally focused on Hispanic and African American patients and controls, whereas those reporting insignificant correlation comprise mainly European American patients and controls. Different reports also cite significantly decreased or increased Gas6 in severe SLE, although these variations may be due to differences in measuring free versus sAxl-bound Gas6. Most report an increase.

Axl signaling varies by cell type, which has therapeutic implications

This study highlights the importance of cell-specific pathway effects in human disease. While Axl ligand Gas6 is elevated in the serum of mice and humans with SLE, its anti-inflammatory activity is blunted in leukocytes by Axl shearing. Conversely, Gas6 in the kidney contributes to nephritis and inflammation through Axl-mediated mesangial cell proliferation. In short, Axl signaling has opposing effects in kidney mesangial cells (pro-disease) versus peripheral leukocytes (anti-inflammatory). In short, an Axl kinase inhibitor may exacerbate SLE by inhibiting Twist-mediated anti-inflammatory signaling in macrophages while a Gas6 homolog may exacerbate SLE by enhancing mesangial cell proliferation. Thus matrix

metalloprotease inhibition, which would directly increase leukocyte but not mesangial cell Axl signaling, may be a more effective intervention.

Matrix metalloproteases are increasingly available and tested for other indications in clinical trials. My data suggest that ADAM10 or TACE inhibition alone will not maximally restore leukocyte Axl function. I found that varying doses of ADAM10/TACE inhibitors exhibit similarly synergistic upregulation of Axl versus either inhibitor alone by flow cytometry. This suggests that the observed Axl rescue is not due to any off-target effects of either inhibitor. Taken together, these data further suggest that dual inhibition using multiple inhibitors or a multi-selective inhibitor that spares other proteases may improve macrophage phenotypes *in vivo* (see Table). Small molecule XL784, for instance, recently completed Phase 2 clinical testing in diabetic nephropathy and selectively inhibits both proteases while sparing other close family members.[404]

Drugs targeting both ADAM10 and TACE

Drug	Company	Notes
XL784	Exelisis	Already in clinical trials for diabetic nephropathy, good half-life for *in vivo* use
TIMP3	n/a	Soluble protein that inhibits broadly, including TACE, ADAM10, and other MMPs
TAPI-2	Generic (Sigma, Santa Cruz, etc)	Broad, including TACE, ADAM10, and other MMPs

Table 16 – Currently-available drugs which target both ADAM10 and TACE.

We previously reviewed how influencing macrophage phenotypes in SLE may offer an effective treatment strategy.[216] Given that TAM family receptor Mer has recently been implicated as marker of M2 macrophages,[302] we hypothesize that Axl may also be such a marker. In addition, other protease targets are known markers of macrophage phenotype.[216] These data suggest that proteases may be an important source of regulation of macrophage phenotype. If so, protease inhibitors may be used to modulate other diseases in which macrophages are important, including rheumatoid arthritis (RA). Continued testing will be required to confirm these effects.

Conclusion

In conclusion, SLE leukocyte proteases ADAM10 and TACE (ADAM17) shear surface Axl and abrogate macrophage anti-inflammatory Gas6-mediated Twist induction. Combined with previous reports, our data suggest that Axl loss contributes to pathology in some SLE patients. Additional study will be needed to determine how many patients this may affect. My study further posits Axl loss as an additional mechanism by which the interferon signature exerts pro-inflammatory effects in SLE. The data may further suggest the exploration of combined ADAM10/TACE inhibition as a treatment for SLE. Additional study is warranted.

FUTURE DIRECTIONS—WNT/B-CATENIN IN SLE

Wnt/β-catenin signaling is depressed in leukocytes from lupus-prone mice as measured by Western analysis and RT-PCR. This loss is most pronounced in CD19+ and CD11b+ cells. I hypothesized that deletion of β-catenin in macrophages would accelerate spontaneous disease in lupus-prone mice, but these were identical in disease progression to sibling controls.

In contrast, I found that Wnt/β-catenin signaling is elevated in lupus-prone kidney by RT-PCR. Endothelial cells appear to be one source of this continued signaling, although these cells appear to have a lower overall level of available β-catenin in the cell as measured by immunofluorescence and immunohistochemistry. Lupus-prone renal podocytes also express β-catenin.

Alternative Effects of Leukocyte B-catenin Loss

β-catenin has previously been implicated in autoimmunity. Restored β-catenin signaling helps reverse joint damage in a mouse model of rheumatoid arthritis.[405] Further, loss of β-catenin in T cells contributes to experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis.[406] In these settings, β-catenin appears to have an important role in regulating NFκB transcriptional activity in leukocytes.[407]

Myeloid cell-specific deletion of beta catenin did not alter major indicators of SLE pathology despite the alteration in beta catenin in these cells in lupus-prone mice. Beta catenin was also lost from CD19+ cells. These cells may respond to beta catenin signaling to alter disease, but this will require a B cell-specific deletion.

Potential Contributions of Renal B-Catenin to Disease

I showed that Wnt/β-catenin signaling and transcription is increased in the lupus-prone kidney (Figure 42, p139). Others have reported that inducing kidney β-catenin transcription by exogenous treatment leads to renal fibrosis and kidney injury.[408] DKK-1 is elevated in SLE and lupus-prone mouse serum,[348,385] which may reflect an insufficient protective feedback mechanism to reduce nephritis development.

It is interesting that β-catenin signaling is elevated (by RT-PCR) while β-catenin levels are decreased in the kidney (by Western analysis, IF, and IHC). This reflects a major theme of β-catenin as a player in two important cellular functions: structure and signaling. B-catenin acts in adherens junctions in adhesive cells like tubular epithelial cells (TECs) to maintain lumen integrity in organs like the kidney. This β-catenin is distinct from β-catenin that acts in signaling by translocation to the nucleus

at TCF/LEF transcription sites. β-catenin that is involved in signaling is not available for adherens junctions, and β-catenin involved in cellular structure is also unavailable for signaling. Thus our data may indicate that β-catenin in lupus-prone tubular epithelial cells is dramatically less available for the formation of adherens junctions, which may contribute to nephritic disease. This appears to be the case from the immunofluorescence staining in Figure 41 (p138), which shows peripheral staining in renal tubular epithelial cells in healthy B6 kidney but more centralized staining in these cells in lupus-prone Mrl-lpr kidney sections. Further study will be necessary to determine how this affects disease. We will use Ksp1.3-Cre as a tool in conjunction with spontaneous mouse models to explore the potential contribution of structural β-catenin loss in SLE.

