AUSTRALIAN NATIONAL UNIVERSITY

The contribution of genetic variation to development of systemic lupus erythematosus

A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

Simon Hee-Tang Jiang

December 2015

Department of Immunology and Infectious Disease

Humoral Immunity and Autoimmunity Group

John Curtin School of Medical Research

Dedicated to my mother, Wilhelmina, for everything.

STATEMENT

This thesis is original work undertaken at the Department of Immunology and Infectious diseases, John Curtin School of Medical Research, Australian National University. The results herein, except where stated otherwise, were performed by me under the supervision of Professor Carola Vinuesa.

Simon H. Jiang

Acknowledgements

To Carola Vinuesa, for her excellent supervision, insights, and for all the support.

To Giles Walters, for his guidance both before and during my PhD, without whom this would have been impossible and whom always underestimates his contribution.

To Vicki Athanasopoulos, for the guidance with experiments and experimental technique and all the support over the years.

To my many collaborators for their assistance with all the varied projects; most significantly Ilenia Papa and Jean Cappello without whom this would not be possible.

And to my wife, Hilde, and my children for keeping me sane.

Table of Contents

Table of Contents

Table of Contents

Table of Contents

Abstract

Systemic lupus erythematosus is the prototypical autoimmune disease characterised by multi-organ involvement. Genetic variance is one of the most potent risk factors for development of lupus yet currently identified variants only account for 15% of genetic risk. Despite a strong genetic basis it does not follow Mendelian patterns of inheritance. A favoured hypothesis is that many common, mildly damaging variants combine to predispose to lupus. An alternative hypothesis is that few rare damaging variants cause disease. Single nucleotide and copy number variation have both been increasingly associated with a diverse range of diseases and this thesis investigates their contribution to lupus in isolation or in epistasis.

This thesis describes the use of whole exome sequencing and SNP-chip arrays to identify genetic variants that associate with systemic lupus erythematosus. Using a combination of molecular tools we demonstrate the translational consequences of these variants and how the alterations to protein function may then disturb cellular function. In the final step of proof of causation we generate bespoke mouse models of the variants to demonstrate and test the effect of these variants in autoimmunity. Specifically we show that inheritance of a rare damaging mutation in *BANK1* together with a second damaging rare mutation in one of three BANK1-interacting proteins: BLK, SAMSN1 or IRAK2, alters B cell responses to TLR ligands increasing secretion of the lupus-promoting cytokines IL-6 and IL-10. Introduction of the human $BANK1^{W40C}$, BLK^{R131W} and $SAMSN1^{T198A}$ variants in mice via CRISPR/Cas9 reveals the mutant alleles increase IL-6 production by B cells and cause lupus-like disease. These results provide support for digenic or oligogenic lupus, bespoke mouse models of human lupus, and novel targets for personalised intervention.

Using a SNP-chip array a large intronic deletion in *VANGL1* was identified that associated with glomerulonephritis in a cohort of SLE. Kidneys from *Vangl1* deficient mice

demonstrated spontaneous IgA and IgG deposition but were normal by light microscopy, with no evidence of systemic autoimmunity. B cell deficient *Vangl1* mice, but not B cell sufficient Vangl1 mice, injected with wild type or *Vangl1*$^{+/-}$ serum demonstrated IgG deposition indicating a kidney intrinsic mechanism for the development of nephritis.

This thesis identifies and demonstrates the contribution of rare single nucleotide variants functioning in systemic autoimmunity. The demonstration of the oligogenic contribution is a first for complex autoimmunity, as are many of the interactions and consequences of the mutations. Further, this thesis identifies genetic mechanisms through which organ-specific manifestations of lupus may occur – relatively common copy number variants in *VANGL1* associates with increased risk of lupus nephritis.

List of Abbreviations

ACR	American College of Rheumatology
ANA	Antinuclear antibody
APC	Antigen presenting cell
BAFF	B-cell activating factor
BANK1	B cell scaffolding protein with ankyrin repeats 1
BCR	B cell receptor
BLK	B lymphocyte kinase
CADD	Combined annotation dependent depletion
CBA	Cytometric bead array
CD	Cluster of differentiation
cDNA	Complementary DNA
CNV	Copy number variation
CR1	Complement receptor 1
CR2	Complement receptor 2
CRISPR	Clustered regular interspaced short palindromic repeats
CRP	C-reactive protein
DLA	Dual luciferase assay
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
GC	Germinal centre
GWAS	Genome wide association study
H&E	Haematoxylin and eosin
HEK	Human embryonic kidney

List of Abbreviations

HLA	Human lymphocyte antigen
IFN	Interferon
ITAM	Immunoreceptor tyrosine-based activatory motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IRAK	Interleukin receptor associated kinase
LCL	Lymphoblastoid cell line
LN	Lupus nephritis
LPS	Lipopolysaccharide
LUMIER	Luminescence-based Mammalian Interactome
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
MACS	Magnetic-activated cell sorting
MAF	Minor allelic frequency
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
OR	Odds ratio
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PPN	Polymorphism phenotyping 2
qPCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RNA	Ribonucleic acid

List of Abbreviations

SAMSN1	Sterile alpha motif domain, SH3 Domain And Nuclear Localization Signals 1
SHM	Somatic hypermutation
SIFT	Sorting intolerant from tolerant
SLE	Systemic lupus erythematosus
SLICC	Systemic lupus erythematosus International Collaborating Clinics
SNV	Single nucleotide variation
SRI	Systemic lupus erythematosus Responder Index
SS	Sjogren's syndrome
TCR	T cell receptor
Tfh	T follicular helper cell
TLR	Toll like receptor
TNF	Tissue necrosis factor
TRAF	TNF receptor associated factor
VANGL1	Van-gogh like 1
WES	Whole exome sequencing
WGS	Whole genome sequencing

Chapter 1: Introduction

Systemic Lupus Erythematosus

Systemic lupus erythematosus is a chronic, debilitating relapsing and remitting autoimmune disease. The clinical presentation of lupus is highly heterogeneous, resulting in the establishment of the 11 criteria of which 4 were required to meet the formal diagnosis of SLE[1]. Prevalence rates range from 20 to 150 per 100,000 and follows clear racial and gender predilections [2]. The mainstay of modern therapy is non-specific anti-proliferative agents and the promise of targeted monoclonal antibodies such as anti-B cell activating factor (BAFF) and anti-CD20 antibodies have largely failed in randomised clinical trials [3] Phase III RCTs that led to approval of anti-BAFF antibodies (belimumab) for SLE reported highly conditional benefits; only at 52 weeks of therapy and not 76, only at higher doses and only for the Systemic Lupus Erythematosus Responder Index (SRI) and other measures of disease activity [4,5]. This may relate to the growing realisation that, like clinical presentation, the underlying pathophysiology of SLE is equally heterogeneous with complement, T, B and myeloid cell biology, and cytokine-based mechanisms all being significantly implicated in the disease process. Additionally, environmental and other risk factors including hormonal influences contribute to induce dysregulation of the immune system.

1.1 Systemic lupus erythematosus – Historical context and presentation

The attribution of *lupus*, Latin for *wolf*, to SLE was due to the destructive cutaneous lesions that were reminiscent of bites from this animal. The earliest descriptions of SLE were by Hippocrates, detailing cutaneous ulcerations as *herpes esthiomenos* (gnawing dermatosis). Although described in various forms, the first clear description of lupus erythematosus is credited to Laurent Biett [6] of the Paris School of Dermatology. His student, Pierre Louis Cazenave, coined the term “lupus erythematosus” and described lupus

as a rare skin condition, appearing most frequently in young females and primarily involving the face [7]. Both Moriz Kaposi [8] and William Osler [9], who possibly coined the term “systemic lupus erythematosus”, built upon these early descriptions to conceptualise lupus as a systemic auto-inflammatory disease affecting multiple organs.

This historic difficulty in studying SLE arises from its rarity and heterogeneous presentation. Virtually any organ system may be affected by SLE, ranging from mild cutaneous lesions to kidney failure. The most common manifestations are cutaneous lesions, arthritis and blood or serological abnormalities [10]. Although these have profound adverse effects on quality of life, with the exception of the lupus anticoagulant these also contribute the least to the mortality burden in SLE. Kidney involvement, particularly end-stage kidney disease, is one of the most potent risk factors for death [11]. Neurological and cardiac disease are also poor prognostic manifestations [12] and some extractable nuclear antigen antibodies are reportedly associated with superior outcomes [11].

In 1971, in an effort to unify observations of SLE, a consensus criterion was established to standardise the diagnostic definition of SLE based upon the descriptions by Osler, Kaposi and others. In 1982, advances in serological testing (anti-nuclear and anti-double-stranded deoxyribonucleic acid (DNA) antibodies) and biostatistical techniques led to a revision of these criteria; the American College of Rheumatology (ACR) revised diagnostic criteria which are used to this day [1]. The ACR criteria defines the syndrome of SLE as requiring 4 or more of 11 clinical or serological diagnostic criteria (Table 1) and was designed as a standard to facilitate research. Several iterations have been proposed since; a modest revision in 1997 by Hocheberg [13] adding antiphospholipid antibodies, the 2012 Systemic Lupus International Collaborating Clinics (SLICC) Classification criteria where clinical and laboratory criteria are categorised separately and requiring a combination of clinical and immunologic criteria [14].

Table 1. 1997 Update of the American College of Rheumatology revised criteria for classification of systemic lupus erythematosus

Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
Non-erosive arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
Pleuritis or pericarditis	1. Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion *OR* 2. Pericarditis--documented by electrocardiogram or rub or evidence of pericardial effusion
Renal disorder	1. Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed *OR* 2. Cellular casts--may be red cell, haemoglobin, granular, tubular, or mixed
Neurologic disorder	1. Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uraemia, ketoacidosis, or electrolyte imbalance *OR* 2. Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uraemia, ketoacidosis, or electrolyte imbalance

Hematologic disorder	1. Haemolytic anaemia--with reticulocytosis *OR* 2. Leukopenia--< 4,000/mm^3 on ≥ 2 occasions *OR* 3. Lymphopenia--< 1,500/ mm^3 on ≥ 2 occasions *OR* 4. Thrombocytopenia--<100,000/ mm^3 in the absence of offending drugs
Immunologic disorder	1. Anti-DNA: antibody to native DNA in abnormal titre *OR* 2. Anti-Sm: presence of antibody to Sm nuclear antigen *OR* 3. Positive finding of antiphospholipid antibodies on: 1. an abnormal serum level of IgG or IgM anti-cardiolipin antibodies, 2. a positive test result for lupus anticoagulant using a standard method, or 3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
Positive antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs

The ACR criteria helped estimate the incidence and prevalence of SLE. It was observed that race and gender not only predispose to SLE, but also influence the types and calibre of organ involvement [15,16]. Incidence rates range from 1 to 10 per 100,000 person-years and prevalence rates generally range from 20 to 70 per 100,000 [17,18]. Asian or African-American populations have significantly higher incidence and prevalence rates of SLE than those of other racial backgrounds [16]. Almost universally these epidemiological studies identified a potent predisposition to SLE in women with a female-to-male ratio of 9:1. Initially typified as affecting women of childbearing age after epidemiological studies performed in the United States (US), this observation has since been proposed to be derived from strong racial influences; namely, that SLE is more common in African-American women of childbearing age, but not other racial backgrounds [19].

This racial predisposition also influences prognosis. Historically, prior to the ACR criteria and introduction of corticosteroids, the 5 year mortality was below 50% [20]. Advances in diagnosis and treatment have improved 5-year survival rates to approximately 95% [20]). Despite these improvements, however, standardised mortality rates are still 2-4 times higher than the general population [21]. Five-year survival varies globally: Whereas European and North American countries report 5-year survival rates of approximately 95%, countries with lower socio-economic status have published 5-year survival rates of 57-86% [22-25] likely relating to disparities in health service, education, and availability of resources considered standard in North America and Europe. It has been reported that American SLE patients of African descent have 3 times higher mortality, and SLE patients of Asian descent had 1.8 to 2.7 times higher mortality, than Americans of European descent [26]. Importantly, it is also observed that the higher mortality risk observed between races crossed health insurance strata suggesting access to health services did not account for this difference [27]. A possible explanation is differing disease manifestations between these racial groups; namely, that those of African American and Asian ancestry are more likely to have kidney

involvement, and kidney involvement is the most consistent and potent predictor of mortality [28]. Together, these studies implicate racial background in strongly influencing development, disease manifestation and prognosis of SLE.

The large scale clinical trials following adoption of the ACR criteria led to several advances in the treatment of SLE. Development of anti-proliferative immunosuppressants such as azathioprine and mycophenolate, which inhibit purine synthesis and prevent lymphocyte proliferation, translated into significant improvements in mortality. Modern therapy ranges from anti-proliferative medications such as hydroxycholoroquine, azathioprine and mycophenolate to potent, toxic alkylating agents such as cyclophosphamide. These agents are often adjuvants to minimise use of corticosteroids, powerful immunosuppressive agents with equally profound side-effects including increased risks of infection, malignancy, osteoporosis, insulin resistance, and Cushing's syndrome. Whilst certain agents may be superior for particular disease manifestations (i.e. mycophenolate for classes of lupus nephritis [29]) the common theme unifying modern immunosuppressive agents is that they non-specifically suppress the immune system.

The age of "biological agents", monoclonal antibodies directed against specific moieties, promised greater precision in therapy. The term "biological agent" refers to the first generation agents which were antibodies raised against specific epitopes, such as Tissue Necrosis Factor (TNF) or cluster of differentiation (CD) 19. These epitopes, expressed on specific lymphocytes or part of particular inflammatory processes, represented precise targets for intervention. Targets trialled in SLE include CD20 and B-Cell Activating Factor (BAFF)**.** However, the agents most promising in concept and murine trials such as the B cell suppressing anti-BAFF [30] and B cell depleting anti-CD20 [31] antibodies have been found to be mildly or not superior to current treatment for the majority of SLE and are only anecdotally successful in cases of SLE resistant to conventional treatment. This disappointing realisation is likely due to the either the highly complex pathophysiology of

SLE wherein virtually all limbs of the immune system may be dysregulated in any one individual, or the heterogeneity in disease pathogenesis between individuals. If the latter is true, lupus may be a collection of different diseases that present with shared clinical manifestations but are caused by different molecular pathways. Monoclonal antibodies against particular cells or cell products may thus only be effective in the subset of patients with a molecular dysregulation that can be targeted by the antibody.

1.2 Overview of the immune system

The immune system is a highly evolved and specialised group of defence mechanisms responsible for protecting individuals from foreign pathogens. It is composed of two broad and linked limbs, the innate and adaptive immune systems. Innate immunity is typified by non-specific defence mechanisms such as physical barriers of the skin, or flow of urine and tears. It also includes leukocytes such as myeloid cells, natural killer cells and innate lymphoid cells, capable of recognising and responding to generic foreign compounds. These include lipopolysaccharide (a component of bacterial endotoxin) and viral or bacterial DNA. Innate cells present this foreign antigen in the presence of additional stimulatory signals to members of the adaptive immune response. Adaptive immunity is mediated by T and B cells and in comparison to the innate, is capable of recognising specific individual pathogens or antigens and generating increasingly potent and precise responses to that pathogen. Further, after refining immunological responses it is capable of preserving that response through long-lived immunological memory – the principle of vaccination.

Antigen, a term for any compound capable of inducing an antibody response is typically part of the invading pathogen. Innate leukocytes such as macrophages or dendritic cells, also referred to as antigen presenting cells (APCs), are capable of recognising, ingesting and processing foreign particles and apoptotic cells within specialised organelles – endosomes and lysosomes. The digested material is then presented by APCs complexed

with major histocompatibility complex (MHC), a protein responsible for regulating recognition of self-tissue. Material originating from outside the cell is processed into small peptides which are presented on MHC-II; foreign material originating from inside the cell is presented on MHC-I [32]. Dendritic cells can also present extracellular material on MHC-I in a process known as cross-presentation [33]. APCs migrate to secondary lymph organs, such as lymph nodes, tonsils, or Peyer's patches, where they present antigen to T cells that bear specific receptors for the peptide presented on MHC molecules.

T (from thymus) cells are a class of lymphocytes which originate as lymphoid precursors in the bone marrow before migrating to the thymus. There, they undergo a process of *positive selection* where the T cell receptor (TCR) is selected to recognise self-MHC. After positive selection, the TCR is then tested for recognition of self-tissue and T cells binding self-peptide-MHC with high affinity become regulatory T cells, or undergo *negative selection* by *apoptosis*, a process of programmed cell death [34]. After this dual selection, T cells circulate as naïve cytotoxic CD8+ or helper CD4+ T cells. When the naïve T cell encounters antigen specific to its TCR, presented by APCs and with appropriate co-stimulation by CD80/CD86 expressed on APCs, it is activated. Cytotoxic CD8+ T cells recognise antigen presented on MHC I, whereas helper CD4+ T cells recognise antigen presented on MHC II. In the case of either CD4+ or CD8+ T cells, recognition by the TCR combined with appropriate co-stimulatory signals results in differentiation into either an effector or memory T lymphocyte [32]. Of the CD4+ helper T cell class, there are an expanding number of recognised subsets including T helper 1, T helper 2, T helper 17, T follicular helper and T regulatory cells [35]. Each promotes immune responses against particular types of pathogen through secretion of specialised cytokines and through cell-cell interactions. Ultimately, CD4+ helper T cells help activate macrophages (Th1), eosinophils (Th2), neutrophils (Th17) and B cells (Tfh) to respectively fight infections by intracellular bacteria and viruses, helminths, fungi and extracellular bacteria and viruses.

B (from bursa) cells are the other major class of lymphocyte. Like T cells, they are derived from bone marrow haematopoietic stem cells [36] and express an antigen recognising receptor, the B cell receptor (BCR). The BCR is composed of heavy and light immunoglobulin chains, with an antigen-binding fragment (Fab) composed of variable(V), diversity(D) and joining(J) segments; the random combination of these components constitutes the diversity of the BCR for antigen. The Fc fragment of the secreted immunoglobulin is bound by Fc receptors present in effector immune cells.

B cells emerge from the marrow into peripheral circulation and secondary lymphoid organs as transitional B cells, which may be subdivided into transitional 1 (T1), transitional 2 (T2), and transitional 3 (T3) B cells [37]. These transitional B cells differentiate into mature-naïve B cells which divide rapidly in response to antigen [38]. B cell receptor engagement and B cell costimulation through cell-surface CD19 and CD21 stimulate activation and differentiation [39,40]. Activated B cells receiving T cell help in secondary lymphoid organs may then become germinal centre (GC) B cells which proliferate and class-switch the BCR constant region from IgM/IgD to another isotype of IgG, IgA, or IgE [41]. A proportion of these may differentiate into antibody secreting cells (plasmablasts or plasma cells) or memory B cells which may rapidly respond to re-exposure to cognate antigen [37]. Typical flow cytometric markers of these stages of development are listed in Table 2.