FUTURE DIRECTIONS—FIBRINOGEN A ALPHA-E IN SLE

Determining the causal link of thrombosis and Fibrinogen Aα-E

We have determined that the rare Fibrinogen alpha chain isoform Aα-E may be elevated in the serum of anti-phospholipid antibody negative (aPL-negative) SLE patients with thrombotic complications. This correlation and the properties of Aα-E chains are suggestive of Aα-E leading to weak clots and thrombotic complications (Figure 51A). If this is the case, several questions remain. Firstly, what causes the increase in Aα-E in these patients? Secondly, will anticoagulant therapy in these patients reduce their risk for thrombotic events? Alternatively, however, thrombosis may cause alternative Fibrinogen chain splicing or a third factor may lead to both Aα-E and thrombosis (Figure 51B and C, respectively). These hypotheses will require a long-term prospective study.

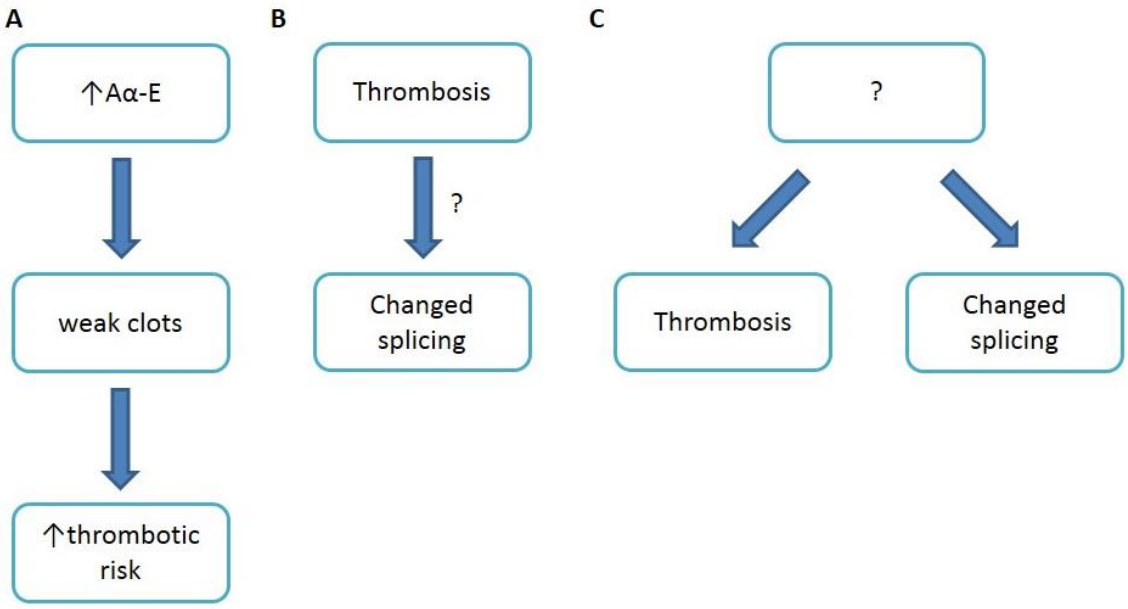


Figure 51 – Alternative explanations for the correlation between Fibrinogen Aα-E and thrombosis. (A) Increased Aα-E may form weaker clots to increase thrombotic risk. (B) Thrombosis may induce differential splicing of the Aα-E variant. (C) Some other phenomenon in SLE may induce both thrombosis and alternative splicing independently.

New monoclonal antibody for ELISA

Clinical testing must be quantitative and consistent between samples. Western blot does not fulfill these criteria as loading, gel runs, gel transfers, and other steps lead to variations. Our polyclonal rabbit sera is similarly unsuitable for clinical use because it is polyclonal, does not react with the folded protein by ELISA, and appears to detect the much-smaller fibrinogen gamma chain on Western blot under non-stringent washing conditions (data not shown).

Enzyme-linked immunosorbance assay provides the necessary quantitation and consistency. Additionally, a monoclonal antibody improves consistency between

samples. We will generate this antibody using larger, folded protein and B cell hybridoma techniques.

Modeling SLE thrombotic complications in mice

No spontaneous lupus-prone mouse model exhibits thrombotic complications seen in SLE. Some scientists model thrombosis in mice by transplanting anti-cardiolipin antibodies from human patients with anti-phospholipid syndrome (APS).[400] As mice lack the fibrinogen isoform, the introduction of exogenous Aα-E by injection may offer a useful tool to determine the role of this isoform in thrombosis. In the event that model Figure 51A is correct, these mice should form weaker clots and exhibit increased incidence of thrombotic complications. Alternatively, some models allow the infusion of human serum into mice. Similar studies could utilize a knock-in Aα-E construct in a spontaneous model.

DISSERTATION SUMMARY

Systemic Lupus Erythematosus (SLE) is a multifactorial disease requiring novel markers for disease diagnosis, treatment, and follow-up. Here I have shown the use of an important set of tools—proteomic screening methods—to discover new markers in SLE. Different markers are likely to have different functions, which is well-illustrated here. Axl tyrosine kinase elevation in SLE appears to be the result of proteolytic cleavage from leukocytes. This has functional consequences in that it

reduces the anti-inflammatory capacity of macrophages and contributes to disease. Moving beyond the screening method, I further determined that proteases ADAM10 and TACE cause of this cleavage. Thus Axl tyrosine kinase not only offers an additional serum marker for follow-up but also offers insights into SLE pathogenesis and a novel treatment regimen. Beta catenin may also contribute to disease, although my work suggests it may be most important in nephritis. Beta catenin signaling is elevated in nephritic kidneys but appears to be less active in adhesion. Further work will be necessary to determine whether this mechanism is plausible, but β-catenin pathway components may be a useful marker of nephritis.

Similarly, fibrinogen alpha isoform Aα-E appears to be a marker of thrombotic complications in SLE. Further work will be needed to determine the causal relationship of this isoform and thrombosis.

APPENDIX A

CATALOG OF MYELOID CELL CHANGES IN SLE

Figure 52 – A catalog of myeloid cell changes in SLE. Figure adapted from *Orme and Mohan* 2012.

	Mo/Mφ		PMN	Notes
	Human	Mouse	Human	
ACTIVATION				
CD40	ALN[152]			
CD40L (CD154)	L[151,153]	ALN[409]		anti-CD40L and CTLA4Ig rescues lupus in BWF1mice[409]
c-mer RTK		L[62]		B6.c-mer^{ko}mice develop lupus with altered macrophage function
FPRL1	L[119]			
LPSR (CD14)	L[241,245]			After isolation and culture[245]
MRP8 (ABCC11)	ALN[150]		ALN[150]	Authors suggest that these results may be due to steroids.
ACTIVATION/PHAGOCYTOSIS/SCAVENGING				
Calreticulin	L[410]		L[410]	Also called CRP55, calregulin, ERp60
CD44	L[411]		L[411]	CD44 is a receptor for hyaluronic acid and other serum factors
C1q		L[412]		Mrl.C1q$^{-/-}$ show accelerated disease and poor phagocytosis
C5aR (CD88)			L[413]	C5aR-blocking antibodies reduce disease in B6.FCγR2B$^{-/-}$ PMNs
CR1 (CD35)	ALN[159]		L[147,414,415]	

			L[157]	
CR3 (CD11b, ITGAM)	AL[155,156]		L[157]	No difference was seen in inactive lupus[155]
	ALN[165,177,416]			
FCγR1 (CD64)	L[159]	ALN[417]	ALN[154]	BWF1.Fcγ$^{-/-}$ mice are protected from spontaneous nephritis[417] Also increased in Mφ in ALN[31]
	L[31,141]			
FCγR2B (CD32)	L[418]	L[162]	L[419]	This study refers to FCγR2A[418] Mrl-lpr.FcγR2B^{hi} Mφ have no effect(unlike FcγR2B^{hi} B cells)[162] (NZB x B6)F1.Yaa^{mut} mice develop disease[420] B6.FCγR2B$^{-/-}$ develop lupus[161]
	L[31,419]	L[420]	L[158]	
	L[17,141,158-160]	ALN[161]	AL[147]	
FCγR3 (CD16)	L[31,159]		AL[147]	Result of polymorphisms. Also shows susceptibility in rats.[18]
	L[141]		L[421]	
	L[160]		L[422]	
	GN[18]			
Mannose Receptor	L[141]			
sLRP1 (CD91)	L[423]			
SRA		L[242]		BWF1.SRA$^{-/-}$ mice develop lupus; Anti-SRA correlates with disease
SRB (CD36)	L[424]			
ADHESION				
CD177			L[425,426]	
ICAM-1 (CD54)	ALN[152,168]			
	L[159]			Authors suggest that this result may be due to corticosteroid treatment.

L-selectin (CD62L)			L[155]	Appears to be shed (soluble) in SLE.
Siglec-1 (CD169)	AL[174]			
Vitronectin	L[164]			CD51/CD61
ANTIGEN PRESENTATION				
MHC II	L[240,241]			
TAP1	L[119]			
APOPTOSIS				
Bim (BCL2L11)			ALN[427]	B6/lpr.BCL2L11$^{-/-}$ mice have increased macrophage activation
Caspase 3			PL[225]	
FADD, Fas			PL[225]	
c-IAP1, cIAP2			PL[225]	
XIAP			PL[225]	
XAF-1	PL[119]			(XIAP-associated factor 1)
TRAIL (CD253)	PL[225]			
	L[119]			
CHEMOKINES				
CCL2 (MCP-1)	PL[175]	ALN[13]		Indicative of poor prognosis[175]
	L[31]			
	ALN[176]			Imiquimod-treated Mrl-lpr mice overexpress CCL2[13]
CCR1 (CD191)	ALN[179]	ALN[428]		CCR1 antagonist in Mrl-lpr mice halts nephritis progression[428]
CCR4		L[177]		Mrl-lpr[177]
CCR5	ALN[179,181]	L[177]		Mrl-lpr[177]
CXCL10	L[223]			
	AL[429]			
CXCL12		ALN[178]	ALN[178]	B6.Sle1Yaa, BXSB, and Mrl-lpr[178]
CXCR2 (IL8R-β)			AL[430]	

CXCR4 (CD184)		ALN[178]	ALN[178]	B6.Sle1Yaa, BXSB, and Mrl-lpr[178]
CYTOKINES				
MIF		L[431]		Mrl-lpr.MIF$^{-/-}$ have attenuated disease
IFN-I	L[191]		L[432,433]	Expression is also induced in normal PMN by SLE serum[433]
IFN-γ	AL[222]	ALN[434]	L[432]	Mrl-lpr.IFNγ$^{+/+}$ Mφ restores disease in Mrl-lpr.IFNγ$^{-/-}$ mice[434] Mrl-lpr mice have high IFNγ mRNA expression Mφ and PMNs[236]
		AL[236]	AL[236]	
IK (MuRED)		ALN[435]		IK cytokine treatment alleviates disease in Mrl-lpr mice
IL-1	L[158]	ALN[436]	L[158]	Mrl and BWF1 mice[436] BWF1 mice[437]
	L[438]	L[436,437]		
IL-1R antagonist	L[158]		L[158]	
IL-10	L[192,233]	AL[236,237]	AL[236]	Mrl-lpr Mφ and PMN[236]; Polycytidylic acid-accelerated BWF1[237] Mrl$^{+/+}$ and BWF1 mice[238]
		L[238]		
IL-12		L[238,239]	AL[439]	Mrl$^{+/+}$ and BWF1 mice [238]; Mrl-lpr and BWF1 mice[239]
IL-15, IL-15R		L[440]		BXSB mice; IV hIL-15R-Fc further reduced disease in BXSB mice
IL-1β		ALN[441]		Mrl$^{+/+}$ kidneys
IL-2		AL[236]	AL[236]	Mrl-lpr mice have high IL-2 expression in Mφ and PMNs[236]

IL-4		AL[236]	AL[236]	Mrl-lpr mice have high IL-4 expression in PMNs[236]
IL-6	L[192]	L[442]	L[443]	Mrl-lpr mice[442]
		L[444]		Mφ-depletion lowers IL-6 and reduces disease in BWF1[444]
IL-8			ALN[445]	
TGF-β	ALN[176]			
TNF-α	L[248]	ALN[441,446]	L[432]	$Mrl^{+/+}$ kidneys[441]
	L[241]	L [234]		TNFα-treated Mrl-lpr mice have accelerated Mφ-related disease[446]
		ALN[447]		
		L[448]		TNFα polymorphisms affect disease progression in BWF1 mice[447]
				Artemisinin-induced TNF blockade reduces lupus in BXSB[448]
ENZYMES				
Carboxypeptidase D	L[449]			
Collagenase	L[156]		L[156]	
MMPs		ALN[237]	L[190]	Polycytidylic acid-accelerated BWF1[237] Shows an inverse correlation with anti-dsDNA antibodies[190]
mPR3			L[425,426,450]	
GROWTH FACTORS				
CSF-1 (M-CSF)		ALN[446,451]		TNFα-treated & untreated Mrl-lpr mice have high CSF-1[451]