Table 2. Surface phenotype defined human peripheral B subsets.

Name	Type	Phenotype
Transitional B cell	T1	$IgD^{+}CD27^{neg}CD10^{+}$ $CD24^{Hi}CD38^{Hi}$
	T2	$IgD^{+}CD27^{neg}CD10^{+}$ $CD24^{Hi/+}CD38^{Hi/+}$
	T3	$IgD^{+}CD27^{neg}CD10^{neg}$ $CD24^{+/low}CD38^{+/low}$
Mature Naïve B cell		$IgD^{+}CD27^{neg}CD10^{neg}$ $CD24^{+/low}CD38^{+/low}MTG^{neg}$
Memory	Double negative	$IgD^{+}CD27^{neg}$
	Non-switched	$IgD^{+}CD27^{+}$
	IgM-only	$IgM^{+}IgD^{+}CD27^{+}$
	Switched	$IgM^{neg}IgD^{neg}CD27^{+}$
Antibody secreting cell	Plasmablast	$IgM^{neg}CD27^{hi}$ $CD38^{hi}CD138^{neg}$
	Plasma cell	$IgM^{neg}CD27^{hi}$ $CD38^{hi}CD138^{+}$

(Adapted from Kaminski et al.)[37]

B cells are also selected during their development to limit self-reactivity. In the bone marrow, B cells recognising self-antigen undergo deletion, receptor editing or become anergic [42,43]. Nevertheless, the mechanisms ensuring self-tolerance for B cells, however, are not as robust as for T cells since immature B cells leaving the bone marrow contain a significant fraction of self- or polyreactive B cells [44]. Further tolerance checkpoints operate at the point of selection into the mature recirculating follicular repertoire to purge a significant fraction of these self-reactive B cells. Amongst them is competition for BAFF, for which

anergic B cells are less responsive [45,46]. Additional checkpoints operate in the periphery after B cell activation by protein antigen, including the need for co-stimulation through CD40 from rigorously self-tolerised T cells [46] which recognise the cognate antigen that has activated the B cell, and potentially mutation away from self-reactivity within germinal centres [47] followed by stringent selection by limiting follicular helper T cells [48].

B cells do have the capacity to activate independently of T cell assistance in response to T cell-independent antigens, but unlike T-dependent responses, these antigens mainly induce extrafollicular B cell differentiation (see below) and thus do not typically induce long-lived antibody responses. Nevertheless, evidence from animal models suggests that these extrafollicular responses may become longer-lived and pathogenic in the context of inflammation. T-independent antigens include those capable of extensive BCR crosslinking with antigen multimers or those capable of delivering toll-like receptor (TLR) stimulation [49]. TLRs are a class of receptors expressed primarily in myeloid cells and B cells which recognise pathogen-associated molecular patterns (PAMPs) [50]. PAMPs are typically core components of foreign pathogens such as lipopolysaccharide from endotoxin (recognised by TLR4), RNA (recognised by TLR7 and TLR8) or viral and bacterial DNA (recognised by TLR9). Ultimately, engagement of TLR or BCR may induce the B cell to either rapidly differentiate into short-lived plasmablasts capable of producing a soluble form of the BCR called immunoglobulin (Ig) [51], which is of limited affinity for the antigen, or to enter the follicle in secondary lymph organs to enhance the affinity of the BCR for the antigen. Within the lymphoid follicle, a specialised structure called the germinal centre forms when T follicular helper (Tfh) cells and their cognate B cells migrate into the centre of the follicles. There, the now called germinal centre B cells undergo rapid expansion and a process called somatic hypermutation(SHM), wherein random mutation edits the BCR resulting in stochastic increases and decreases in affinity [52]. The process may also induce autoreactive autoantibodies. Selection by limiting Tfh cells promotes competition between B cells for survival signals, so those capable of presenting more antigen to T cells – and thus

expressing higher affinity receptors – are selected. It is thought that Tfh cells and possibly T follicular regulatory (Tfr) cells that derive from Tregs, represent a tolerance check-point and may prevent selection or promote death of B cells that have acquired self-reactive receptors through SHM.

The complement system is an intricate network of effectors, receptors and regulators that are involved primarily in opsonisation, clearance of cellular debris and apoptotic cells, and recognition of foreign microbes. In these roles it is involved in both innate and adaptive responses, and dysregulation of several of its diverse functions has been implicated in SLE pathogenesis. Complement has a significant role in removing immune complexes from the circulation, achieved by the binding of complexes to the complement receptor 1 (CR1) expressed on erythrocytes. There are three main pathways to activation of complement; the classical pathway which is antibody dependent, the lectin pathway mediated by mannose-binding lectin (MBL), and the alternative pathway mediated by 'tickover' of C3 [53].

The classical pathway is strongly initiated by IgM/IgG clusters but the pattern recognition molecule C1q also activates complement by recognising distinct structures directly on microbial and apoptotic cells. The C1 complex consists of C1q bound to C1r and C1s, with bound C1s cleaving C4 into C4a and C4b, and cleaving C4b-bound C2 into C2a and C2b – resulting in formation of C4b2b, the classical pathway C3 convertase. The lectin pathway similarly uses assembly of MBL–associated serine proteases that are structurally similar to C1r/C1s to similarly cleave C4 and C2 to generate the CP C3 convertase C4b2b.

In contrast to both the lectin and classical pathways, the alternative pathway represents three distinct but partially overlapping processes. First, a small fraction of the relatively inert C3 is hydrolysed to $C3_{H20}$ which binds factor B (fB) and is in turn cleaved by factor D (fD) resulting in an initial solvent-based C3 convertase ($C3_{H20}Bb$) that activates complement by cleaving C3 into active components C3a and C3b. This cleavage exposes a

reactive thioester in C3b which is quickly amplified by foreign cells but regulated by human cells - the 'tick-over' phenomena. This positive feedback loop can rapidly amplify a complement response. The alternative pathway also includes a PRM-based initiation mechanism wherein properdin recognises several pathogen and damage-associated molecular patterns that allows recruitment of C3b to recognised surfaces and allows C3b convertase assembly [53]. All C3b convertase complexes can induce amplification of the alternative pathway via activation of C3. Furthermore, although named the alternative pathway it accounts for 80-90% of total complement activation [54].

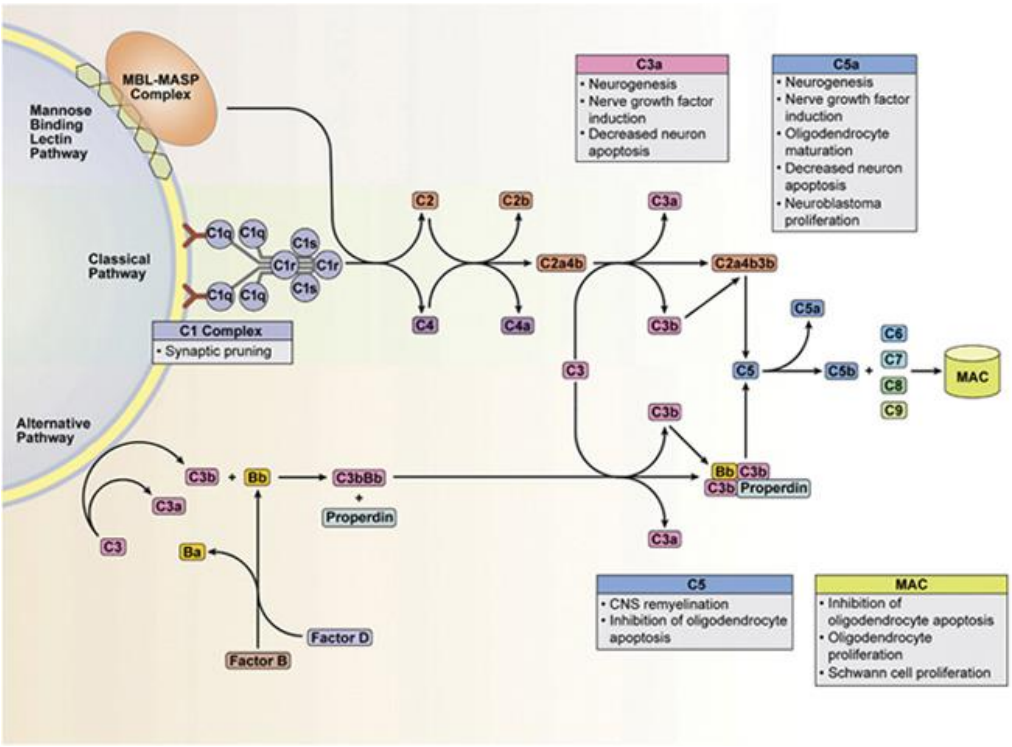


Figure 1. The classical, alternative, and lectin pathways for complement activation[55]

1.3 Pathophysiology of SLE

Almost all components of the innate and adaptive immune systems have been implicated in the development of SLE. Despite being classically considered a disease based on B cell production of auto-antibodies, abnormalities have also been reported in not just B cell biology, but T cell biology, complement activation, uptake of apoptotic cells by

macrophages, neutrophil nets, autophagy, post-translational antigen modifications, DNA repair and cytokine signalling. Many of these pathways, however, may ultimately be implicated in inducing B cells to break tolerance through excessive or altered auto-antigen production, presentation, TLR or cytokine-mediated costimulation, or T cell help.

1.3.1 Complement in SLE

Several observations implicate complement strongly in SLE pathogenesis. First is the characteristic finding of complement deposition in organs with SLE involvement. For example, SLE nephritis is typified by a “full house” of immunological deposition with IgG, IgA, IgM and both C3 and C1q. Secondly, serum complement levels are often perturbed during active and quiescent disease and extensive work has attempted to determine the use of these fluctuations in predicting disease flare [56]. Typically, levels of classical pathway components (C4, C2) are low but, especially in patients with severe disease, may also be accompanied by a reduction in C3 levels. However, in some individuals C4 may remain low even when the patient is well [57]. It has been proposed that a panel of complement activation products – C1r, C1s, C1 inhibitor, and C4a-d (classic), Bb (alternative), and C3a-d, C5a, and C5b-9 (terminal complement complex) – may be superior to C3 or C4 alone. In early studies elevated C3a, but not C5a, were seen in patients with more active disease and rises in C3a preceded flares in ~40% [58], though in inactive/stable disease C4d may be a more sensitive marker of subsequent flares [59]. However, for disease activity scores, elevation of C5b-9 had the strongest correlation with disease activity [60,61]. Ultimately the imprecise correlation between these various complement factors and disease activity limits their clinical utility.

Third and most strikingly, genetic complement factor deficiency strongly predisposes to SLE. Deficiencies of the factors in the classical pathway, such as C1r, C1s, C4 and C2 are associated with development of SLE and SLE-like disorders but not autoimmune disease

in general (i.e. rheumatoid arthritis (RA) and Sjogren's syndrome (SS)). Further, deficiency of C1q, C1r/C1s or C4, but not C2, have equal male-female incidence and disease usually starts earlier in life than sporadically-occurring SLE. C1q deficiency and C1r/C1s deficiency are associated with a very high risk of developing disease whilst C4 and C2d deficiency have lesser risk (90% and 20%, respectively) [62]. Less than 70 cases of C1q deficiency have been reported, but all patients develop SLE [63] and in one case study bone marrow transplantation ameliorated SLE in a patient with C1q deficiency [64]. The correlation between deficiency of C1q and development of a disease process mediated in large part by complement is paradoxical but is thought to be related to impaired clearance of apoptotic debris. C1q has also been suggested to ensure the 'silent' removal of apoptotic cells by preventing 'inflammasome' assembly [65,66]. Additionally, C1q antibodies are a common observation in SLE; resulting decreased C1q levels may mimic a C1q deficient state. These antibodies have been proposed as a predictive marker for LN [67,68].

C2 deficiency is found in around 1 in 20000 and increases the risk of infection and SLE with antibodies against C1q and cardiolipin [69] and a 28 base pair (BP) deletion in C2 was found to be overrepresented in SLE patients of European descent [70]. Complete C4 deficiencies are rare but CNV of C4 is common and the degree of expression affects plasma levels [69]. C4A, but not C4B, CNV are associated with SLE although the predisposition is likely to be modified by HLA haplotypes [71] as C4A is deleted in the extended haplotype *HLAA1*, *B8*, *DR3* which is common in Caucasians and even more common in SLE [72] as are *HLA CW-*, *C4*B1*, *C2*C*, *B*fS*. C4A and C4B are two tandemly duplicated genes in the class III MHC region and are highly polymorphic with variants including non-expressed or null alleles (such as *C4AQ*0* and *C4BQ*0*). C4AQ*0 has strong association with SLE in Caucasians [73] with SLE risks of 9.7 – 16.8 [74,75]. A C2 null *C2Q*0* typically occurs in association with the haplotype *HLA-A25*, *B18*, *CW-*, *DR2*, *C4A*2*, *C4B*2*, *Bf*s* [76].

In comparison, alternative pathway and terminal sequence deficiencies are all associated with increased risk for Neisserial infection but not SLE. Deficiency of C3 associated with a high frequency of recurrent infection and an immune complex manifestation like GN observed in a post infectious background. There is no evidence linking genetic deficiency in MBL in SLE.

Complement inhibitor mutations, such as in *CFH*, are found in numerous kidney diseases such as atypical haemolytic uraemic syndrome but weaker associations with SLE have also been made[63]. CR3 polymorphisms have been associated with SLE with a nonsynonymous allele observed to reduce phagocytic abilities of macrophages, neutrophils and monocytes with no difference in CR3 expression, cell migration or release of inflammatory markers [77].

Complement also plays an important role in directing B cell-mediated SLE processes. Complement is involved with presentation of antigen to B cells and it has been proposed that complement deficiency may result in less exposure of autoantigens to B cells with a corresponding reduction in tolerisation by these same B cells [78]. This theory proposes a protective role for complement in systemic lupus erythematosus and could in part explain how complement deficiencies would have strong associations with SLE. Locally produced C4 is also directly involved in the negative selection of autoreactive B cells by inducing anergy and $C4^{-/-}$ mice produce more autoreactive B cells [79]. Much of this signalling is mediated through the complement receptor 2 (CR2) which is expressed on B cells and CR2 expression is altered in B cells of SLE patients.

In aggregate, observations in human SLE suggest that complement deficiency predisposes to SLE. There are three main hypotheses: Firstly, that CP deficiency results in a reduced capacity for immune complex handling with reduced C1q and C4 in SLE accompanied by decreased density of complement receptor CR1 (CD35) on erythrocytes

[80]. Antibody binding to CR1 is an important mechanism for clearance of immune complexes which trigger inflammatory responses. However, polymorphisms causing CR1 repression do not predispose to SLE [81]. Secondly, waste disposal hypothesis proposes that the main source of auto-antigen in SLE is apoptotic cells and several proteins play a role in scavenging of apoptotic cells, such as C1q together with CRP and natural IgM. Complement deficiency results in excess apoptotic cellular debris overwhelming clearance mechanisms [53]. Three, aberrant tolerance induction proposes that complement is required for elimination of self-reactive lymphocytes during maturation of the immune system and self-antigens are normally coated with complement fragment and delivered to specific B cells by binding to CR1 (CD25) and CR2 (CD21). Loss of this process of tolerisation against self-antigen will impair elimination of self-reactive B cells [53].

1.3.2 B cells in SLE

The characteristic feature of SLE is the production of auto-antibodies against nuclear complexes. Autoantibody production may be reflective of abnormal B cell activation and differentiation that is characteristic of both human lupus and murine models of SLE [82], and/or or may also reflect a defined breach in tolerance by which self-reactive B cells are not properly tolerised or anergy is selectively broken. B cell generation occurs continually throughout adult life and is subject to several tolerance checkpoints, defects in which may predispose towards autoimmunity. These may be grouped into *central tolerance* checkpoints, tolerance occurring during B cell development, or *peripheral tolerance* checkpoints, mechanisms controlling auto-reactivity after B cells have developed[83].

In the bone marrow, B cells develop from stem cells through precursor stages, pro-B cell expressing immunoglobulin heavy chain (Igμ) to pre-B cell which express a pre-BCR complex comprised of Igμ, surrogate light chains, and two signal-transducing proteins Igα and Igβ. The pre-BCR and IL-7 receptor promote survival of the pre-B cell until

downregulation of the pre-BCR. Rearrangement of the light chain leads to expression of a BCR and therein developing diverse antigen specificity in the B cell compartment. Random rearrangement of V(D)J segments in the BCR results in vast numbers of antigens recognised by B cells. The stochastic nature of V(D)J rearrangement that leads to such vast receptor diversity also results in creation of a large number of autoreactive B cells [44]. Even though these auto-reactive B cells can be detected at an early stage in B cell development, they are largely suppressed through deletion, anergy, or receptor editing [44,84].

Clonal selection was first proposed by Burnet in the 1950s, postulating that self-reactive lymphocytes were eliminated to prevent immune responses against self [85]. Subsequently it was shown in transgenic mouse models of B cells that when immature B cells react with a self-antigen with high avidity, such as a highly expressed membrane-bound protein, B cell differentiation arrests and the B cell undergoes apoptosis within 2 to 3 days [86,87]**.** Low affinity interactions permitted further differentiation but also a shorter lifespan resulting in deletion in the periphery [84]. However, it also became apparent that clonal deletion was the fall back after failure of receptor editing to induce tolerance.

In the early 1990s three papers proposed that the immune system could remove antibodies reacting with high affinity to self antigen whilst preserving the cells that produce their specificities [88-90]. In transgenic mouse models against specific antigen, autoreactive immature B cells were shown to ‘edit’ their antigen receptors away from auto-reactivity. In this program, autoreactive immature B cells reactivate immunoglobulin rearrangement processes resulting in expression of a new light chain that pairs with the existing heavy chain to for a new, non-autoreactive BCR. Therein, the B cell is capable of being selected to the periphery. Depending on the avidity of the BCR for self-antigen, editing B cells can completely down-modulate surface expression of IgM (and resemble pre-B cells) or express low to normal levels of sIgM [91,92]. So efficient is this process that, based on chimeric studies of autoreactive B cell mouse models, autoreactive B cells with or without their self-

antigen are capable of producing a normal B cell population[93]. Indeed, partial or complete inhibition of receptor editing programs results in defaulting to clonal deletion, with marked reductions or absence of B cell development [93].