Hbegf, Pdgfc		ALN[237]		Polycytidylic acid-accelerated lupus in BWF1 mice[237]
INFLAMMATORY MEDIATORS				
Cathelicidin (LL-37)	L[119]			
Defensins	L[119]		ALN[452,453]	
Lactoferrin			L[454]	
COX-1, COX-2	ALN[195]	ALN[196]		SWR x NZB F1 mice hyperexpress COX-2 and develop lupus[196]
LTB4	L[254]		L[254]	
PG synthases			AL[204]	
PGE_2		ALN[194]		LPS-stimulated pristane-induced BALB/c mice[194]
		L[455]		BXSB mice[455]
		ALN[198]		ω-3 fatty acid-treated Mrl-lpr express less PGE_2 and disease [198]
NETOSIS				
DNAse 1	L[456]	L[457]	L[189]	BWF1 urine measurements[457]
		ALN[458]		B6.Dnase1$^{-/-}$ mice develop lupus nephritis[458]
DNAse1L3		L[459]		Missense mutation found in both Mrl-lpr and BWF1 mice[459]
Elastase			L[460]	α1-α-trypsinase is found unchanged
REACTIVE OXYGEN SPECIES				
H_2O_2		L[461]	AL[204]	Peptone-induced Mrl-lpr mice[461]
MPO			L[462]	
Nitric Oxide (NO)		L[194]		Pristane-induced lupus in BALB/c mice[194]

O_2^-		L[455]	L[149,201-203]	Increase superoxide production in BXSB mice with age[455] SLE serum factors induce O_2^- in normal neutrophils[201] Superoxide generation is LTB4-independent[149] SLE serum induced O_2^- production, even after heating[202] SLE serum induces hyperresponsiveness of PMNs to stimuli [203]
			ALN[146]	
			AL[204]	
OTHERS				
Neopterin	AL[222]			Levels correlate with atherosclerosis[463]
	L[463]			
Osteopontin		L[464]		Observation in Mrl-lpr; this may be due to tubular expression[464] Increased Mφ OPN expression in IFN-accelerated BWF1 lupus[237]

Abbreviations in alphabetical order: BCL2L – B cell lymphoma 2-like protein; BWF1 – NZB/NZW cross F1; CCL – CC chemokine ligand; CCR – CC chemokine receptor; CD – cluster of differentiation; CR – complement receptor; CSF – colony stimulating factor; CXCL – CXC chemokine ligand; CXCR – CXC chemokine receptor; COX - cyclooxygenase; FADD – Fas-associated protein with death domain; FCγR – gamma immunoglobulin receptor; FPRL - formyl peptide receptor like-1; Hbgef – heparin-binding EGF-like growth factor; IAP – inhibitor of apoptosis; ICAM – inter-cellular adhesion molecule; IFN – interferon; IL – interleukin; LPSR – lipopolysaccharide receptor; LRP – low density lipoprotein receptor-related protein; LTB4 - Leukotriene B4; MIF – macrophage migration inhibitory factor; MHC – major histocompatibility complex; MMP – matrix metalloprotease; mPR3 – proteinase 3; MPO - myeloperoxidase; MRP - multidrug resistance protein; Pdgf - platelet-derived growth factor; PG - prostaglandin; R – receptor; RTK – receptor tyrosine kinase; Siglec - sialic acid binding Ig-like lectin; SR – scavenger receptor; TAP – antigen peptide transporter; TGF – transforming growth factor; TNF – tumor necrosis factor; TRAIL - TNF-related apoptosis-inducing ligand; XIAP – X-linked inhibitor of apoptosis.
An updated version of this table is found at http://www.mohanlab.org/SLE_BASE/myeloid_cells/.

KEY FOR APPENDIX A

Expression

decreased	unchanged	increased	polymorphic

deletion-induced or rescuing	deletion-rescued

Pathological State

L[ref]	AL[ref]	ALN[ref]	PL[ref]
Lupus	Active (non-nephritic) lupus	Lupus nephritis	Pediatric lupus

Colors refer to expression as listed. Letters refer to pathological state (Lupus is L, non-nephritic active lupus is AL, lupus nephritis is ALN, pediatric lupus is PL).

APPENDIX B

AXL CONSTRUCTS

Below are mouse protein and mRNA sequences of Axl, respectively. When the protein sequence [432]QPLHHLVSEPPPRA[446] (brown below, coding sequence in yellow) is deleted, Axl locates normally to the membrane but *is cleaved much less readily than wild-type Axl*.[295]

NP_033491 - Protein sequence of Axl:

```
  1 mgrvplawwl alccwgcaah kdtqteagsp fvgnpgnitg argltgtlrc elqvqgeppe
 61 vvwlrdgqil eladntqtqv plgedwqdew kvvsqlrisa lqlsdageyq cmvhlegrtf
121 vsqpgfvgle glpyfleepe dkavpantpf nlscqaqgpp epvtllwlqd avplapvtgh
181 ssqhslqtpg lnktssfsce ahnakgvtts rtatitvlpq rphhlhvvsr qptelevawt
241 pglsgiyplt hcnlqavlsd dgvgiwlgks dppedpltlq vsvpphqlrl ekllphtpyh
301 iriscsssqg pspwthwlpv ettegvplgp penvsamrng sqvlvrwqep rvplqgtllg
361 yrlayrgqdt pevlmdiglt revtlelrgd rpvanltvsv taytsagdgp wslpvplepw
421 rpgqgqplhh lvsepppraf swpwwyvllg alvaaacvli lalflvhrrk ketrygevfe
481 ptvergelvv ryrvrksysr rtteatlnsl giseelkekl rdvmvdrhkv algktlgege
541 fgavmegqln qddsilkvav ktmkiaictr seledflsea vcmkefdhpn vmrligvcfq
601 gsdregfpep vvilpfmkhg dlhsflysr lgdqpvflpt qmlvkfmadi asgmeylstk
661 rfihrdlaar ncmlnenmsv cvadfglskk iyngdyyrqg riakmpvkwi aiesladrvy
721 tsksdvwsfg vtmweiatrg qtpypgvens eiydylrqgn rlkqpvdcld glyalmsrcw
781 elnprdrpsf aelredlent lkalppaqep deilyvnmde ggshleprga aggadpptqp
841 dpkdscsclt aadvhsagry vlcpstapgp tlsadrgcpa ppgqedga
```

NM_009465 – mRNA sequence of Axl

```
  1 agagggggag ccaggggcgg ggaaagaagt ctgggagtga gagaatgagg cagggtagcc
 61 gggaaggcgg ctagctgcgg aggagttgag ccagccgagg ggctcccgct gtgccaggcg
121 ggcagtgcca aatcccagga gcccaggggt ggggggaggg ccggggacaa cccggccctg
181 ccccctttcc tagcgaggtg cccatcaact tcggaagaaa gtttggcatc aatctgagct
241 gttggtgtct ggaggatggg cagggtcccg ctggcctggt ggttggcgct gtgctgctgg
301 gggtgtgcag cccataagga cacacagacc gaggctggca gcccgtttgt ggggaaccca
361 gggaatatca caggtgccag aggactcacg gggacacttc ggtgtgagct ccaggttcag
```

421 ggggaacccc ctgaggtggt gtggcttcga gatggacaga tcctagaact ggctgataac

481 acccagaccc aggtgcctct gggcgaagac tggcaagatg aatggaaagt tgtcagtcag

541 ctcagaatct cagccctgca actttcagat gcaggggagt accagtgtat ggtgcatcta

601 gaaggacgga cctttgtgtc tcagccgggc tttgtagggc tggaaggtct cccgtacttc

661 ctggaggagc ctgaggacaa agctgtgcct gccaacaccc ctttcaacct aagctgccag

721 gcccagggac ccccggaacc cgtgacccta ctctggcttc aagatgctgt ccccctggcc

781 ccagtcacag gacacagctc ccagcacagt ctgcaaactc caggcctgaa caagacatct

841 tctttctcat gtgaagccca caatgccaag ggagtcacca cctcccgcac agccaccatc

901 acagtgctcc cccagaggcc tcaccatctc cacgtggttt ccagacaacc tacggagcta

961 gaggtagctt ggacccctgg cctgagtggc atctacccgc tcacccactg caacctgcag

1021 gccgtgctgt cagacgatgg ggtgggtatc tggctgggaa agtcagatcc tcctgaagac

1081 cccctcacct tgcaagtatc agtgcccccc caccagcttc ggctggaaaa gctccttcct

1141 cacaccccgt atcacatccg gatatcctgc agcagcagcc agggcccctc accttggacc

1201 cactggcttc ctgtggagac cacagaggga gtgcccttgg gtccccctga gaacgttagc

1261 gccatgcgga atgggagcca ggtcctcgtg cgttggcagg agccaagggt gcccctgcaa

1321 ggcaccctgt tagggtaccg gctggcatat cgaggccagg acacccccga ggtacttatg

1381 gatatagggc taactcgaga ggtgaccttg gaactgcggg gggacaggcc tgtggctaac

1441 ctgactgtgt ctgtgacagc ctatacctcg gctggggatg ggccctggag ccttcctgtg

1501 cccctagagc cctggcgccc agggcaagga cagccactcc accatctggt gagtgaaccc

1561 ccacctcgcg ccttctcgtg gccttggtgg tatgtactgc tgggagcact tgtggctgcc

1621 gcctgcgtcc tcatcttggc cctgttcctt gtccatcgga ggaagaagga gactcgatat

1681 ggggaggtgt ttgagccaac cgtggaaaga ggtgaactgg tagtcaggta ccgtgtccga

1741 aagtcctaca gccggcggac cactgaagcc accttgaaca gtctgggcat cagtgaagag

1801 ctgaaggaga aactacgaga cgtcatggta gatcggcata aggtggcctt ggggaagacc

1861 ctgggagaag gagaatttgg cgctgtgatg gaaggtcagc tcaatcagga tgactccatc

1921 ctcaaggtcg ctgtgaagac catgaaaatt gccatctgca caagatcaga gctggaggat

1981 ttcctgagtg aagctgtctg catgaaggaa tttgaccacc ccaacgtcat gaggctcatt

2041 ggcgtctgtt ttcagggctc tgacagagag ggtttcccag aacctgtggt catcttgcct

2101 ttcatgaaac acggagacct acacagtttc ctcctgtact cccggctcgg ggaccagcca

2161 gtgttcctgc ccactcagat gctagtgaag ttcatggccg acattgccag tggtatggag

2221 tacctgagta ccaagagatt catacatcgg gacctggctg ccaggaactg catgctgaat

2281 gagaacatgt ccgtgtgtgt ggcagacttc gggctctcca agaagatcta caacggggat

2341 tactaccgcc aagggcgcat tgccaagatg ccagtcaagt ggattgctat tgagagtctg

2401 gcagatcggg tctacaccag caagagcgat gtgtggtcct tcggtgtgac aatgtgggag

2461 atcgccaccc gaggccaaac tccctatcca ggggtggaga acagtgagat ttacgactac

2521 ctgcgtcaag gaaatcggct gaaacagcct gtggactgtc tggacggcct gtatgccctg