Despite the significant fraction of self-reactive B cells leaving the bone marrow as transitional B cells, a large proportion of these cells are purged from the mature recirculating follicular repertoire [44]. One of such mechanisms is anergy, causing reduced ability to compete for limited BAFF. BAFF is a homotrimer that was discovered by several groups as part of a concerted search for members of the TNF superfamily [94-96] that arose based on increasing recognition of the role of TNF receptors and their ligands in regulation of lymphocyte survival [97,98]. BAFF is found either on the cell surface as a type II transmembrane protein or is released in a soluble form after cleavage. It is produced by macrophages, monocytes, and dendritic cells in response to IFN-y [94,95,99] and IL-10 [99]. BAFF has structural similarities to APRIL, a proliferation-inducing ligand, and these ligands may share several biological activities [100,101]. BAFF binds to three separate receptors – BCMA (B-cell maturation antigen), TACI (transmembrane activator and calcium-modulator and cyclophilin ligand) and BAFF-R (BAFF receptor) [102]. These three receptors have different expression profiles; BCMA is expressed on mature B cells and plasma cells [103,104], TACI on B cells and a subset of activated T cells [105,106], and BAFF-R which is restricted to B cell lineages [106]. Whilst APRIL shares TACI and BCMA as receptors with BAFF, BAFF-R is selectively ligated by BAFF [106,107].

BAFF is important in the maturation of B cells. After migrating from the bone marrow to the spleen, immature B cells pass through two transitional stages – transitional type I (T1) and transitional type II (T2) - before differentiating into naïve mature B cells [108], although very few make this transition [109]. BAFF-deficient mice have impaired T1 – T2 maturation [110,111] as does induced BAFF inhibition [111] as evidenced by normal T1 populations in these mice but no T2 [110,111]. It has been postulated that BAFF may induce transitional B

cells to overcome apoptotic (clonal deletion) signals triggered through the BCR [102]. To support this is evidence that *Baff*-transgenic mice express higher levels of the anti-apoptotic protein Bcl-2 than wild-type mice and develop autoimmune disorders similar to Bcl-2 transgenic mice [112-114]. BAFF prolongs the survival of T2 B cells and all mature B-cell subsets in the periphery, regardless of whether they are resting or activated [115,116]. When BAFF is overexpressed, T2 and marginal zone B cells are increased [114] and the reciprocal phenotype is observed in BAFF deficiency [110,111]. Furthermore, BAFF may potentiate antibody responses and increase the number of plasma cells [115,117], as well as increasing serum immunoglobulin in the blood [117], particularly IgA [115].

Therefore, although essential to B cell homeostasis, BAFF is also strongly associated with autoimmunity. Overexpression in mouse models induces B cell hyperplasia, elevated serum immunoglobulins, autoantibodies and renal involvement reminiscent of SLE [114,118] as well as exocrine autoimmunity reminiscent of Sjogren's Syndrome [114]. Marginal zone B cells are a prominent phenotype of the *BAFF*-transgenic mice, and this phenotype is a T cell independent phenomena [119]. It appears this process is a B cell intrinsic phenomena and is driven by TLR7/9 stimulation [119]. In NZB/W mice a marked autoreactive population in T2 and MZ B cells is observed [116,120] and the marginal zone compartment is implicated in oestrogen-induced models of autoimmunity [121]. In human disease, such as RA and Sjogren's syndrome an association was found between elevated BAFF in the blood and severity of disease [122,123]. The strongest evidence for the importance of BAFF is the discovery that competition for BAFF is used to induce anergy in autoreactive B cells [45] and is therefore a mechanism through which therapeutic inhibition has been shown to be of some benefit.

It is clear that in SLE these mechanisms are impaired. Even SLE patients with inactive SLE demonstrate an inability to remove B cells expressing self-reactive BCRs [124]. Later in development, failure to select against entry of autoreactive B cells into the mature

repertoire is a hallmark of lupus [125]. It is unclear what leads to failure of these main central tolerance checkpoints. Evidence supports roles for both BCR and TLR signalling in maintaining central tolerance and limiting activation of anergic B cells. Genetic disruption in important BCR signalling genes such *PTPN22* and *LYN* lead to expansion of auto-reactive B cell clones [83] and genome-wide association studies (GWAS) have implicated several B-cell specific genes immediately after BCR signalling such as *BANK1* and *BLK* with SLE [126]. All types of TLR except TLR3 use IRAK4 and MyD88 to signal and patients deficient in IRAK4 and MyD88 all have elevated frequencies of poly-reactive new emigrant/transitional B cells which indicate a break in central B cell tolerance [127]. Indeed, it is postulated that B cells with both BCRs recognising nucleic acid auto-antigens and nucleic antigen recognition by TLRs may be selected against, and any mechanism breaking this co-recognition may lead to increased generation of autoreactive B cells [83]. Furthermore, it has been demonstrated that not just abnormalities within the pathways themselves, but uncoupling of TLR and BCR-ERK signalling permits autoimmunity [128] and it is likely that either abnormalities within these signalling pathways, or abnormalities in their interactions, permit breaks in tolerance.

Several mechanisms of peripheral tolerance for B cells also exist. Immature CD10+ transitional B cells expressing IgM/IgD emigrate from bone marrow to peripheral blood wherein they mature into naïve B cells [129]. After encountering antigen and T-cell help within follicles of secondary lymphoid organs, mature naïve B cells migrate to germinal centres (GC) to undergo clonal expansion, somatic hypermutation and Ig heavy-chain class-switch recombination. During the GC reaction, naïve antigen specific B cells mature into either memory B cells or Ig-secreting plasma cells [129], although the affinity maturation is stochastic in nature and susceptible to generation of auto-antigen specific antibodies. Recent work would suggest that the rates of SMH are lower in antibodies from SLE patients [130]. Co-stimulation from CD4+ T cells has been implicated in maintaining peripheral tolerance. Indeed, the GC reaction itself is implicated as a tolerance checkpoint [52]. This

concept may be best exemplified with the 9G4 idiotype antibody, encoded by VH4-34 heavy-chain gene rearrangements that bind B220 self-antigen, and are commonly found in autoantibodies [131]. This idiotype is normally negatively selected in GCs: healthy individuals do not contain 9G4+ memory B cells despite being present in the mature recirculating repertoire. In SLE individuals, 9G4+ cells can circumvent negative selection in GCs and expand into a memory B-cell and plasma population, and 9G4-expressing B-cells as well as 9G4-containing anti-dsDNA antibodies correlate with disease activity [132]. Further, backwards mutation to germ line antibody results in loss of DNA binding activity [133]. Indeed, the VH4-34 heavy-chain itself is intrinsically autoreactive and antibodies encoded from this heavy-chain will react against carbohydrates located on red blood cells [134]. Interestingly B cells with VH4-34 based BCRs largely do not pass through the GC [135] and are heavily selected against and therefore do not appear to undergo SHM [130].

Abnormalities in central and peripheral tolerance can lead to loss of anergy and auto-reactivity, often associated with reproducible B cell phenotypes. Some of the described abnormalities of SLE include increases in memory B cells, particularly “atypical memory B cells” - CD27- IgD- memory B cells [136-138] together with a decrease in IgM+ non-switched memory B cells [139]; IgD-only memory B cells; Cd11b+ B1 cells [140]; T1 B cells [141]; CD19hi CD21lo [139] and plasmablast populations [132,136,142].

Within the memory compartment several subsets have been shown to be abnormally expanded. An expanded population of transitional B cells and switched CD27+ B cells as well as a distinct population of CD27-IgD- B cells with a memory phenotype in normal circumstances [129]. This is supported by molecular data that peripheral B-cell repertoire in SLE can be shaped by somatic hypermutation, whereas the Ig repertoire initially generated by V(D)J recombination appears similar to normal controls[143]. Furthermore, the frequency of memory B cells is increased in the peripheral blood of SLE patients undergoing

immunosuppressive therapy, potentially reflecting a pool of treatment-resistant B cells that will serve as a reservoir for future flares [142].

Plasmablasts have also been implicated in SLE pathogenesis. Short-lived plasmablasts ($CD27^{high}$/$HLA\text{-}DR^{high}$) appear capable of producing anti-DNA antibodies and their frequency in blood correlates with disease activity whereas long-lived plasma cells ($CD27^{high}$/$HLA\text{-}DR^{low}$) produce stable autoantibody titres to extractable nuclear antigens such as –Sm, -Ro, or –La [144]. $HLA\text{-}DR^{high}$ plasmablasts are likely freshly generated plasmablasts and most strongly correlate with disease activity. Several lines of evidence suggest that plasmablasts are derived from GC reactions: they are reduced after inhibiting GC reactions [145] and circulating plasma cells in patients with active SLE expressed highly mutated IgVH genes with mutations consistent with GC hypermutation [142].

Additional mechanisms that may contribute to breaching peripheral B cell tolerance may be dysregulated Fc gamma receptor signals. Fc gamma receptors form a family of receptors that recognise immune complexes for the purposes of either suppressing or enhancing immune responses [146]. Four different classes of Fc receptors have been identified: Frei (CD64), FcɣRII (CD32), FcɣRIII (CD16), and FcɣRIV [147]. Functionally there are two different classes of Fc, activating and inhibitory receptors which transmit their signals via immunoreceptor tyrosine-based activation (ITAMs) or immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and balance of these two forms of Fc allows balanced immune responses [147]. The inhibitory FcɣRIIb is expressed on all cells of the immune system – prominently in B cells which do not express any other Fc gamma receptors - with the exception of T cells and natural killers and is part of the family of immune inhibitory receptors. It was hypothesised to play a role in regulating BCR signalling, the strength of which will determine whether a B cell will undergo proliferation and differentiation into an antibody-secreting plasma cell, or to induce apoptosis in inappropriately activated B cells. Indeed, $Fc\gamma RIIb^{-/-}$ mice spontaneously develop a lupus-like disease characterized by the

production of autoantibodies and severe glomerulonephritis [148]. Polymorphisms within human FcγRIIb have been strongly linked to SLE [148]. Notably, the *Fcgr2b*$^{-/-}$ mouse did not develop problems with early events, such as in receptor editing in bone marrow or the development of IgM+ autoreactive B cells. After class switching to IgG, however, FcγRIIb was essential to prevention of expansion of autoreactive B cells and their maturation into plasma cells [148], highlighting a late stage role in prevention of the initiation of severe auto-reactivity.

These combined data allow several theories for the role of B cells in SLE. First, that abnormalities in central B cell tolerance checkpoints allow emergence of autoreactive clones due to defective negative selection [149]. Second, that defects that allow differentiation of anergic B cells through TLR and/or BCR signals may lead to T-independent forms of the disease. Also, indirect evidence of therapeutic disruption of GC reactions and GC patterns of antibody mutation suggest that SLE may result from dysregulated GC activity and potentially preferential selection by autoantigen [149]. Further to this, it has been suggested that this increased GC activity may result from ectopic generation of GCs [150] or possibly SHM developing autoreactive clones [151].

1.3.3 T cells in SLE

Although classically considered a B cell mediated disease, emerging lines of evidence implicate T cells in SLE pathogenesis. Numerous mouse models have described the need of T cells or T cell-derived molecules for SLE development, regardless whether diseases is thought to originate from follicular or extrafollicular B cells. For example, depletion of CD4+ T cells in NZB/BXSB F1 mice ameliorated disease [152]. MRL.*lpr* mice made deficient in CD40L, which abolishes T cell help for B cells, did not develop anti-dsDNA antibodies not kidney damage [153]. Deletion of SAP, which is expressed by T cells and NK cells and is required for optimal TCR signalling and Tfh formation, prevents lupus in a

number of mouse models; MRL.lpr [154], *sanroque* [48] and pristane-induced lupus [155]. T cell-derived products have also been shown to be instrumental for SLE pathogenesis. For example, IFN-γ is the key driver of lupus in *sanroque* mice [156] and is also required for lupus nephritis in NZB/W F1 mice [157] and MRL.lpr mice [158]. Another T cell derived cytokine, IL-21, has been shown to be required for SLE development in BXD2 [159] and BXSB-*Yaa* mice [160]. Tregs – defined in mice as CD4+ Foxp3+ T cells - have also been shown to be reduced in mouse models of lupus including NZB/W F1 [161] in which exogenous Treg transfer reduced disease progression and mortality [162] and the production of autoantibodies [163]. The latter was proposed to occur through a role of Tregs in inducing B cell anergy. Treg transfer from young (disease-free) lupus-prone NZM2328 mice also prevented anti-dsDNA antibodies but not kidney disease in thymectomised recipient mice from the same strain [164]. It has also been suggested that in some lupus-prone mice such as MRL.*lpr* that despite Tregs being functional, effector T cells may be less sensitive to suppression [165]. In the case of *sanroque* mice, Tregs also appear to be functional [166], but T effectors may be resistant to suppression due to excessive IFN-γ production [156].

Despite this evidence for a role of T cells in lupus development in mice, there are also lupus-prone mouse models such as BAFFR transgenic mice in which lupus can develop in the complete absence of T cells [119], again suggesting SLE is heterogeneous with T-dependent and T-independent pathways of B cell dysregulation.

A number of T cell abnormalities have been reported in human SLE. Although for most it is not possible to conclude whether they are a cause or consequence of the disease, they provide evidence of dysregulation and support the notion they may contribute to some aspects of pathology. Some of the T cell phenotypes found in human SLE are summarised here.

Lipid rafts are micro-domains that form on the membrane of T and B cells. In T cells, these rafts bear TCR-CD3 complexes and associated signalling molecules. In normal T cells, TCR stimulation leads to clustering of these rafts to aid formation of the immunological synapse, allowing for cognate interactions with corresponding molecules on APCs [167]. SLE T cells, however, display pre-clustered lipid rafts indicating T cells are primed for activation. Further, the composition of these rafts is abnormal, with greater expression of FcRγ, Syk, and phospholipase C γ (PLCγ), with reduced expression of the lymphocyte kinase Lck [167]. Although cytotoxic T lymphocyte antigen 4 (CTLA4), a co-stimulatory molecule that is an important negative regulator of TCR signalling, is generally found to have increased expression in SLE T cells it is unable to suppress aberrant T cell activation [168]. A trial of atorvastatin, an inhibitor of 3-hydroxy-3methylgluteryl CoA reductase that disrupts lipid rafts, reduced co-localisation of CD45 and Lck with reduction in IL6 and IL10 production [169]. Abnormalities in the TCR-CD3 complex have been identified wherein the CD3ζ chain is decreased and, structurally and functionally, replaced by an FcRγ chain which binds the spleen tyrosine kinase (Syk) instead of ZAP70 which is conventionally bound by the CD3ζ chain. This results in exponentially increased signalling with a markedly increased calcium flux [170].

Activated T cells express CD40 ligand (CD40L) and provide cognate help to CD40-expressing B cells via the CD40-CD40L interaction. T cells from SLE patients have increased baseline CD40L and increased and prolonged expression of CD40L upon activation [171,172]. Reciprocally, hyperactive B cells can stimulate T cells to upregulate CD40L which can, via positive feedback, provide help to autoreactive B cells resulting in autoantibody production. A possible suggestion for the observation of elevated CD40L relates to the increased nuclear factor of activated T cells, NFAT, found in SLE T cells. NFAT itself targets the CD40L promoter to drive expression, and its elevation is likely related to the enhanced calcium flux that occurs in SLE T cells (as discussed earlier) [173].

Regulatory T cells (Tregs) are a subset of CD4+ T cells that constitute approximately 1-2% of human PBMCs [174]. Human Tregs are characterised by expression of CD25, and high expression of FOXP3, a transcription factor essential to the development and function of Tregs [175]. Indeed, after the discovery of the important role for Tregs in maintaining tolerance and the role of FOXP3 in their development, point mutations in FOXP3 were identified as the genetic basis of the disease called immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) [176]. CD25, a component of the IL-2 receptor, is highly expressed on Tregs, yet may also be found on recently activated CD4+ T cells [177]. Apart from naturally occurring thymically derived Tregs, stimulation of peripheral CD4+ T cells with anti-CD3 and anti-CD28 can induce *Foxp3* expression [178] and therefore Tregs have been classified as inducible (iTregs) or natural (nTregs) thymically derived Tregs and it is thought that expression of *Helios* may distinguish these subtypes in the periphery [179]. In 2006, low expression of the alpha chain of the IL-7 receptor (CD127) was found to be a consistent feature of bona fide human Tregs [177,180], and is now used routinely for Treg identification in human studies.

Several studies have been conducted addressing the role of Tregs in human SLE with conflicting results. Until CD127 was described to better identify human Tregs [180], it is likely that using CD25 and/or FOXP3 was not identifying *bona fide* Tregs due to the limitations discussed previously. Whether measuring those CD4+ expressing either CD25+ or $CD25^{high}$, thought to have greater repressive ability, both subsets have been reported to be reduced in SLE [181-183] although this has been disputed [184,185]. It has been reported that SLE patients with active disease have reduced numbers of $CD4+CD25^{high}$ cells compared with SLE patients with inactive disease [182]. FOXP3 expression is equally confounding, with reports that CD4+CD25+FOXP3+ Tregs are reduced in SLE patients compared with healthy individuals [186-188] and other studies reporting they are equivalent [184,185]. It is also possible that it is either Treg function that, as seen in some mouse

models, is impaired, or the sensitivity of effector T cells or APC's to Treg suppression that is altered.

1.3.4 Cytokine signalling in SLE

Several cytokines have been reproducibly associated with SLE. The classic association is with type 1 interferon, but other significantly associated cytokines are IL-1, IL-2, IL-6, IL-10, IL-21 and IFN-y.