2581 atgtctcggt gctgggaact gaaccctcga gaccggccaa gttttgcgga gctccgggaa

2641 gacttggaga acacactgaa ggctctgccc cctgctcagg agccagatga aatcctctat

2701 gtcaacatgg atgagggcgg aagccacctt gaaccccgtg gggctgctgg aggagctgac

2761 cccccaaccc aacctgatcc taaggattcc tgtagctgtc tcactgcagc tgacgtccac

2821 tcagctggac gctatgtcct ttgtccttct acagccccag gacccactct gtctgctgac

2881 agaggctgcc cagcacctcc agggcaggag gacggagcct gagacaatct tccacctggg

2941 acatcctctc aggacccaag ctaggcactg ccactggggg aaagctcacc cccccactcc

3001 gtcactccag gccttctccc cagatgcaga atggccttcc ctcccttctc agatgcagtc

3061 catgccttat gcaccctatc cataacagtt tcaagggatc gtctcacatc ttccatccca

3121 gcgttctaga ttttaaggtt tgagtttaga gttcaaagtt ctcaaagatg atgagtcttt

3181 ggaccgagat gcttgtttct aggtctgcag cgctgttgct atagacaggc ccactgctcg

3241 aaggctctga gattctatgg ctctagattt ttctggctct ataattcgtg gcaatgctcc

3301 catggtttta ggttgcacga ctctgagatt ccaggaccta aggcttctag actttatttt

3361 tctggagcca ggggtcctgt cagtggaaga ttgtagattt ttaaattcta aagattctag

3421 gcatgaaggt tctaaggcat actgcttctc cagtttaaca gtttagggct catgttggaa

3481 tactccagat cataatgttt caaactttta ttttttttaa tttctaagac cccagtgatg

3541 gtcaactaca gattctgaag ccttatgacc atagattctt ttatataaaa atcctgtatc

3601 tcaaggaaat atgattctag actctgaaat tccaaagctt taagagtctc cagatggagt

3661 ttctaagcta tgatgtggtg ataatctaaa gtttagtcca aggttctaga ttcctaagct

3721 tccacgtcat ctgctcccag gattccagat tattaaactc taaaactcta atgttggcct

3781 gatcttcgtc tcaggccctg taggatgctg tgggtcctca gcatctaagt cacaagaggc

3841 tccagttaac gaggactaat gagacaccaa agttctaacc acttctaatg ctggacacct

3901 ctaggttcta tgctgctttt tgcctttcta gcacataatt aaatgcccaa gaatacatat

3961 gtctaaagat cttaaatctc taagcactat ggagccaatg ttttgagtgt ctgagattct

4021 aaaggtccac agtctagagt attaggtacg actccaaggg tgggcgcttg tagccatcct

4081 aagtcctttc cctccttaag cacctatgct cctcctctcc ttgtgtgggg tacaccccac

4141 cttaagcctg tgcgatgcac tgggaatgcc tgctttcctc caagggatgg gtcatctccc

4201 ctcatttggg gccatgttgc cccttgagcc agtcccctat gcctgttctg aagtgtggac

4261 tctggtgcct ccagagaggc tcagatcaca taaaactttt gtcagtcact attctgtctc

4321 ttgtgtccat tcaactgtgc tacgtcccct ctccctagag attcggtttc cccactgtga

4381 gaagaaaggc tcctgtaatc ccaacacctg agcggcaaga ggatggaaag ttagttgcag

4441 gccatcgtgg cttacctaga gggaccctga cctcaataaa aagaataaga agagtttgag

4501 ggacacatgg gaatgttttc attagtgaga gtagacatga accatgcaca tagacaaata

4561 tatacaacca aggaaagtca gactctcaga aagccctgag gaatactccc tctctgtcag

4621 agagagagag agagagagag agagagagac agagagagag agccagacag agagagcgag

4681 ccagacagag agagagagag agggatggag ggagagagcg atccccgaca gggtcttaca

4741 caaacattcc aaagtaacag acatgtgaat tagtgcacat gctcaggcaa acctagccac

4801 aaatgcaaag gcccagttat gcactcacag taacagaaac atataaaccc acacttgtaa

4861 atctgcaaac atcagcaggc aggtactcac cagcaatagg aagaaccttt agtaacagac

4921 acagaacaag cattacacac actcaagaat ccaacatggt gcacataatc ccggcgcttg

4981 agaggtataa gcagaaagat caagacttca aggttgacct tgatttcaca gagagatgca

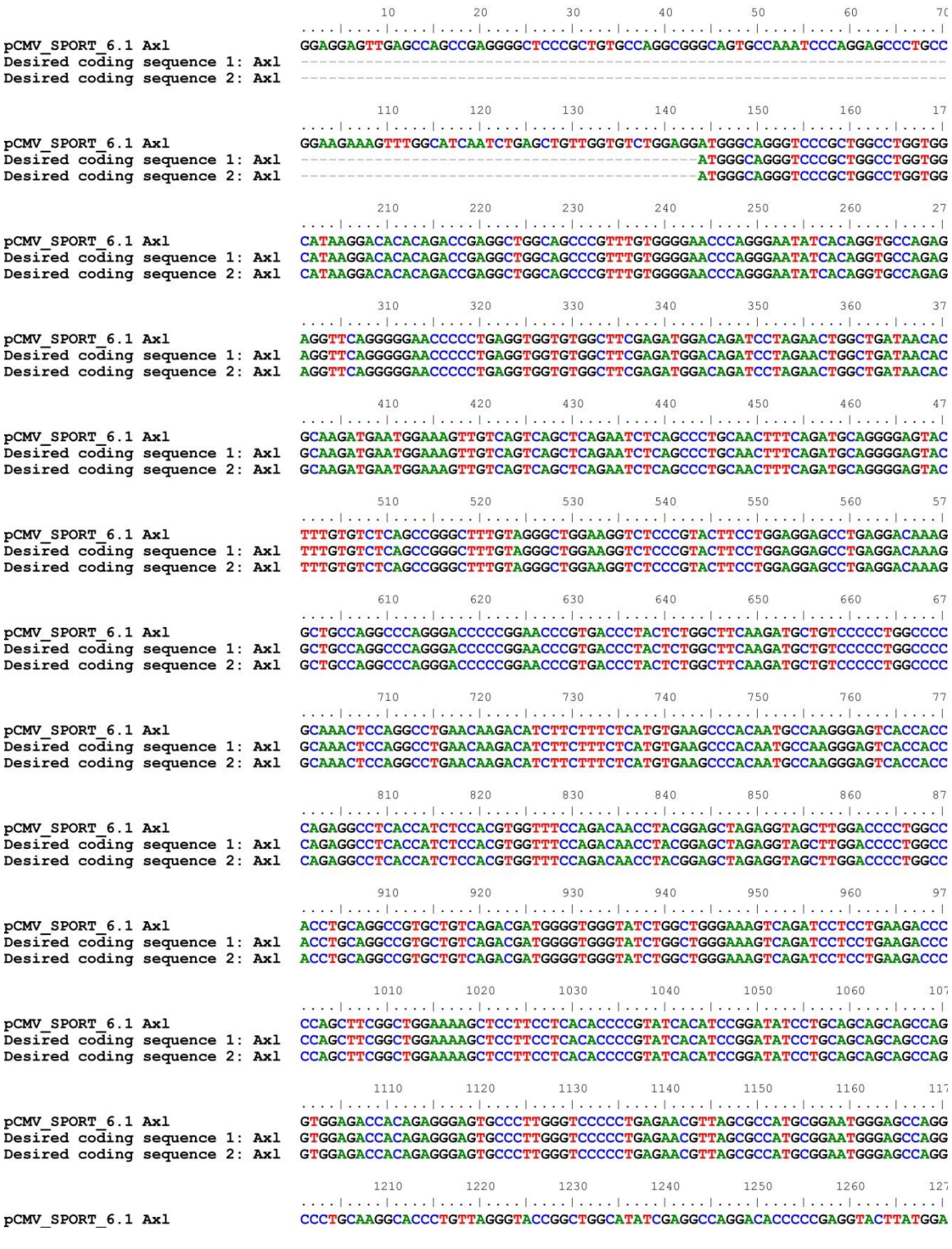
5041 ggccagcctg ggctacatga gactgtctta ataaagaaga aaat

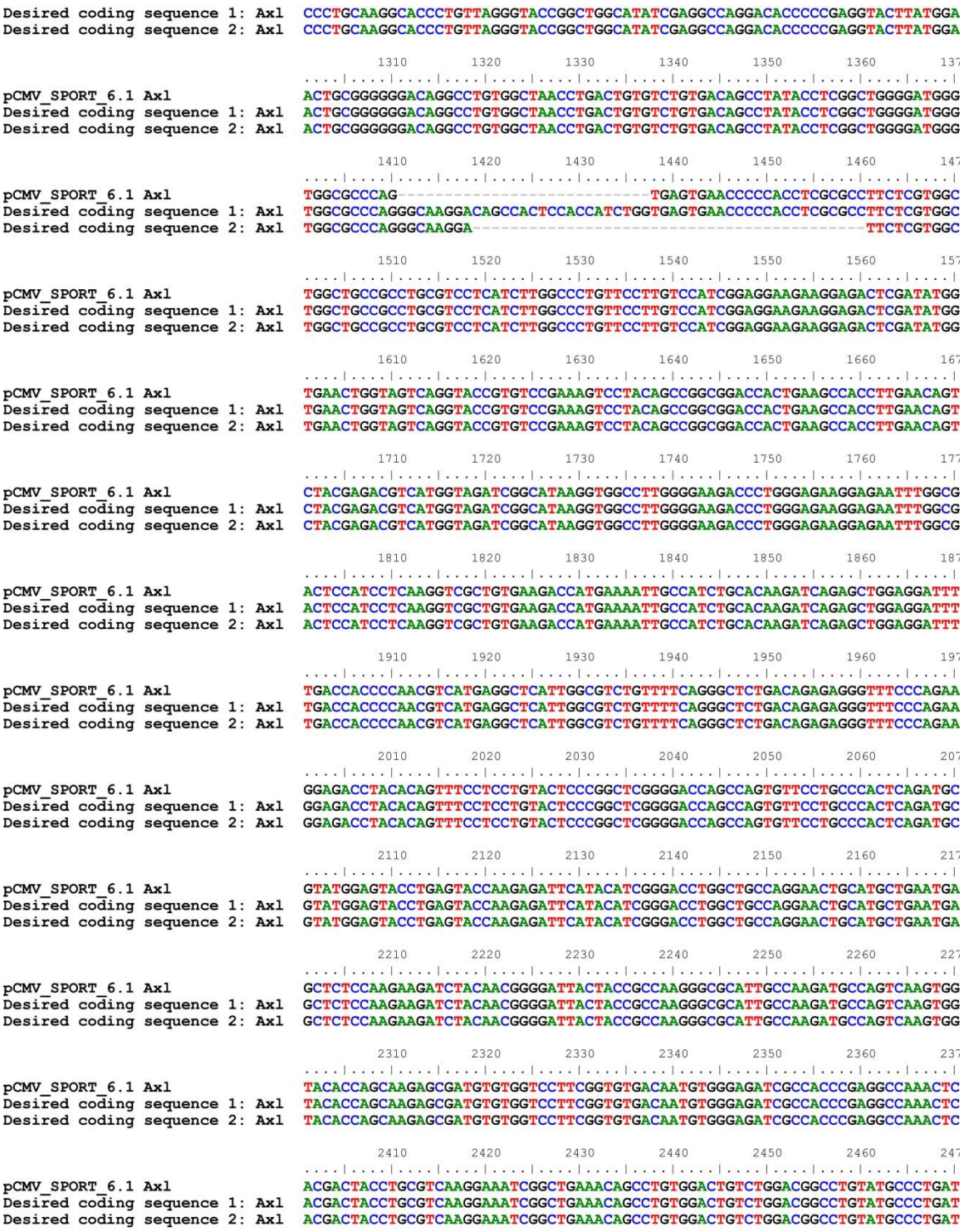
ALIGNMENT OF SEQUENCES:

As shown below, we ordered pCMV_SPORT6.1 Axl from the Harvard/Dana Farber PLASMID core (Cat #MmCD00319729). The sequence is to be mutated in two steps with a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Cat #200521) using the following primers:

Name	Sequence (5’ -> 3’)
Primer ins 1410-for	CCCTGGCGCCCAGGGCAAGGACAGCCACTCCACCATCTGGTGAGTGAACCCCC
Primer ins 1410-rev	GGGGGTTCACTCACCAGATGGTGGAGTGGCTGTCCTTGCCCTGGGCGCCAGGG
Primer del 1276-for	GCCCAGGGCAAGGATTCTCGTGGCCTTG
Primer del 1276-rev	CAAGGCCACGAGAATCCTTGCCCTGGGC

Table 17 – Primers sets used for preparing wild-type and uncleavable Axl, respectively.