Interferons are cytokines produced in response to foreign pathogen which function to enhance immune responses. Based on the receptor they signal through, there are three major types of IFN; type 1, type 2, and type 3 interferon. Type 1 interferons include IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω which are capable of binding IFNAR [189]. In humans IFN-γ is the sole known type II interferon [190]. Type 3 interferons include IFN-l, although this has only recently been proposed as separate class of IFN [190]. Of these, IFN-α is considered the key IFN in lupus pathogenesis [191]. It is often elevated in the serum of SLE patients and its long-term administration in other diseases (i.e. viral hepatitis) is associated with the development of SLE-like symptoms[191]. Peripheral leukocytes from SLE patients largely overexpress a large number of IFN-stimulated genes [192]. Whilst IFN-α may be produced by numerous leukocytes, plasmacytoid dendritic cells (pDCs) have the capacity to rapidly produce large quantities of IFN-α [193]. One of the most potent inductors of this IFN-α response in pDC is TLR7/9 and TLR 9 stimulation by RNA and DNA, respectively [193]. pDCs are derived from circulating monocytes, often in response to IFN-α, and indeed wild type monocytes cultured in the serum from SLE patients may rapidly differentiate into pDCs [191]. It is therefore apparent that excess IFN-α in SLE patients may reflect a positive feedback loop wherein increased IFN-α initiated by TLR activation leads to pDC differentiation and therefore more IFN-α production [191]. Beyond the effects on pDC

differentiation, type 1 IFNs can affect adaptive immune responses. In human B cells, pDC-derived IFN-α generates non-Ig-secreting plasmablasts in conjunction with IL-6 [194]. In T cells, type 1 IFN prevent activated T-cell death during inflammatory responses, particularly CD8+ effector T lymphocytes. The result of this is further release of nucleosome SLE autoantigens, therein sustaining the type 1 IFN cycle [195].

IL-1 has potent anti-inflammatory properties, IL-1 is an important mediator of tissue damage in chronic autoimmune diseases. IL-1 receptor antagonist (IL-1Rα) blocks IL-1 in a dose dependent manner, and its production is regulated at least partially through FcγR dependent pathways. Monocyte-derived IL-1β production was found to be reduced, regardless of disease activity, and in active disease monocytes had deficient FcγR - mediated production of IL-1Rα [196] but increased amounts of IL-1Ra in response to adherent IgG [197]. High levels of IL-1Rα were observed during disease flares not involving the kidney whereas low levels characterised renal flares [198]. Similarly implicated is IL-2, a T cell and B cell growth factor. Absence of IL-2 in *Il2*$^{-/-}$ mice results in lymphoid hyperplasia and inflammatory bowel disease. SLE T cells have been found to produce decreased amounts of IL-2 following antigenic stimulation which is more pronounced in patients with active disease [199] although other cohorts do not report a difference in baseline or induced IL-2 [200]. Its receptor, sIL-2R is released by activated lymphocytes and has generally, but not universally, been found to be elevated in patients with active SLE [201-203]. Elevation in sIL-2R seems to predict and correlate with disease flares, particularly those with nephritis [204,205].

IL-6 has been strongly implicated in B cell hyperactivity and immunopathology in human SLE. Lymphoblastic lines from SLE patients produce increased levels of IL-6 and inhibition of IL-6 reduced anti-dsDNA production by lymphoblastoid cells *in vitro* [206]. Basal and stimulated IL-6 messenger RNA (mRNA) levels in PBMCs are reproducibly increased in

SLE patients and correlate with disease activity [207,208]. Furthermore, SLE patients have elevated serum levels of IL-6 although the correlation between disease activity and IL-6 is imperfect[209-212]; a contrarian study reported few SLE patients having elevated serum IL-6 [213]. In aggregate, IL-6 tends to be elevated in PBMCs and immortalised B cell lines from patients with SLE although higher levels do not necessarily correlate with disease activity.

IL-10 is a pleiotropic cytokine with both potent anti-inflammatory effects but also the capacity to induce B stimulation and autoantibody production. IL-10 is elevated in both human and murine SLE and may be either contribute to disease, or increase in an attempt to regulate Th1 immune responses. Knockout of IL-10 in MRL/Fas^{lpr} mice results in more severe lupus and higher mortality [214] although conversely therapeutic anti-iL-10 antibodies significantly improve survival in NZB/W mouse models [215]. In SLE patients both baseline and stimulation-induced IL-10 production are elevated, but do not correlate with disease activity [207,211,216].

IL-21 is increasingly implicated in the development of SLE. IL-21 was initially reported to be produced by activated CD4+ T cells [217], with both Th2 [218], Th17 [219-221] and Tfh [222] cells implicated as the source of Il-21. In mice, it is involved in late B cell development inducing mature B cells to differentiate into Ig-secreting PCs. IL-21 receptor deficiency in mice results in impaired IgG responses [223] but essentially normal B cell populations, whereas in humans IL-21 is the major cytokine that induces B-cell activation, PC differentiation and immunoglobulin production [224,225]. With anti-CD40, IL-21 is capable of inducing PC differentiation whist inhibiting anti-IgM and IL-4 induced proliferation [217]. These diverse effects are likely mediated through sustained expression of Bcl-6 and Blimp-1 in GC B cells [226]. Given its important role in mediating plasma cell physiology and influencing the GC reaction it is unsurprising that IL-21 is increasingly implicated in SLE. In patients with SLE higher IL-21 and IL-21 mRNA levels are observed [227,228] and case-control analysis suggests that mutation in the IL-21 gene is a risk factor for SLE [227]. In the

SLE mouse model BXSB-*Yaa* mouse have elevated serum and transcriptional IL-21 [229] and genetic ablation of IL-21R in these mice led to SLE-like disease [160].

IL-12, IL-18 and interferon-γ have been associated with SLE. Elevated serum levels of IL-12, IL-18 and IFN-γ have been observed in small cohorts of patients with active SLE [230,231], although again levels do not appear to associate with disease activity. However, significant data exists that IL-12 production is not elevated in SLE [232]. Further, interferon gamma production itself was not found to be elevated in SLE at basal or stimulated levels [200] and in a further study was reportedly found at reduced levels [216].

1.3.5. Deoxyribonucleic Acid and Ribonucleic Acid in SLE

Lupus is characterised by production of autoantibodies directed against nuclear antigens, particularly DNA [1]. It is therefore unsurprising that DNA, and the antibodies against DNA, have been shown in several different ways to be important in disease pathogenesis. These autoantibodies are thought to contribute directly to the pathogenesis of SLE as they precede the development of clinical disease in most, but not all, patients and levels of anti-DNA antibodies correlate with disease activity [233,234]. The development of an immune reaction to endogenous DNA is thought to result in disease by permitting deposition of antigen-antibody complexes in tissue, and therefore leading to local activation of complement and recruitment of inflammatory lymphocytes. The strongest evidence of the pathogenic nature of DNA is observed in SLE pedigrees involving genes implicated in both local and systemic DNA and/or RNA degradation. Polymorphisms in DNA processing genes such as *TREX1* [235], *DNASE1* [236] and *RNASEH2* [237] family members are recognised causes of monogenic lupus and overlapping diseases such as Aicardi-Goutiere syndrome. These defects in DNA processing are thought to contribute to SLE pathogenesis as excessive DNA and or RNA overwhelms tolerance mechanisms in the immune system. CpG motifs in DNA derived from either bacteria or self can activate TLR9 [238,239], and RNA

activates TLR7, possibly in association with autoreactive BCR [240]. Evidence of an important role of TLR7 in lupus comes from the finding that lupus in BXSB-*Yaa* mice is caused by a TLR7 duplication [241,242].

1.3.6 Human Leukocyte Antigen (HLA) in SLE

Major histocompatibility complex (MHC), termed human leukocyte antigen (HLA) in humans, is located in the short arm of chromosome 6. The strong association of HLA with multiple autoimmune diseases has been largely elicited from genome-wide association studies (GWAS). Indeed, in meta-analyses of these genes HLA is reproducibly the most potent genetic risk factor when studying genetic risk with GWAS [243]. HLAs may be divided into three classes. Class I HLAs are responsible for presentation of peptide from inside the cell, such as viral fragments, and induce CD8+ cytotoxic immune responses. Class II HLAs are responsible for presenting peptide from outside cell to CD4+ T –lymphocytes for the purposes of eliciting helper T responses and subsequent B-cell antibody responses. Class III HLAs region encodes classical complement components, C2, C4A, and C4B [244]. The most consistent HLA associations with SLE are in the class II alleles HLA-DR3 (DRB1*0301) and HLA-DR2 (DRB1*1501) in predominantly white populations [245]. Inherited deficiencies of C4A and C4B, part of the class III HLA region, are strongly associated with SLE [246,247] which is unsurprising as monogenic forms of SLE exist relating to C4 mutations [248]. Ultimately, due to limitations in GWAS, disease associations are limited to regions encompassing HLA class II and II, and more specifically DR3- and DR2-bearing ancestral haplotypes [244]. HLA subsets may also influence disease manifestations, such as development of specific antinuclear autoantibodies Ro/SSA found in 25-50% of SLE cases, which associate with the HLA-DR2/DR3 and HLA DQw1/DQw2.

Several non-mutually exclusive mechanisms have been proposed to explain the reproducible association of DR/DQ with autoimmunity. First, that variation in binding regions

of associated DR/DQ molecules could lead to preferential presentation of limited, specific sets of self-peptide, allowing autoreactive T cells to escape tolerance and enter the periphery [249]. The increasing evidence of the post-translational modifications of known autoantigens - i.e. citrullination, deamidation in RA and celiac disease respectively -, make this a very attractive mechanism to explain how T cells tolerised against self-antigens, can be activated by modified self-peptides that are now seen as foreign. Second, that DR/DQ could select autoreactive T cells or fail to select appropriate Treg populations [250]. Thirdly, that preferential binding in DR/DQ heterozygous subjects could occur through epitope stealing by one MHC over another which could affect whether an immune response is mounted [251]. Finally, it is postulated that misfolded class II MHC may inappropriately present endogenous antigen [252]. Ultimately several mechanisms are likely to contribute yet it is unclear exactly the quanta that each contributes to overall predisposition.

1.3.7 Conclusion

Diverse mechanisms are implicated in the pathogenesis and pathophysiology of SLE. These mechanisms may be universally linked, perhaps as a result of confirmation bias, to the development of B cell auto-reactivity. Complement may exert its effect through several mechanisms; complement deficiency may result in failure to clear apoptotic or nuclear debris leading to excess auto-antigenic stimulation of B cells; or failure to repress or regulate complement activation, particularly of the classical pathway, may precipitate tissue damage and subsequent auto-inflammation. Defects within B cells themselves are also strongly implicated through several possible mechanisms including BCR or TLR signalling defects that permit breaks in central or peripheral tolerance, or B cell predisposition to recognition of auto-antigen or differentiation into plasmablasts. Within T cell lineages, excessive T cell help or abnormalities in enforcing tolerance within or outside germinal centres have been implicated, as the possibility of impaired Treg-mediated suppression. Further, increased antigen production through either complement or failure to appropriately clear or process

DNA may result in excessive auto-antigen presentation to B cells. Excess auto-antigen presentation may also be a feature of B cells themselves, or resulting from excessive type 1 interferon driving APC differentiation and therefore B and T cell activation, or even failure of clearance of nucleic debris. Finally auto-antigens may be aberrantly selected for presentation, post-translationally modified so as not to be recognised as self any longer, or aberrantly presented by particular or misfolded MHCs, resulting in selection of auto-reactive B cells.

1.4 The genome and genetic mutations

The basic structure and function of the human genome in biology is well established, based on sequences of deoxyribonucleic acid (DNA) purines, *guanine* (G) and *cytosine* (C), or pyrimidines, *adenine* (A) or *thymine* (T), which are bound to a backbone comprised of alternating phosphate and sugar residues. The pyrimidine *uracil* (U) may replace thymine in ribonucleic acid (RNA). These strands are then paired through complementary base pairing where adenines will only bind to thymine and guanine will only bind to cytosine through hydrogen bonds. This complementation permits all genetic information to be held on each strand. Together, these bases constitute the genome, and the human genome is composed of over three billion bases of DNA encoding 19,000 genes [253].

.

The genome may be divided into *introns* and *exons*, or non-protein coding and protein coding segments, respectively. Only 1.5% of the human genome consists of protein-coding exons, however, with the majority as intronic or non-protein coding sequence [254].This non-protein coding sequence is constantly changing, a process permitting evolution and adaptation to the environment [255]. Furthermore, it contains significant and yet poorly understood regulatory regions, that consist not only in gene promoter an enhancing regions, but also encodes for both small and long non-coding RNAs that control multiple aspects of gene expression and function[256].

The chemical structure of DNA biases the patterns of mutation. *Transitions*, where the class of nucleotide is preserved (such as purine to purine mutation) are more common than purine to pyrimidine (or vice versa) *transversions*. Once occurred, any mutation will then either increase or decrease in frequency through *positive selection* or *negative selection*. Mutation that confers a selective advantage may become enriched due to positive selection – an example being the low frequency of lactose intolerance in European populations due to expansion of a lactase variant that was positively selected in the dairy-heavy diet of this group [257]. The benefit in nutrition promoted survival of this genetic variation. Conversely, genetic variants that impair fitness of the organism by >1% will be under negative selection and be observed at uncommon frequencies. Over multiple generations and depending on potency, these variants will ultimately disappear [258]. The majority of variants, however, only modestly alter gene function in either a positive or negative polarity, if at all. These variants experience *neutral selection*, not being selected for or against as they do not alter the individual's fitness. Furthermore, patterns of mutation may also bias according to gene function, an example being the immune system where functional diversity may translate into a significant host advantage upon immune challenge [259].

Genetic variation exists on a spectrum of size; from cytogenetically apparent segments [260] to individual single nucleotide variants (SNVs). Advances in sequencing technology and mathematical modelling have permitted increasing detection of intermediate sized genetic variation in the form of copy number variation (CNV), defined as any insertion or deletion of genomic material greater than 1000 base pairs. It is estimated that there are over 10 million common (minor allele frequency >1%) [261] SNVs in the genome, although only a small subset are likely to result in phenotypic differences between populations.

1.4.1 Single nucleotide variation

SNV are single base-pair changes in DNA sequence that can occur within introns, exons, and regulatory elements. Once considered “junk DNA”, it is increasingly apparent that intronic sequences have numerous critical functions including splicing, gene activation, or generation of regulatory non-coding RNAs. Variants within introns can therefore have significant consequence due to changes to gene splicing, resulting in loss of segments of translated protein, or due to changes to gene activators or repressors resulting in altered gene expression. Furthermore, many observations regarding non-coding genome remain unexplained such as the presence of ‘gene deserts’ which are regions up to 3 Mb that are devoid of genes and the loss of which has no obvious effect [262], the impact of high alternative splicing rates (35-60%) for coded genes [263], or even the exact role of ultra-conserved elements which are extraordinarily highly conserved regions between evolutionarily distant species [264]. The basic overview of the genome described above does not encompass the growing complexity and importance that is recognised for intronic sequences.

SNV in exons, the portion of the genome coding for amino acids, can have profound effect on protein structure and function. Exonic SNV fall into two broad classes; *synonymous* variants wherein the SNV does not change the encoded amino acid and which therefore have no functional consequence, and *non-synonymous* variants which change the encoded amino acid and can potentially alter protein function [258]. The potential impact, and basis of that impact, for each amino acid change is large. These range from loss of residues with critical functions (such as tyrosines or serines) to changes to secondary structure of the protein from alterations in electropotential charge. SNVs typically have two alleles, meaning within a population there are two commonly occurring base-pair possibilities at a particular genetic position. The frequency of the SNV is given in terms of *minor allele frequency* (MAF), or the frequency of the less common allele. A MAF of 0.4 estimates that 40% of the

population carries one base type, whereas 60% of the population carries the other base type (the major allele).

Although this most likely reflects historical biases towards studying coding regions of the genome, it is estimated that non-synonymous variation accounts for approximately half of the known genetic lesions responsible for human inherited disease [265]. However, recent advances in CNV detection has also increased the recognition of the contribution of CNV to human disease [266].

1.4.2 Copy number variation

Current estimates suggest around 12% of the genome is subject to copy number variation [267]. CNV, large gains or loss of >1000bp of DNA, can arise both meiotically and somatically, as evidenced by the finding that identical twins can have different CNVs [268] and that repeated sequencing in different organs and tissues from the same individual can vary in copy number [269]. Indeed, CNV seems to be as important as SNV in determining differences between individuals and is a major driving force for evolution[270]. As mentioned previously, in the case of CNV there exists a bias for mutation; CNVs are enriched in genes involved in olfaction, immunity and secreted proteins [271]. It is uncertain whether this due to greater adaptation in these genes (genes relevant to interaction with the environment) or whether CNV is better tolerated in these genes, and therefore they are purged at a lower rate than in other areas of the genome. The major issue confronting current research is the unavailability of adequate tools that can accurately detect CNV. Microarrays and chromatin precipitation arrays are used predominantly, although advances in bioinformatics analysis mean that smaller CNVs are now detected by WES or WGS. Larger CNVs, due to alignment of small read fragments in WES/WGS, are more difficult to detect. However, improving recognition of CNV as a form of genetic variation has resulted in discoveries of susceptibility

to complex genetics diseases such as psoriasis [272], Crohn's disease [273] and glomerulonephritis [274].

1.4.3 Chromosomal variation

With the 1956 discovery of correct chromosome numbers in humans, rapid flow of discoveries in the era of clinical cytogenetics followed. Classic and relatively common examples include chromosomal variation in chromosome 21 (trisomy 21, Down Syndrome) and chromosomal variation in the X chromosome (Loss of X, 45 X, Turner syndrome or gain of X, 47 XXY, Klinefelter syndrome) [275]. As a class, chromosomal disorders are often highly penetrant and typically associated with first-trimester abortions. Those foetuses that survive through to birth often exhibit a spectrum of disease including developmental delay and multiple organ pathology. In relation to autoimmunity, chromosomal variation is of interest given the association of *Yaa* mice with the development of systemic autoimmunity. *Yaa* mice have duplication the TLR7 encoding portion of the X chromosome to their Y chromosome, and therefore male mice have increased expression of TLR7 which is sufficient to induce systemic autoimmune disease [276]. Indeed, although numbers are low an association between additional X chromosomes (Klinefelter's syndrome) and SLE has been reported [277].

1.5 Studying mutation in human disease

1.5.1 Genome wide association study, whole exome sequencing and whole genome sequencing

With the exception of limited case studies and focused sequencing methodologies such as linkage study and candidate gene sequencing, two large-scale methods of analysing complex genetic disease have been used. *Genome-wide association studies* (GWAS) use

capture chips or beads conjugated with small DNA sequences. Alleles are then detected by differential hybridisation with the sample DNA. This technology permitted rapid and simultaneous detection of over a million SNVs and resulted in advances in our understanding of genetically complex disease. However, although a major advance it also had significant limitations. Firstly, given the large number of variants being analysed and the requirement for these SNVs to be sampled in large SLE cohorts, the risk of false positive associations is high [278]. That is, when a large number of variants is tested in a large cohort, the likelihood that any detected difference is due to random chance rises. To account for this, various rigorous statistical tests such as Bonferroni and Sidak tests are need to correct for multiple sampling, and probability cut-offs for significance are restrictive (in the order of $x10^{-8}$). The statistical rigour needed to be confident of genetic association with disease therefore limits the type of SNVs that can be used as probes. As a consequence, rare variants will occur at too low rates to adequately power an association, and therefore only common alleles can be used as detectors. There has been, however, significant success in identifying single gene disorders using linkage and GWAS [279,280]. GWAS studies have uncovered multiple genetic loci that illuminate disease-causing pathways in polygenic conditions, although a large fraction of genetic heritability remains unaccounted for in most complex diseases. This has raised the possibility that the genetic architecture of complex disease may consist on a spectrum of common and rare variants. In the case of complex autoimmunity there is much debate as to what fraction of genetic risk do GWAS alleles contribute to [281], with a large recent studies suggesting that rare variants will not explain the missing heritability [282].

Whole exome sequencing (WES) and whole genome sequencing (WGS) are relatively recent advances in sequencing that have allowed greater resolution and examination of individual genomes. The process is based on fragmentation of DNA with redundant sequencing of millions of DNA fragments in lengths of 25 – 1000 bases. These reads are then assembled into large contiguous segments that can be ordered and oriented

against a reference genome to construct the individual genome. The process of alignment is computation-intensive but allows resolution of novel and rare mutations [283]. Whole exome has been, until recently, the mainly used platform primarily due to cost. However, the cost of sequencing whole genomes has declined significantly and, with modest improvements in exon coverage, is likely to become the gold standard of massively parallel sequencing. The greatest value of either WGS or WES is their capacity to identify both rare and novel SNV. Unfortunately, in comparison with issues of adequate detection faced by GWAS, WES and WGS face the inverse problem, generating vast amount of genomic data; how to predict or rank those SNVs poses a significant challenge.

1.5.2 Computational prediction of the effects of a mutation

Several methods and computational programs have been created in an effort to predict whether SNVs may affect protein function. These predictive programs compute at genomic or protein levels, typically relying on conservation or chemical change to predict the effect of variation. Commonly employed programs are *Sorting Intolerant from Tolerant* (SIFT), *Polymorphism Phenotyping2* (Polyphen2; PPN), and the *Combined Annotation Dependent Depletion* (CADD) tool.

To predict the effect a mutation may have on protein function, the SIFT program was developed to prioritise substitutions for further study [284]. It relies heavily on conservation of amino acids. If a specific residue is always conserved, any change is considered deleterious. If a specific type of amino acid residue is conserved at the site (i.e. hydrophobic amino acids), any class of that amino acid will be tolerated and any change of type (i.e. from hydrophobic to charged) will be predicted to be deleterious.

Polyphen2 differs from SIFT by imputing additional information besides charge conservation [285]. The sequence containing the SNV is compared against a database of

domains and functions. If the amino acid is annotated as a binding site or another important functional residue it is scored. Also, important residues beyond the substituted amino acid in the sequence are recorded and if a 3D structure is known, PPN checks whether the mutated residue is in proximity to the important residues in that structure. Therefore, for sequences in relatively unknown regions, there will be minimal differences in predictive ability between PPN and SIFT.

The most recent iteration of bioinformatics prediction of damage is CADD, designed to overcome several limitations to bioinformatics prediction such as circularity (discussed below). CADD integrates diverse genomic predictions to contrast scores on fixed alleles (alleles with no variation) in humans with simulated *de novo* variants [286].

Although the above tools can be useful for enhancing discovery of pathogenic variants, a number of significant limitations exist. First, predictions vary widely with input and output; conservation predictions are genome-wide but do not use functional information and are not allele-specific. Protein-based predictions (i.e. SIFT, Polyphen) only deal with coding variants and therefore do not consider the majority (>99%) of genetic variation. Both SIFT and PPN programs are significantly better at predicting loss-of-function than gain-of-function and derived most of their predictive ability from conservation [287]. Each predictive tool has its own scoring system and it is difficult to compare or reconcile these scores. Most significantly, these programs are trained on known pathogenic mutations and are subject to major ascertainment biases. This concept, referred to as *circularity*, describes how these programs are used to evaluate variants or proteins that were themselves used to train the programs' predictive models [288]. The natural consequence of this is that the generalisability is poor.

These combined limitations, therefore, impair the predictive ability of SIFT, PolyPhen and CADD. SIFT and PPN have poor sensitivity (69% and 68%, respectively) as well as

poor specificity (13% and 16%, respectively) [287]. In a recent study, CADD, which was thought to bring significant improvements, did not score better [289]. It is therefore recommended that all three prediction tools are used to limit the risk of missing potentially-damaging variants.

1.6 Genetic theory for SLE

Twin studies report disease concordance of 24-75% and 2-5% for identical and non-identical twins, respectively [290,291], indicating a significant genetic contribution towards SLE. Epigenetic modifications account for part of the disease discordance in monozygotic twins[292]. Despite the well-recognized familial clustering of SLE [293,294], only rarely do single nucleotide variants (SNVs) associate with SLE in a Mendelian pattern [295] with most genetic predisposition following complex patterns of inheritance [294]. Genome-wide association studies (GWAS) have identified numerous loci which confer modest risk for SLE [296-298] and whose molecular and physiological consequences are uncertain [299,300].

The cumulative consequences of genetic variants identified by GWAS might account for sporadic cases of lupus, although available data leave a substantial component of the estimated genetic risk unaccounted for by common variants. Furthermore, common variants are unlikely to account for familial clustering of lupus as this would require consistent co-segregation of an improbably large number of weak-acting variants. An alternative theory is that a small number of variants with highly deleterious effects act in concert to cause SLE. These variants would be expected to be rare and therefore may have evaded analysis in GWAS, although it is possible that rare variants are in linkage disequilibrium with common GWAS-identified variants. Rare variants are now identifiable by whole-exome sequencing (WES) or whole-genome sequencing (WGS). Although studies in other non-immune complex diseases such as autism [301] and epilepsy [302] have suggested a significant proportion may be caused by novel or rare variants, whether this mechanism applies to

lupus remains unknown [282,300]. The rare variant hypothesis is supported by results from ethyl-nitrosurea mutagenesis programs, which have identified single point mutations that result in SLE-like phenotypes in mice [166] and spontaneous lupus-prone models in which single point mutations in genes such as Fas [303] also cause disease. Since single gene mutations can cause human SLE [304] as mentioned in 1.3.1, it is plausible that the disease could arise from oligogenic defects. Furthermore, as mentioned previously, larger structural variation such as CNV has been show to predispose to SLE [274] nephritis and autoimmunity. Regardless of the possible contribution of rare variants, the discovery of multiple common variants associated with SLE through GWAS has provided significant advances in our understanding of the genetic susceptibility to SLE.

1.6.1 Genes implicated in SLE

The evident familial association with SLE has resulted in lengthy investigation into possible causative gene variants. Over several decades increasingly sophisticated technology has permitted increasingly broad and unbiased methods of identifying risk variants. 4 major groups were initially, and now reproducibly, associated with SLE. The first gene associations with SLE were with the major histocompatibility complex (MHC), which encode the human leukocyte antigen (HLA)[305]. Indeed, class II alleles *HLA-DR2 (DRB1*1501)* and *HLADR3 (DRB1*301)* are reproducibly associated with SLE and the *HLA-DQ* and *HLA-DR* alleles have strong associations with SLE autoantibodies[306]. Second, rare variants manifesting in Mendelian patterns led to the discovery of the importance of complement. As noted in 1.3.1, *C1q* and *C1r/C1s* deficiency were found to have exceptionally high risks for SLE[62] and *C4* and *C2d* deficiency were also substantially associated with risks for SLE[62]. A further striking observation is that SLE associated with these complement deficiencies has earlier onset, typically more severe disease, as well as afflicting males equally to females[62]. Third, the Fc gamma Fcγ receptor family were strongly and reproducibly associated with SLE. *FCγRI* (*CD64*), *FCγRII* (*CD32*), and *FCγRIII* (*CD16*) are particularly implicated[307], and perhaps unsurprisingly as these genes function

to bind and clear IgG antibodies and IgG-containing immune complexes from the circulation. The pathophysiolgocial importance of circulating antibody-antigen complexes to end organ damage is further highlighted by the association of FCγRIIa and FCγRIIIa in particular with lupus nephritis[308]. The last major group of initial genes discovered associated with SLE were those associated with type 1 interferon. As noted in 1.3.4, type 1 interferon activity is strongly linked to the pathogenesis SLE, and early targeted associations of interferon genes identified *interferon regulatory factor 5* (*IRF5*) and *tyrosine kinase 2* (*TYK2*) genes with risk alleles for SLE[309]. Subsequently, both original and subsequent IRF5 mutations have been confirmed to increase risk and indeed, several IRF5 mutations in combination are capable for additively increasing this risk of SLE[310].

More recently, GWAS have permitted unbiased ascertainment of alleles associated with SLE. It is apparent, as stated earlier, that these alleles may be grouped under several broad categories of complement activation, interferon signalling, antigen presentation, and B cell signalling. A summary of these genes has been listed in Table 3.

Table 3. List of known genetic variants associated with SLE

Gene	Location	Odds Ratio	Variation	Discovery
HLA-DR2, HLA-DR3	6p21.32	2.4	Haplotype	1971
C2	6p21.32	5.0	Deletion	1972
C4	6p21.32	4.3	CNV	1974
C1q	1p36.12	10	Deletion	1981
FCGR2A	1q23.3	1.6	H131R	1996
FCGR3A	1q23.3	1.4	F176V	1997
FCGR2B	1q23.3	1.7	I232T	2002
PDCD1	2q37.3	1.2	PD1.3G/A	2002
PTPN22	1p13.2	1.4	R620W	2004

IRF5	7q32.1	1.6	SNP	2005
FCGR3B	1q23.3	1.2	CNV	2005
STAT4	2q32.2	2.2	SNPs	2006
IRAK1	Xq28	1.5	SNPs	2007
TREX1	3p21.31	25	SNPs	2007
MECP2	Xq28	1.3	SNPs	2007
TNFSF4	1q25.1	1.4	SNPs	200/8
CRP	1q32.2	1.3	-707 SNP	2008
ATG5	6q21	1.2	SNPs	2008
PTTG1	5q33.3	1.2	SNPs	2008
UBE2L3	22q11.21	1.2	SNPs	2008
PXK	3q14.3	1.2	SNPs	2008
PHRF1	11p15.5	1.2	SNPs	2008
ICA1	7p21.3	12.	SNPs	2008
NMNAT2	1q25.3	1.1	SNPs	2008
ITGAM	16p11.2	1.6	R77H	2008
TNFAIP3	6q23.3	2.0	SNPs	20008
BLK	8p23.1	1.3	SNP	2008
BANK1	4q24	1.2	R61H	2008
TNIP1	2q35	1.3	SNP	2008

Adapted from Ramos, P et. Al 2010

Not only have specific gene loci been reproducibly associated, but these loci also offer an insight into the complex pathogenesis of SLE itself. Broadly, it is apparent these genes may be clustered into cellular defects or signalling pathways as discussed in 1.2 pathogenesis of SLE. Gene loci with the highest risk (by odds ratio) for SLE are classical complement components *C1q* [311], *C4A* [312], *C4B* [313], and *C2* [70], followed by type 1

interferon genes *IRF5*, *ITGAM*[297,298], and B cell signalling genes *BANK1* [296] and *BLK* [298].

Within the cluster of B cell signalling genes, two major patterns are observed. First, *BANK1* and *BLK* are essential to BCR signalling. BLK is a src-kinase family member which is activated upon BCR engagement. The primary known consequence of BLK activation is phosphorylation of BANK1 with subsequent activation of Phospholipase C Gamma 2 (PLCG2) and intracellular calcium flux. Therefore, these two genes would appear to be directly related to BCR-mediated signalling. The other two risk genes which are prominent in B cell biology are *IL10* and *PR domain zinc finger protein 1* (*PRDM1*) which encodes the master transcriptional regulator BLIMP1. As noted in 1.3.4, IL-10 is important in the development of antibody-secreting plasma cells and antibody production. BLIMP1 is essential to the transformation of activated B cells into antibody-secreting plasma cells. This would suggest that genetic lesions affecting BCR signalling and plasma cell differentiation are critical to development of SLE.

1.7 Hypothesis and aims of this thesis

As reviewed in this chapter, SLE is a genetically complex condition in which disturbances in virtually all limbs of the immune system have been implicated. These disturbances cluster at a genetic and physiological level around disturbances in regulation of B cell tolerance, or abnormalities in auto-antigen handling or presentation. Using genetic leads established by GWAS, and the easing of technical and financial limitations to identifying and proving the effect of rare SNV, we now have the capacity to investigate the role of genetic variation in SLE. This thesis hypothesises that rare or novel SNV and CNV are capable of contributing substantially to SLE pathogenesis.

Chapter 2: Materials and Methods

2.1 Human patients and DNA sequencing

Written informed consent was obtained as part of the Australian Point Mutation in Systemic Lupus Erythematosus study (APOSLE). This research cohort recruits patients with SLE and their families residing within Australia. The diagnosis of SLE is made by the treating physician and does not require the ACR criteria definition. The study was approved by the Australian National University and ACT Health Human Ethics Committees. From the cohort, patients were prioritised for WES based on early age of onset, severity of disease, or familial clustering. Saliva was collected in Oragene™ DNA self-collection kits and purified using PrepIT™ DNA purification kits (Oragene) and treated with RNAse out (Ribonuclease A, Qiagen Cat# 19101). DNA samples were enriched with Human SureSelect XT2 All Exon V4 Kit and sequenced by Illumina HiSeq 2000 (Illumina, Inc.). Bioinformatics analysis was performed at JCSMR, ANU by the team of Dan Andrews, Matt Field and Vicki Cho. Raw sequence reads were aligned to the reference genome (Hg19) and single nucleotide variants and small insertions and deletions called using SAMTools. All SNVs of interest were confirmed by PCR and Sanger sequencing. Amplifluor to detect *BANK1*^{W40C} in the APOSLE cohort was performed using the CHEMICON Amplifluor SNPs HT Genotyping System Fam-Joe kit S7909 (Merck-Millipore).

2.2 Expression vectors and mutagenesis

The following expression vectors were obtained: untagged Blk (OriGene Technologies), SAMSN1-MycDDK (OriGene Technologies), myc-Lyn (GeneCopoeia, Inc), Traf3-FLAG (GeneCopoeia, Inc). IRAK2-FLAG was a gift from Rob Brink and BANK1-V5 was a gift from C. Castillejo-López (Uppsala University). Site-directed mutagenesis was performed using the Quikchange I and II Site directed mutagenesis protocols (Agilent Technologies).

2.3 Antibodies

Antibodies for western blotting, immunofluorescence imaging and co-immunoprecipitation studies were as follows: mouse anti-human Blk (SC-65980; Santa Cruz), Rabbit anti Blk (K-23; Santa Cruz); Mouse anti V5 (clone SV5-Pk1, MCA1360, AbD Serotec); mouse anti FLAG M2 (F1804; Sigma); mouse anti-myc (9E10; Merck Millipore); rabbit anti-TRAF3 (c-20). (Santa Cruz), mouse anti-CD71 (DF 1513, Sigma); mouse anti phosphotyrosine-HRP (R&D Systems). Secondary antibodies were conjugated to HRP (Jackson ImmunoResearch), Alexa 568 or Alexa 488 (Molecular Probes, Invitrogen).

2.4 Transfection, immunoprecipitation and Western blotting

HEK 293T cells were transfected (Lipofectamine 2000; Life Technologies) with the relevant plasmids as per manufacturer's recommendation. Cells were lyzed using NP-40 lysis buffer and immunoprecipitated with the relevant antibody using Protein G Sepharose (GE Healthcare) and the relevant antibody. For co-immunoprecipitation experiments transferrin receptor was used as isotype control. Immunoprecipitants were resuspended in SDS-buffer and boiled prior to electrophoresis on 8% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes (BioRad Laboratories), blocked overnight (TBST + skim milk powder; or 5% BSA for phosphotyrosine blots) and probed with the relevant primary and secondary antibodies. Membranes were developed with enhanced chemiluminescence developer (Western Lightning Plus ECL; Perkin Elmer). TLR-9-expressing HEK293 cells (InvivoGen) were transfected as above with the relevant plasmids and mCherry reporter plasmid using Lipofectamine 2000 (Life Technologies). TLR9-HEK239s were cultured for 24 hours before being sorted by mCherry expression using a

FACS Aria II, and 2.5 x10^5 mCherry positive TLR9-HEK293s were plated and stimulated with 5µg/ml CpG (ODN 2006, InvivoGen).

2.5 Analysis of cytokine secretion

Supernatants from human cell lines and mouse B cells were analysed by BD™ Cytometric Bead Arrays (CBA) using the Human Inflammatory Cytokine kit (BD Biosciences) and the Mouse Inflammation kit respectively, as per manufacturer's recommendation at time points indicated.

2.6 Dual luciferase assays (DLAs)

HEK293T cells were transfected with a firefly luciferase reporter (100ng; Stratagene), pRL-Tk (10 ng; Promega), Renilla luciferase control reporter, pC1-EGFP (100 ng; Clontech) and IRAK2 Strep-HA plasmids (as indicated). 48h later DLAs were performed as per published protocols [314,315].

2.7 Isolation of PBMCs and generation and activation of human LCLs

Patient PBMCs were isolated using Ficoll Paque density gradient (GE Life technologies) and cryopreserved at 2x10^6/ml using complete RPMI + 10% DMSO. PBMCs were transformed into lymphoblastoid cell lines (LCLs) through transfection with Epstein-Barr Virus with addition of Cyclosporine A. LCLs were stimulated with 1:1000 R848 (InvivoGen), 5µg/ml CpG (ODN7909 from Invivogen), 1µg/ml *E. Coli* lipopolysaccharide (Sigma-Aldrich), or 20µg/ml anti-IgA+IgM+IgG F(ab')2 (Jackson Immunoresearch).

2.8 LUminescence-based Mammalian IntERactome (LUMIER)

LUMIER was done by Alex Weber and Hui Wei as described for a recent Myddosome study [314]. In brief, 10000 HEK cells/well in triplicate 96-wells were transfected with 20 ng Protein A and Renilla fusion plasmids each (Lipofectamine 2000; Invitrogen). 48 h post-transfection cells were lyzed, raw Renilla activity measured and Protein A-tagged proteins (and Renilla-tagged interactors) purified using magnetic, IgG-coated beads. Bound Renilla luciferase activity was measured and an interaction signal was processed by dividing the activity of bound Renilla luciferase to the amount of raw Renilla activity for each transfection, and then normalized to the specific signal for a Protein A-only (no bait) condition.