pCMV_SPORT_6.1 Axl
Desired coding sequence 1: Axl
Desired coding sequence 2: Axl


Desired coding sequence 1: Axl CCCTGCAAGGCACCCTGTTAGGGTACCGGCTGGCATATCGAGGCCAGGACACCCCCGAGGTACTTATGGA
Desired coding sequence 2: Axl CCCTGCAAGGCACCCTGTTAGGGTACCGGCTGGCATATCGAGGCCAGGACACCCCCGAGGTACTTATGGA
1310 1320 1330 1340 1350 1360
pCMV_SPORT_6.1 Axl ACTGCGGGGGGACAGGCCTGTGGCTAACCTGACTGTGTCTGTGACAGCCTATACCTCGGCTGGGGATGGG
Desired coding sequence 1: Axl ACTGCGGGGGGACAGGCCTGTGGCTAACCTGACTGTGTCTGTGACAGCCTATACCTCGGCTGGGGATGGG
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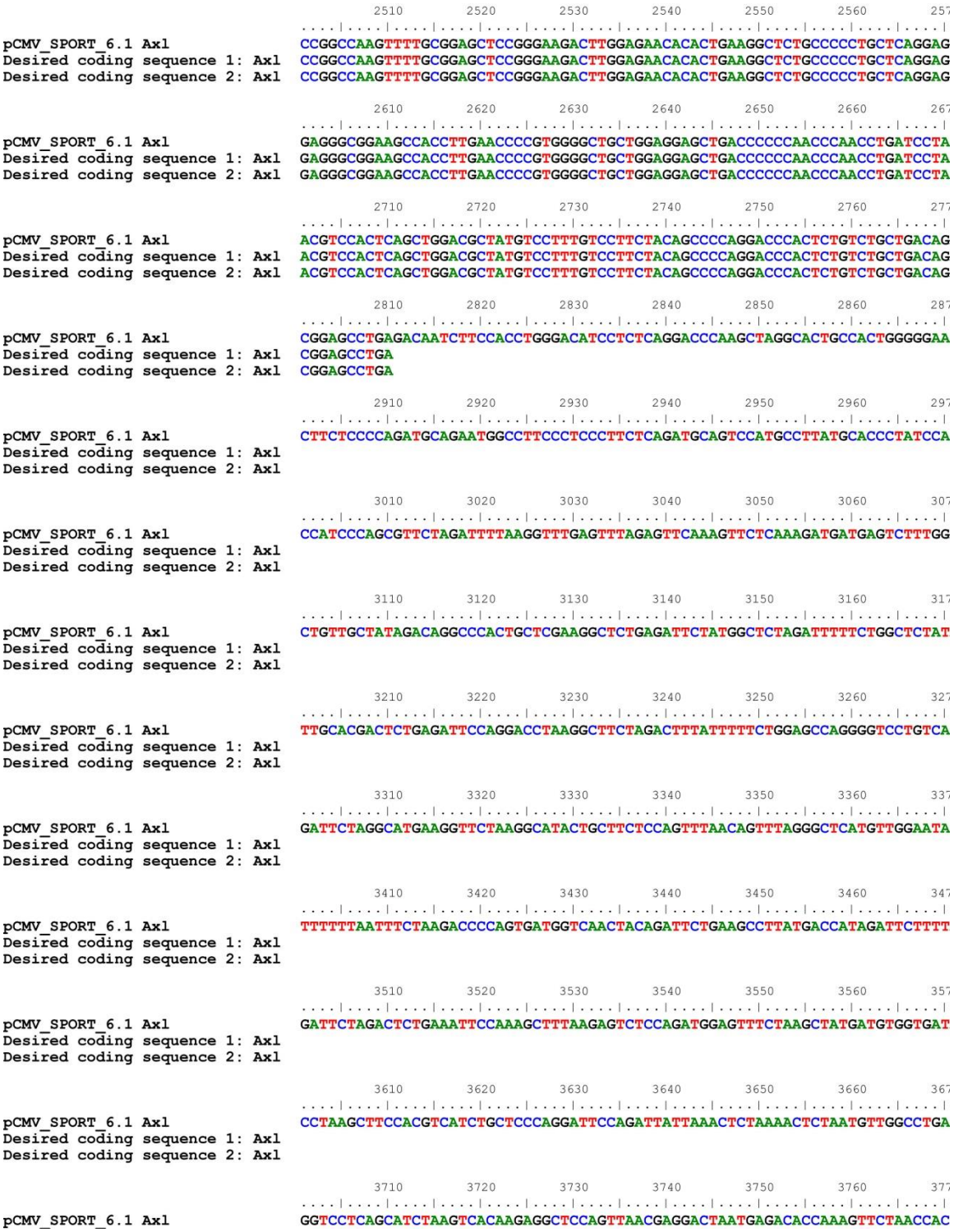
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Desired coding sequence 2: Axl
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Desired coding sequence 2: Axl
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Desired coding sequence 1: Axl
Desired coding sequence 2: Axl
3210 3220 3230 3240 3250 3260
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Desired coding sequence 1: Axl
Desired coding sequence 2: Axl
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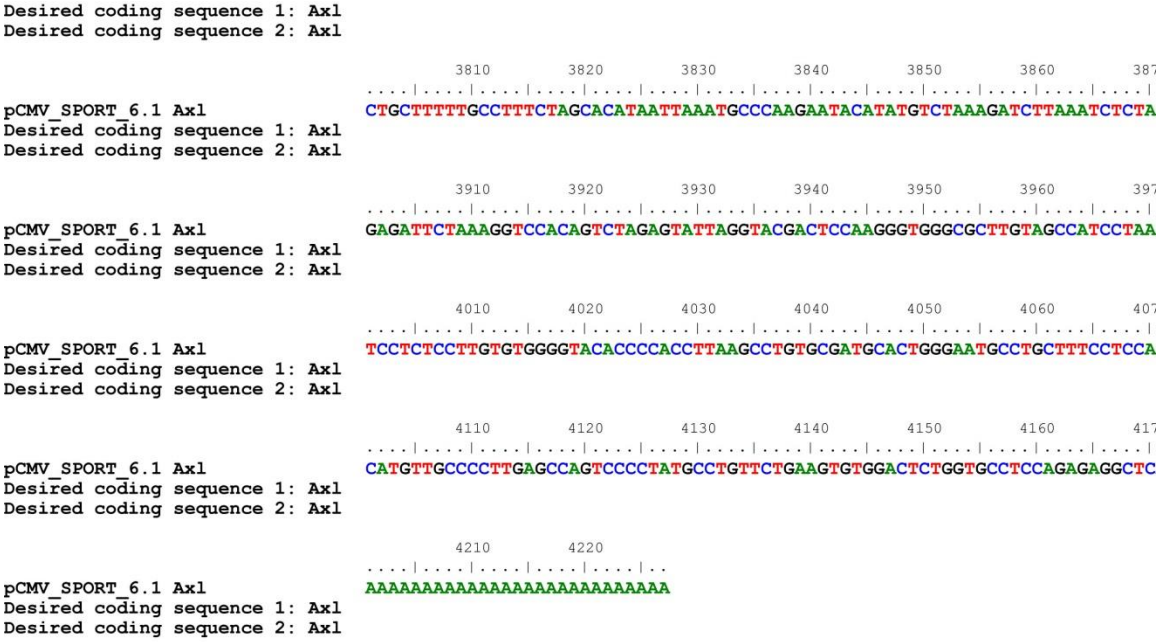
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Desired coding sequence 1: Axl
Desired coding sequence 2: Axl
3910 3920 3930 3940 3950 3960
pCMV_SPORT_6.1 Axl GAGATTCTAAAGGTCCACAGTCTAGAGTATTAGGTACGACTCCAAGGGTGGGCGCTTGTAGCCATCCTAA
Desired coding sequence 1: Axl
Desired coding sequence 2: Axl
4010 4020 4030 4040 4050 4060
pCMV_SPORT_6.1 Axl TCCTCTCCTTGTGTGGGGTACACCCCACCTTAAGCCTGTGCGATGCACTGGGAATGCCTGCTTTCCTCCA
Desired coding sequence 1: Axl
Desired coding sequence 2: Axl
4110 4120 4130 4140 4150 4160
pCMV_SPORT_6.1 Axl CATGTTGCCCCTTGAGCCAGTCCCCTATGCCTGTTCTGAAGTGTGGACTCTGGTGCCTCCAGAGAGGCTC
Desired coding sequence 1: Axl
Desired coding sequence 2: Axl
4210 4220
pCMV_SPORT_6.1 Axl AAAAAAAAAAAAAAAAAAAAAAAAAA
Desired coding sequence 1: Axl
Desired coding sequence 2: Axl

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