2.9 Transcriptome profiling of human PBMCs by microarray

Transcriptome profiling was performed by Virginia Pascual's team (Baylor institute Texas and analysed by Romaine Bancherau from her team. In brief, amplified RNA from BANK1$^{W40C/+}$ PBMC and healthy controls was hybridized to Illumina HT-12 V4 beadchips (47,231 probes) and scanned on an Illumina Beadstation 500. Illumina's GenomeStudio version 2011.1 with the Gene Expression Module v1.9.0 (Illumina, San Diego, CA) was used to generate signal-intensity values. After background subtraction, the average normalization recommended by GenomeStudio was used to rescale the difference in overall intensity to the median average intensity for all samples across multiple arrays and chips. In addition, conventional adult SLE PBMC samples were obtained from the public domain (GEO, GSE24706) for comparison. Each dataset was normalized to the median of its own group of healthy controls. IFN modules were obtained from a previously defined blood module framework [316]. Differentially expressed transcripts between conventional adult SLE PBMC and BANK1$^{+/W40C}$ PBMC were identified by Welch's t test, with Benjamini-Hochberg FDR < 0.05.

2.10 CRISPR-Cas9 genome editing of human B cell lines

CRISPR/Cas9 editing of the Ramos B cell line was performed with help from Dr Vicki Athanasopoulos. Dr Athanasopoulos performed cloning of guide RNA and transfection of Ramos cells and I performed cell sorting and sequencing of sorted cells. The vector px330[317] (Addgene), which expresses Cas9 and the sgRNA, was linearized with *Bbs*I and gel-purified. A pair of complementary 18mer oligomers targeting a single site within the SAMSN1 genome (primer sequences available upon request) was annealed and ligated to the linearized vector. pX330 expressing the sgRNA of interest was transfected along with an mCherry plasmid into the Burkitt's lymphoma cell line Ramos using the NEON transfection system (ThermoFisher Scientific) according to the manufacturer's instructions. $mCherry^{+}$ single cells were sorted 24-48hrs later into 96-well plates and individual clones expanded until confluent. gDNA was extracted by digesting cells with proteinase K (0.1% Tween20, 100μM EDTA, 500ug/mL Proteinase K, 1x HF Phusion buffer) at $56^{O}C$ for 40 mins, followed by incubation at $95^{O}C$ for 8 mins. A region of approximately 300 base pair flanking the variant was PCR amplified (primer sequences available on request) using Phusion DNA Polymerase II (ThermoFisher Scientific) and presence of the mutation confirmed by Sanger sequencing. All sequencing was carried out at the ACRF Biomolecular Resource Facility and Genome Discovery Unit, Australian National University.

2.11 CRISPR-Cas9 mediated genome-editing of mouse zygotes

C57BL/6 mice were housed under specific pathogen-free conditions. All mouse procedures have been approved by the Australian National University Animal Experimentation Ethics Committee. (AEEC A2014/058 and A2014/016) under the NHMRC Australian code of practice. gRNA from Samsn1 and Bank1 were synthetically generated and obtained from Sigma while Blk gRNA and Cas9 protein were obtained from PNABio. Oligomers and ssOligomers were purchased from IDT. C57BL/6Ncrl female mice (4-5 weeks old) were super ovulated with Pregnant Mare Serum Gonadotrophin (PMSG) 5UI day 1 and

Human Chorionic Gonadotrophin hormone (HCG) 5UI day 3. After detection of a vaginal plug of the super ovulated females, mouse zygotes were harvested from the ampullae and were placed in KSOM medium (Sigma). Cas9n protein (100 ng/µl) was co-injected with a mixture of sgRNA (50 ng/µl each) and ssOligomer (100 ng/µl) into the cytoplasm of the fertilized eggs into M2 medium (Sigma). After micro-injection, the zygotes were incubated overnight at 37ºC and 5% CO2 and 2 cell-stage embryos were surgically transferred into the uterus of pseudopregnant CD1 recipient females at 2.5 dpc. Three weeks after birth mouse ears were punched. DNA was extracted and Sanger sequencing performed to confirm the mutations. Selection and testing of gRNAs, mouse zygote preparations and micro-injections were carried out at the Australian Phenomics Facility, Australian National University by a team from the Ausralian Phenomics facility initially led by Stuart Read and more recently by Dr Gaetan Burgio; microinjections were performed by Nikki Ross. The sequencing was carried out at the ACRF *Biomolecular Resource Facility* and Genome Discovery Unit, Australian National University.

2.12 Antinuclear antibodies and kidney immunofluorescence

Four month-old mice were sacrificed and serum and kidneys were harvested. Dilute plasma (dilution 1:40) was incubated on Hep-2 substrate. Bound antibody was detected with anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC). Immunoglobulin and C3 deposition analysis was performed on cryopreserved kidney sections. 7 µm acetone fixed sections were blocked with 3% bovine serum albumin and stained with anti-IgG Alexa Fluor 488 (Invitrogen), anti-IgA FITC (Southern Biotech), anti-IgM FITC (BD Pharmingen) and anti-C3 FITC (MP Biomedicals). Fluorescence intensity was evaluated with an Olympus XI 71 microscope.

2.13 Electron microscopy

Samples from Kidney were fixed in 2% Glutaraldehyde (overnight) in Sorenson's phosphate buffer (0.1M) then postfixed with 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M sodium phosphate buffer for 2 hours. En bloc staining with 2% uranyl acetate preceded dehydration through a graded series of ethanols. Specimens were infiltrated with low-viscosity epoxy resin (Spurr's replacement; TAAB Laboratory and Microscopy, Berkshire, UK) using a mix of 50:50 ethanol to TAAB resin for 2 hours followed by TAAB resin for 3 hours, embedded, and set overnight at 70 degrees C. Ultrathin (80 nm) sections were cut from several blocks (3) per sample. Sections were mounted on 150-mesh copper/palladium grids, stained with Reynold's lead citrate, and viewed on a transmission electron microscope (JEOL 1011; JEOL Ltd, Tokyo, Japan). Images were captured using a digital camera (MegaView G2; Olympus, M¨unster, Germany) and software (iTEM; Olympus). Stains and electron microscopy were performed by Mark Koina at the Canberra Hospital.

2.14 Single cell suspension and flow cytometry

Single-cell suspensions were prepared from spleens of indicated mice. Suspensions were prepared in RPMI 1640 media (Gibco) by mechanical disruption and gentle pipetting through 70 mm nylon mesh filters (BD Biosciences). To stain for surface markers, cells were incubated with each antibody layer for 30 min in the dark at 4 C and washed thoroughly with ice-cold FACS buffer (2% FCS and 0.1% NaN3 in PBS) between each layer. Intracellular staining was performed using the Intracellular Staining Kit (eBioscience) according to the manufacturer's instructions.

Chapter 3: Single nucleotide polymorphisms in *BANK1* and interacting partners predisposes to SLE

3.1 Development of a scoring system for WES variants

We first sequenced the exomes of several individuals with childhood onset disease or members of multiplex families. As with complement deficiencies we expected that familial segregation and early onset/male onset may represent more potent genetic lesions. WES data were assembled against a reference genome using the Raijin supercomputer at the National Computation Infrastructure, Australian National University (ANU) [235]. Bioinformaticians (D Andrews and M Field) at the JCSMR then aggregate data from several public databases to create a report on the identified SNVs, including frequency, *in silico* predictions, gene expression, human disease association and murine association. The approach adopted in this thesis to handling the WES variants was to attempt to filter SNVs that were highly unlikely to associate with disease and therefore enrich for disease causing variants. As the central hypothesis of this thesis is exploration of rare variants, scores were attributed to minor allelic frequency. Initial algorithms also include weighting to Polyphen2 score, and gene expression. All variants were then ranked and variants in the top three ranks (scoring >4 out of 7) were reviewed.

The strategy for proof of causation for these variants was established. First, that variant inheritance would suggest epistatic interaction to explain familial clustering of autoimmunity. Secondly, that we test the ability of the SNV to alter protein function. Thirdly, that we may test the consequence of this altered protein function in changing physiology. Finally, that we may test how the alterations in protein function and cell physiology alter complex system biology, ultimately leading to autoimmunity.

3.2 Rare variants in *BANK1*, *BLK*, *IRAK2* and *SAMSN1* identified in SLE patients

In the exome from the first proband sequenced (proband A – A.II.2 in Fig.4a), two variants attained the highest score (score 7, Fig. 2), a rare (minor allele frequency (MAF) = 0.004) heterozygous missense mutation in *BANK1* (4:102751014, G/C, rs35978636) encoding a tryptophan to cysteine substitution at amino acid position 40 predicted to be damaging (Polyphen2 (PPN) =0.98) and a rare (MAF= 0.006) heterozygous missense mutation in *IRAK2* (3:10177883, C/A, PPN:0.98, rs11465864) encoding a serine to tyrosine change at amino acid position 47. In addition to their high score, both genes were implicated in SLE pathogenesis. First, *BANK1* was reproducibly associated with SLE and complex autoimmunity through GWAS as mentioned previously. Although *IRAK2* has not directly been implicated, family member and signal complex partner *IRAK1* has been previously implicated in SLE[318]. Further analysis identified a second mutation in *IRAK2*, $IRAK2^{T508M}$ (3:10238797, C/T, MAF=0.001, PPN:0.65, rs144752184, $IRAK2^{T508M}$) on the maternal allele, indicating $IRAK^{S47Y}/IRAK2^{T508M}$ compound heterozygosity in proband A.

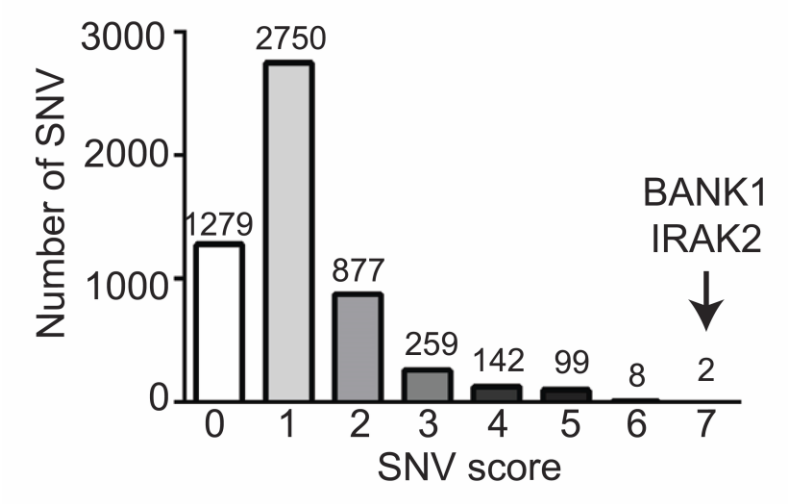


Figure 2. Numbers of non-synonymous SNV by score. Scoring is awarded for allele frequency, bioinformatic prediction of damage, association with immune function and immunological disease. A high score indicates the strongest correlation. Scoring of variants in proband A identifies *BANK1*^{W40C} and *IRAK2*^{S47Y} as top scoring SNV.

An Amplifluor™ was performed on the APOSLE cohorts by the Australian Phenomics Facility, ANU, to test two concepts. First, if GWAS-identified alleles may be not be the risk alleles, but rather associate with disease through sharing of a common haplotype with a deleterious variant. Second, if *BANK1*^{W40C} is significantly associated with autoimmunity then it should be significantly enriched in an autoimmune population. Indeed, we identified the *BANK1*^{W40C} allele in nine of 150 SLE patients (6%) and three individuals from 222 unaffected controls (1.3%), odds ratio (OR) of 4.66 (95% CI: 1.1 - 27.1, p=0.017). In comparison, the common GWAS *BANK1* risk haplotype identified by rs10516487 (R61H, MAF 0.25, OR=1.4 (95% CI: 1.3-1.5, p=3.74 x10^{-10})[296] and which encompasses W40 (Fig. 3) is predicted to be benign (PPN=0). To replicate these findings the Amplifluor™ was repeated in a second, albeit smaller, SLE cohort (n=99) and *BANK1*^{W40C} was observed at a lesser frequency in two of 99 patients (2%). A possible explanation is racial differences in the cohorts as *BANK1*^{W40C} has not been reported in Asian populations and the second cohort was comprised of 47% individuals of Asian background, whereas only 24% of individuals from the original cohort were of Asian descent. The rarity of the allele and smaller cohort may have resulted in insufficient power to detect the variant. A lack of clinical data in the second cohort precluded a detailed examination of phenotypic differences.

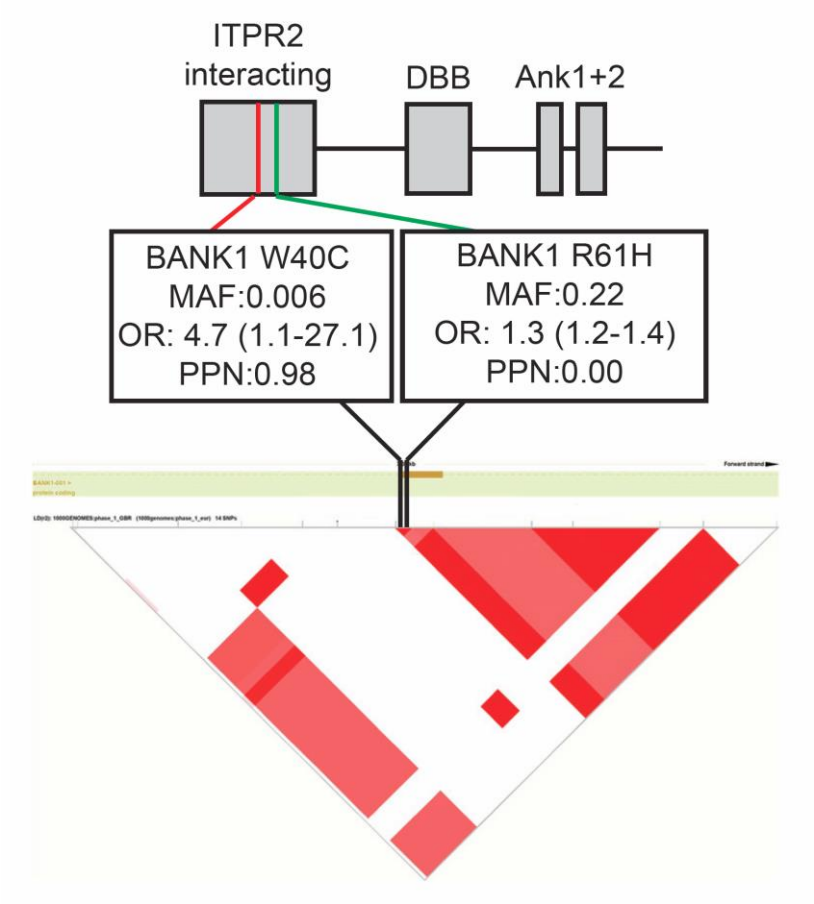


Figure 3. Schematic of protein structure of BANK1 and juxtaposition of *BANK1*^{W40C} and the common SLE risk *BANK1*^{R61H} variants in areas of linkage disequilibrium

To investigate the possibility of oligogenic lupus, as possibly observed with proband A, we sought epistatic interactions with the *BANK1*^{W40C} variant in the other SLE patients carrying *BANK1*^{W40C}. We proceeded to perform WES on all nine SLE probands carrying the *BANK1*^{W40C} SNV. In all 9 individuals, we identified rare or novel heterozygous SNVs in genes encoding proteins known to interact directly with BANK1 or in BANK1-regulated signalling pathways – BCR, TLR, and CD40 – amongst the highest scoring SNVs. Kindred DNA was available to construct a pedigree for 3 of the 9 SLE probands (Fig. 4). The clinical phenotype of all probands are listed in table X. The segregation of the different variants with disease suggested predisposition to SLE was determined by co-inheritance of *BANK1*^{W40C} with a second rare SNV in genes involved in B cell activation.

In Proband A, as stated, we identified *BANK1*^{W40C} and both *IRAK2*^{S47Y} and *IRAK2*^{T508M}. Proband B had inherited *BANK1*^{W40C} and a heterozygous *BLK*^{R131W} variant (8:11550181 C/T, MAF:0.009, PPN:1, rs73663163) from each parent. Proband C carried the *BANK1*^{W40C} allele and a heterozygous *SAMSN1*^{T198A} variant (21:14500705 T/C, MAF:0.01, PPN:0.98, rs62227165) (Fig. 5 and Fig. 6). Each variant encoded amino acid changes within important domains of the translated protein (Fig. 7) and all were predicted to be damaging by Polyphen2 (Fig. 7). This implicates *BANK1*^{W40C} in SLE pathogenesis and suggests that a second pathogenic SNV in a gene associated with BANK1 function is required for induction of SLE in this subset of patients.

Table 3. Clinical phenotype of probands and SLE patients with *BANK1*^{W40C} variant

Gender	Birth year	Ethnicity	Renal	MSK-skin	Oral	Vessels	Thrombosis	Serositis	Serology	Complement
Proband A										
Female	1999	Caucasian	No	Tenosynovitis Photosensitive rash	Ulcers	Raynauds	No	No	ANA: 1:1280 Speckled DsDNA negative	Low C4
Proband B										
Female	1971	Caucasian	No	Arthritis Photosensitive rash	Sicca	Raynauds	No	No	ANA> 1:1280 Speckled Lymphopaenia DsDNA negative SS-A positive SS-B negative HGG	Normal
Proband C										
Female	1974	Caucasian	Class III LN	Photosensitive rash	Sicca	Raynauds	No	No	ANA>1:1280 Homogeneous DsDNA (>500iu/L) SS-A positive SS-B negative Anticardiolipin +	Low C3 Low C4
APOP30										
Female	1988	Caucasian	No	Arthritis Photosensitive rash	No	Raynauds	No	No	ANA 1:1280 Speckled DsDNA negative HGG	Low C3 Low C4

Gender	Birth year	Ethnicity	Renal	MSK-skin	Oral	Vessels	Thrombosis	Serositis	Serology	Complement
APOP37										
Male	1923	Caucasian	No	Arthritis	Ulcers	Raynauds	DVT	Pleural	ANA 1:1280 Homogeneous Lymphopaenia DsDNA (<100iu/L) Anticardiolipin +	Low C4
APOP49										
Female	1961	Caucasian	No	Arthritis Photosensitive rash	No	Raynauds	No	No	ANA >1:1280 Homogeneous Lymphopaenia DsDNA (<500iu/L) Anti-RNP HGG	Low C3 Low C4
APOP69										
Female	1957	Caucasian	No	Alopecia	Ulcers Sicca	No	DVT	No	ANA + DsDNA negative Anticardiolipin +	Normal
APOP85										
Female	1974	Caucasian	No	Arthritis Rash	Sicca	Raynauds Seizures	No	No	ANA >1:1280 Homogeneous DsDNA positive SS-A positive SS-B positive	Low C3

Gender	Birth year	Ethnicity	Renal	MSK-skin	Oral	Vessels	Thrombosis	Serositis	Serology	Complement
APOP89										
Female	1939	Caucasian	CKD	Rash	Sicca	No	DVT	No	ANA >1:1280 Homogeneous DsDNA (<100iu/L) Anticardiolipin + SS-A positive SS-B negative	Low C3 Low C4

ANA=Antinuclear antibodies; DsDNA=double stranded DNA antibodies; DVT= deep vein thrombosis; CKD=chronic kidney disease; HGG=Hyperga

mmaglobulinaemia.

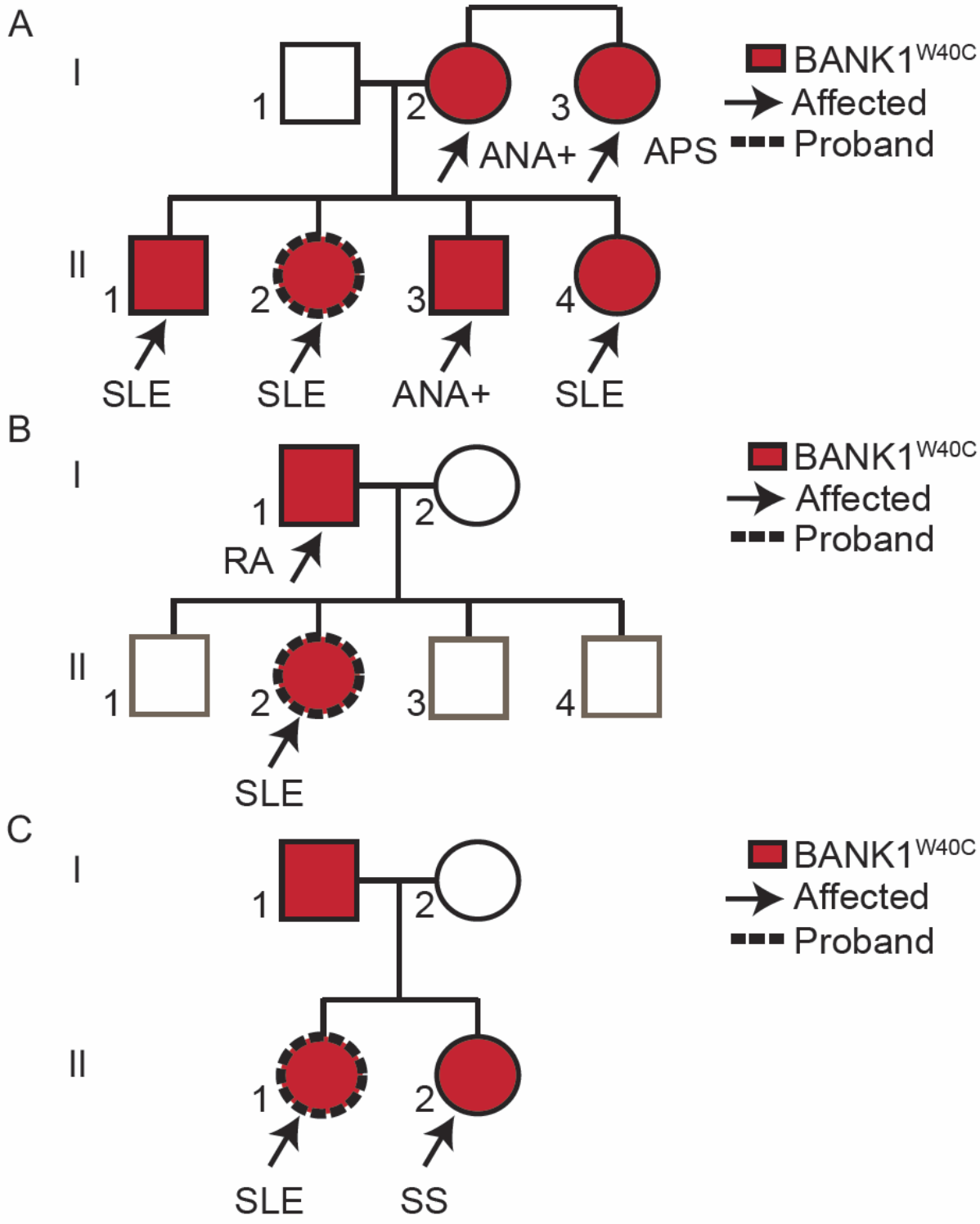


Figure 4. Pedigrees of SLE probands and segregation with BANK1 and associated genotypes. (ANA = antinuclear antibody, SS = Sjogren's Syndrome, SLE = Systemic Lupus Erythematosus, APS = Antiphospholipid Syndrome, RA = Rheumatoid Arthritis)

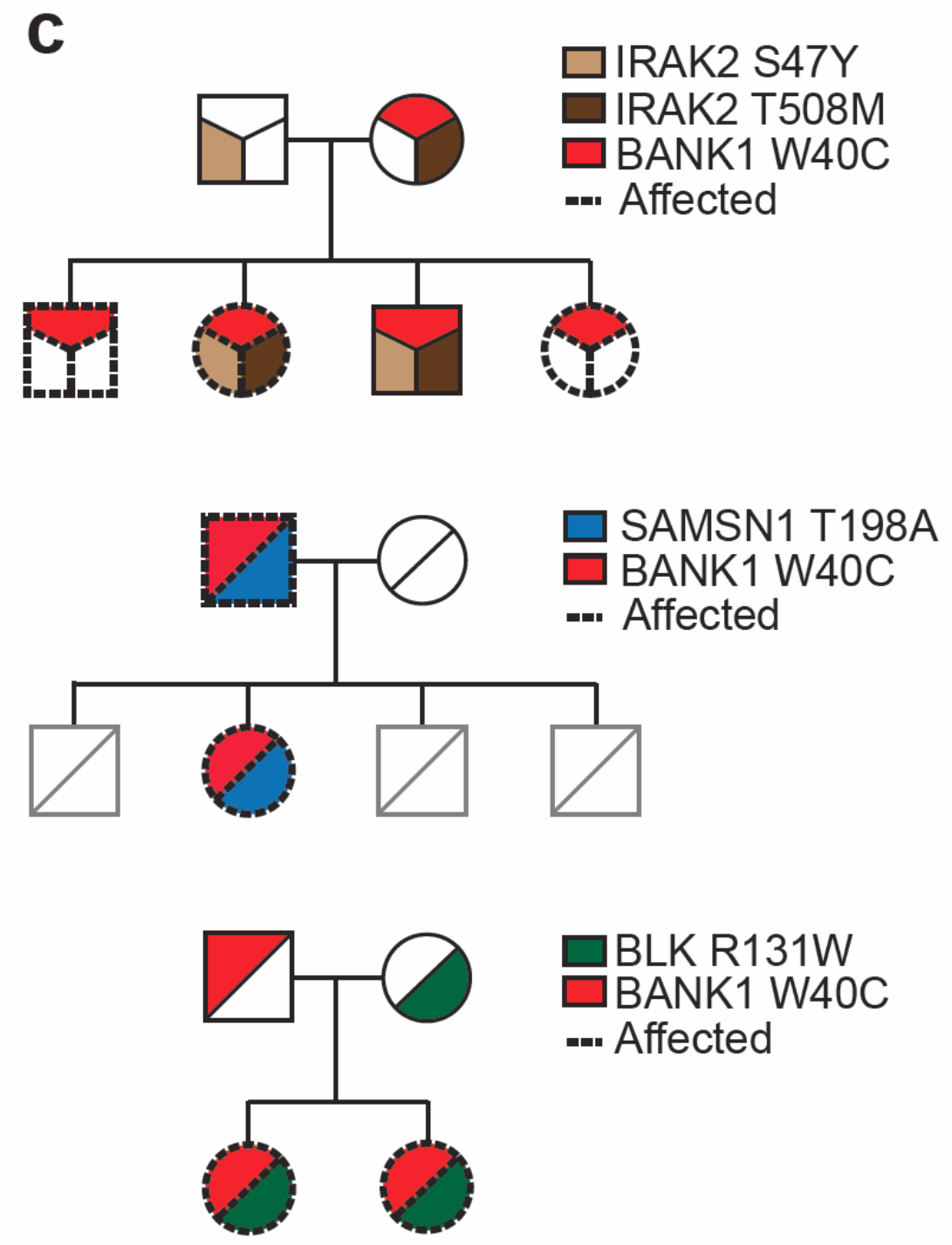


Figure 5. Pedigrees of SLE probands and segregation with BANK1 and second variants

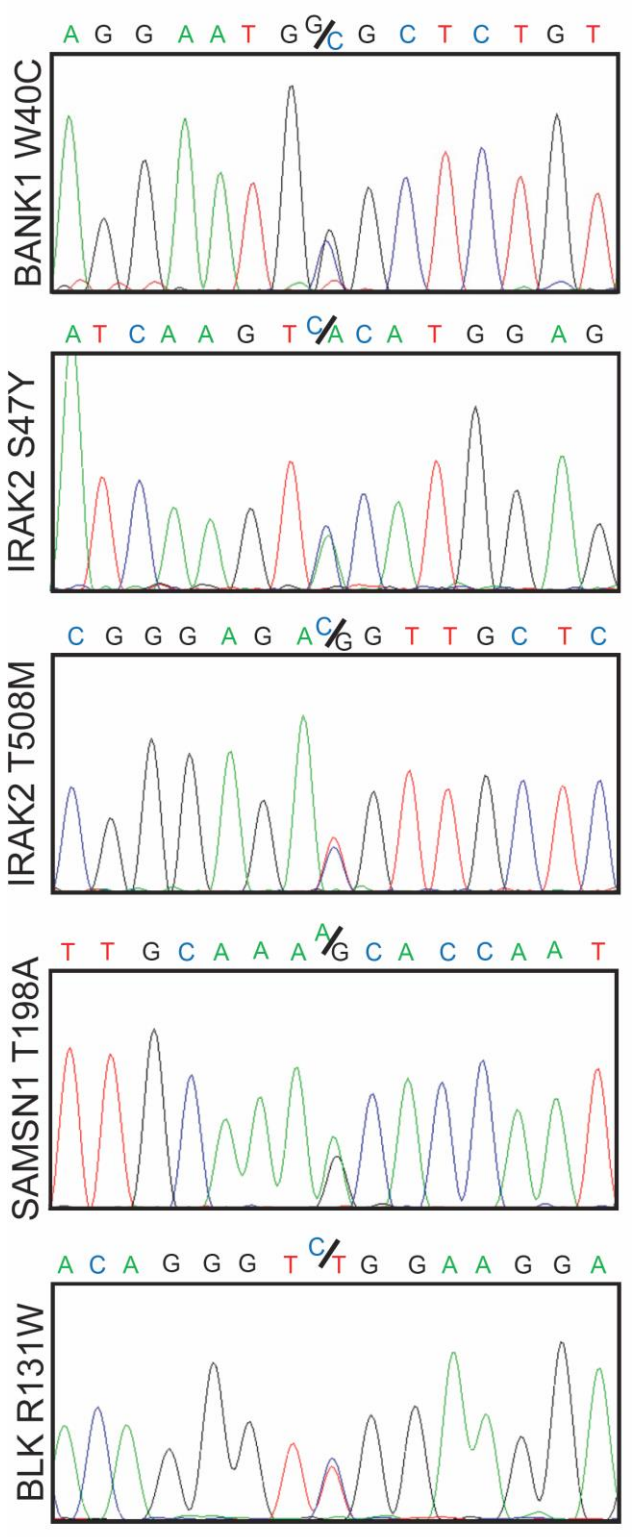


Figure 6. Chromatograms of heterozygous SNVs *BANK1*^{W40C}, *IRAK2*^{S47Y}, *IRAK2*^{T508M}, *BLK*^{R131W}, and *SAMSN1*^{T19A}.

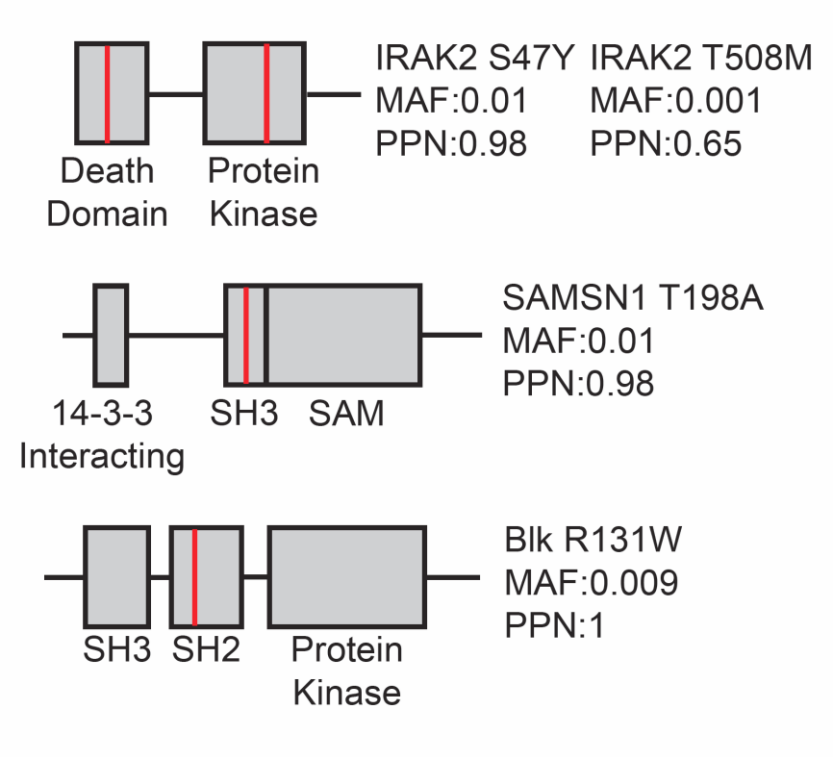


Figure 7. Schematics of protein structures and relevant domains and position of residue changes as a result of identified variants

3.3 Rare gene variants alter protein function

3.3.1 *IRAK2*^{S47Y} and *IRAK2*^{T508M} are gain of function mutations and IRAK2 interacts with BANK1

In addition to *BANK1*^{W40C}, proband A had compound heterozygous novel mutations in *IRAK2*. IRAK2 is an important component of the TLR-MyD88 signalling complex. In response to TLR ligation, IRAK2 forms homo- and hetero-dimers with family members IRAK4 and IRAK1. MyD88 complex formation via TLR ligation results in IRAK family member phosphorylation, with subsequent recruitment of TRAF6 and NF-κB activation [319]. The S47 residue lies in the death domain of IRAK2, which directly interacts with the death domains of other IRAK family members and of MyD88 (PDB 3MOP) (Fig. 8). Modelling of the structure formed by these complexes was performed by J. Babon at the Walter and Eliza Hall Institute of Medical Research, Melbourne University, which identified Serine 47 (Fig. 8) located at the interfaces between the four monomers of the IRAK2 and IRAK4 molecules. In every case there was a cavity between the sub-units immediately adjacent to S47 that could accommodate the larger tyrosine side chain in the IRAK2^{S47Y} mutation with little, if any, re-orientation. Given the more bulky nature of the tyrosine side-chain and the fact that it contains a potential hydrogen bond donor moiety at its distal end, we predicted this mutation may stabilise the overall complex. To test this hypothesis, we used SDM and generated plasmids expressing the *IRAK2*^{S47Y} and *IRAK2*^{T508M} variants. Both variants were tested using LUMIER interactions performed in the lab of A. Weber, University of Tuebingen, Germany, and displayed increased binding affinity for wild type IRAK2, IRAK4 and TRAF6 suggesting increased recruitment to the MyD88 signalling complex (Fig. 9). Our collaborators at the Weber lab then tested whether the enhanced interaction resulted in a change in TRAF6-mediated NF-κB activation and observed that both *IRAK2* variants increased NF-κB transcriptional activity, an effect increased by the combination of these variants (Fig. 10). *BANK1* has also been implicated in negative regulation of TLR7/8 and TLR9 signalling and

postulated to interact with MyD88 through its putative TIR domain [320]. We therefore hypothesized that BANK1 and IRAK2 may coexist as part of the MyD88 complex. To test this, *BANK1* and *IRAK2* were expressed in HEK293T cell lines and co-immunoprecipitation analyses demonstrated a novel physical interaction between the two proteins (Fig. 11), which was not perturbed by the BANK1^{W40C} variant. In summary, BANK1 forms a complex with IRAK2, and proband A is heterozygous for two gain of function mutations in *IRAK2* which increase interaction with other IRAK family members and TRAF6 resulting in increased NF-κB signalling.

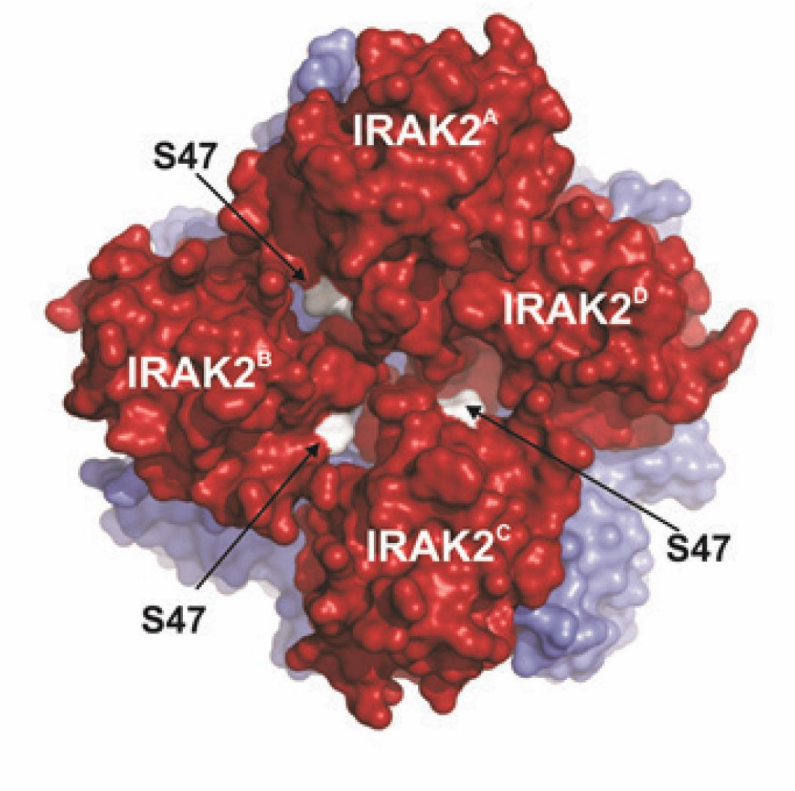


Figure 8. Structure of death domains of IRAK2 (red) in complex with IRAK4 (blue) (PDB 3MOP). The death domains of the two proteins form a left-handed helical super-assembly with the death domains of MyD88 (not shown). Four copies of both IRAK2 and IRAK4 are present (labelled A-D above). In three of the four monomers, Serine 47 (white) is visible in this orientation and is located at an IRAK2:IRAK2 interface whilst in the fourth it is located at the IRAK4:IRAK2 interface (not shown). Note the cavity between the sub-units immediately adjacent to each S47, indicating the importance of the serine at position 47 as a site for docking within the TLR signalasome Modelling by J. Babon.

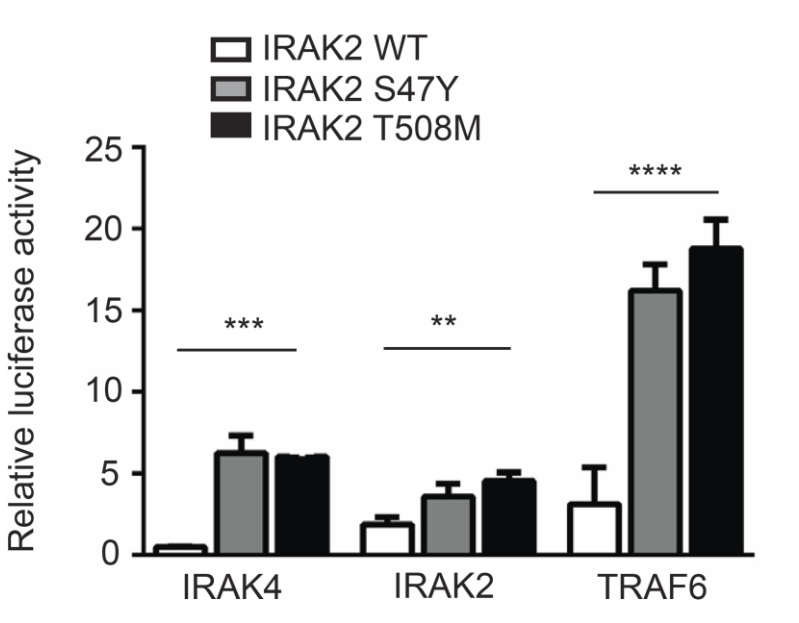


Figure 9. LUminescence-based Mammalian IntERactome (LUMIER) quantifying the interaction between IRAK2 signalling partners (IRAK4, IRAK2 and TRAF6) with IRAK2^{S47Y} and IRAK2^{T508M}. Luciferase intensity indicates strength of interaction between proteins on the x-axis and the variants in IRAK2 as indicated. Each condition is the median of 3 replicates. ** = p<0.01, **=p<0.001, ****=p<0.0001. LUMIER performed by A. Weber.

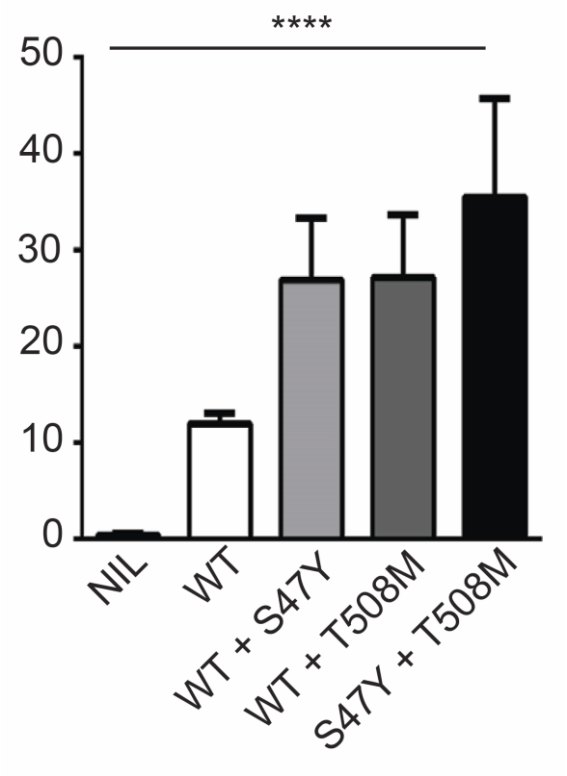


Figure 10. Dual Luciferase reporter assays to quantify NF-κB activation in response to IRAK2 variants. Bars represent median of triplicates for each condition. Representative of 3 repeat experiments (p=0.002) ELISA by A. Weber.

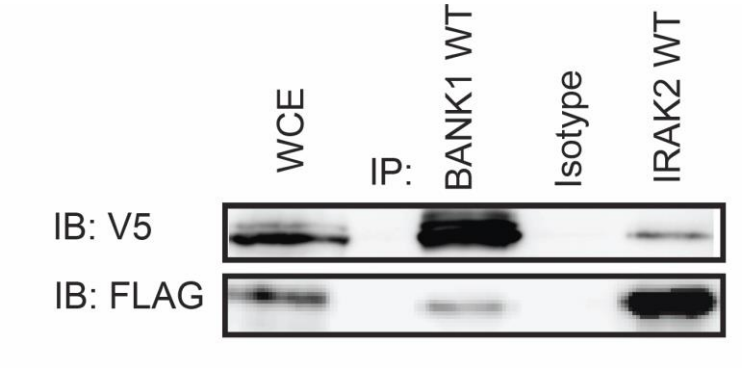


Figure 11. Co-immunoprecipitation of BANK1 and IRAK2 HEK293T lysates 48 hours after co-transfection, blotted with antibodies against IRAK2 and BANK1 demonstrate a two-way interaction between the proteins. Representative of at least 3 separate experiments

3.3.2 *BLK*^{R131W} is kinase inactive

We next set out to test whether the identified variants altered protein function. BANK1 was initially identified as a substrate of Lyn [321] and is known to interact with the Src-family member BLK after BCR crosslinking [322]. In proband B, in addition to the *BANK1* variant we identified a rare heterozygous mutation in *BLK* (*BLK*^{R131W} rs144615291). As with *BANK1*, a common *BLK* variant, rs13277113, has been shown to segregate with SLE by GWAS [298,323]. CD79a and CD79b, which form part of the BCR complex, phosphorylate BLK C-terminal tyrosine residues after BCR crosslinking [324] altering the secondary structure to expose the catalytic domain responsible for the phosphorylation of BANK1 [299]. Therefore, BANK1 is directly linked to BCR signalling through interaction with BLK and Lyn. BLK kinase activity depends on interactions between SH2 domain arginine residues and C-terminal phospho-tyrosines [325]. Arginine 131 is located on Helix αA (αA2), as modelled by J Babon, and is known to co-ordinate phosphorylated tyrosine residues in a number of SH2 domain:ligand structures alongside the conserved arginine at αB5 (Fig. 12). This co-ordination will be disrupted in the presence of the R131W change. To test whether *BLK*^{R131W} alters the conformation and kinase activity of BLK, we used site-directed mutagenesis (SDM) to generate cDNAs encoding the *BLK*^{R131W} variant as well as a constitutively-active *BLK*^{Y501F} variant [326]. When expressed in HEK293T cells, BLK^{R131W} was unable to adopt the high molecular weight kinase active form observed consistently with the BLK^{Y501F} variant and also present in WT BLK (Fig. 13), suggesting that BLK^{R131W} is kinase inactive.

As BANK1 is a known substrate of BLK and BLK is known to require activation through the BCR to phosphorylate BANK1, we predicted *BLK*^{R131W} would impair phosphorylation of BANK1. When co-expressed in HEK293T cells we confirmed that BLK^{R131W} had significantly impaired ability to phosphorylate BANK1 compared with wild-type BLK or BLK^{Y501F} (Fig. 14). In summary, BLK^{R131W} is unable to acquire an open active form and is therefore unable to phosphorylate BANK1.

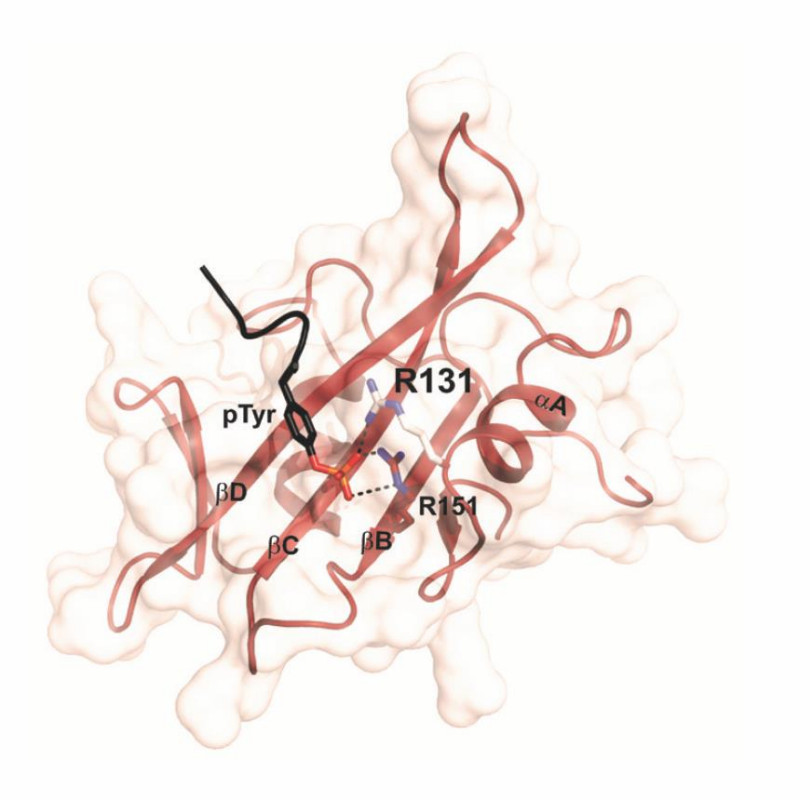


Figure 12. Predicted BLK Arginine 131 (shown in white stick representation) is located on Helix αA (αA2) and is known to co-ordinate phosphorylated tyrosine residues in a number of SH2 domain:ligand structures alongside the conserved arginine at αB5 (R151 in BLK, red stick representation). Modelling by J. Babon.

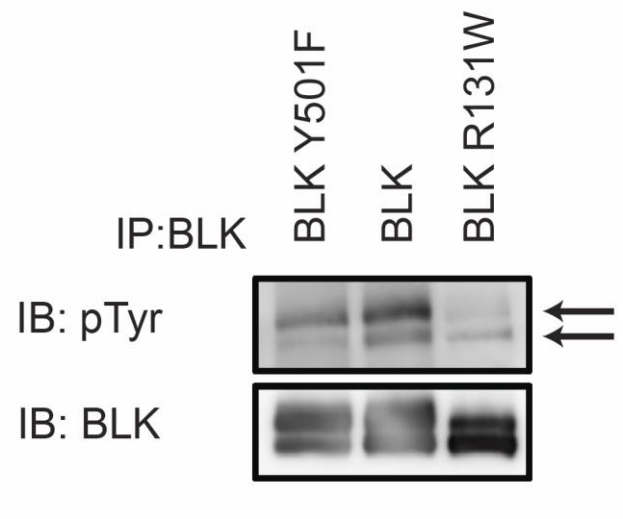


Figure 13. α-phosphotyrosine blotting of expressed BLK^{WT}, BLK^{Y501F} and BLK^{R131W} 48 hours after transfection with 2ug of indicated DNA. Arrows indicate a high and low molecular bands correlating with phosphorylation fo BLK. The lower molecular weight band corresponds to the inactive form (closed configuration due to lack of phosphorylation). Representative of 3 repeat experiments

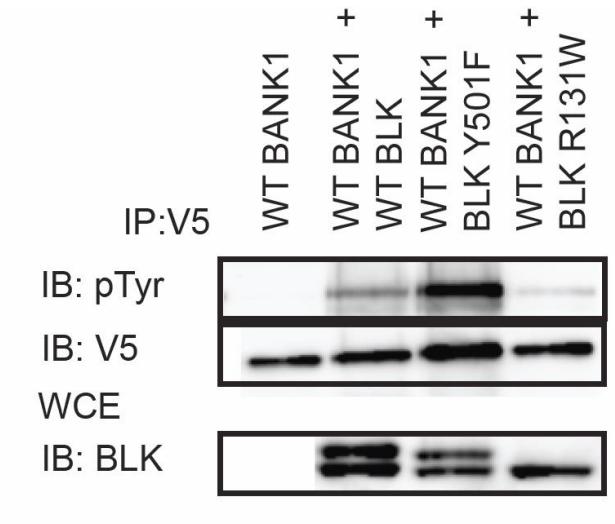


Figure 14. α-phosphotyrosine blotting of precipitated BANK1 72hours after co-transfection with BLK of indicated genotypes. Immunoblotting of pTyr demonstrates impaired phosphorylation of BANK1 by BLK^{R131W}, but appropriately increased phosphorylation by BLK^{Y501F}. Representative of 3 repeat experiments

3.3.3 SAMSN1 is a substrate of BLK and BLK^{R131W} is unable to phosphorylate SAMSN1

Proband C carries *BANK1*^{W40C} allele and a rare heterozygous *SAMSN1*^{T198A} variant (rs62227165). *SAMSN1* has been implicated in the negative regulation of BCR signalling [327] as *Samsn1*$^{-/-}$ mice have enhanced B responses and impaired repression of Lyn activation [327], a Src family member critical in BCR and BANK1 signalling [328]. We therefore hypothesized that a *SAMSN1* loss-of-function mutation would compound BANK1-associated changes to B cell responses through changes in Src family signalling. The T198 residue resides in an SH3 domain and may be necessary to stabilize the conformation of the SAMSN1 n-Src loop (Fig. 15). We tested whether the *SAMSN1*^{T198A} variant altered the ability of SAMSN1 to repress Lyn activation. SAMSN1^{T198A} did not change LYN phosphorylation (Fig. 16). Given the absence of data regarding the function of *SAMSN1*, we therefore could not observe a functional effect of the *SAMSN1*^{T198A} variant. However, we found that LYN was capable of phosphorylating SAMSN1 by co-expressing an SDM-generated constitutively active version of *LYN*, *LYN*^{Y501F}, with *SAMSN1*^{WT} (Fig. 17). The Src-family kinases LYN, BLK and LCK are known to share several substrates. We thus hypothesized that BLK would also be capable of phosphorylating SAMSN1. Indeed, co-expression of *BLK* and *SAMSN1* in HEK239T cells revealed that SAMSN1 is also a substrate of BLK (Fig 18). We then tested whether BLK^{R131W}would, as with BANK1, be unable to phosphorylate SAMSN1. As predicted, when co-expressed, BLK^{R131W} was unable to phosphorylate SAMSN1 (Fig 18). Although the consequence of the SAMSN1^{T198A} variant on normal SAMSN1 function is unclear, these results identify SAMSN1 as a novel substrate of Lyn and BLK and demonstrate that the *BLK*^{R131W} variant disrupts this interaction. Therefore, SAMSN1 is involved in the post-BCR BLK signalling pathway.

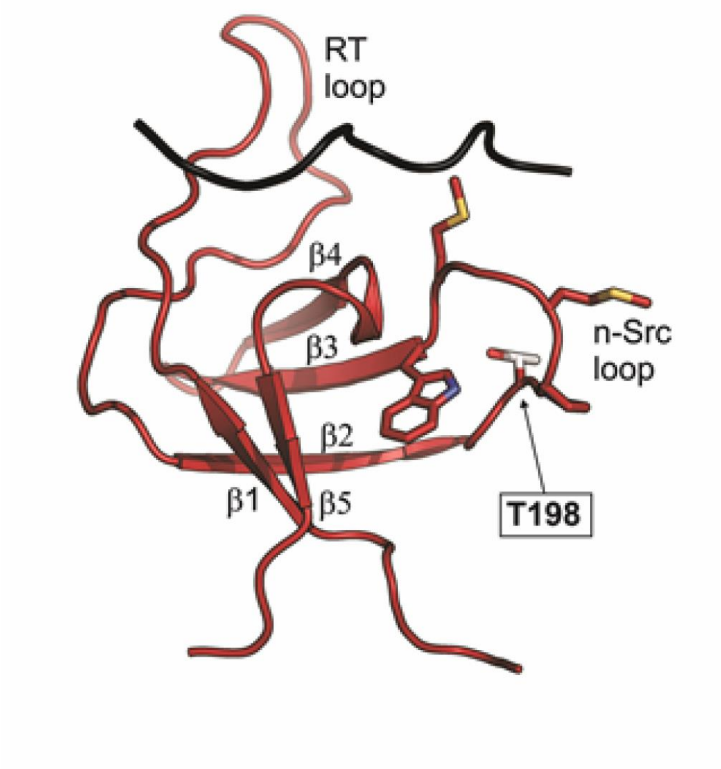


Figure 15. Structure of the wild-type SAMSN1 SH3 domain (PDB 2KEA) shown with a bound ligand modelled (from PDB 1CKA). Threonine 198 (shown in white stick representation) is located on the n-Src loop. Residues at the distal end of this loop form part of the ligand-binding pocket in a number of SH3 domains. Modelled by J. Babon.

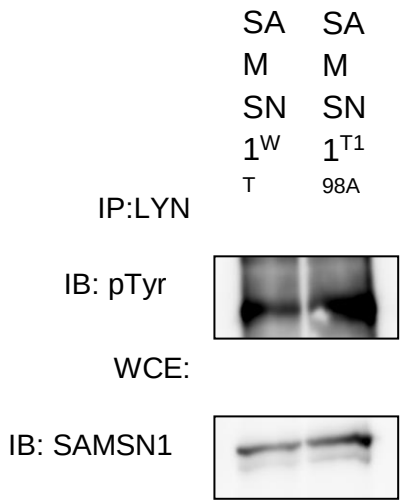


Figure 16. α-phosphotyrosine blot of Lyn co-transfected with *SAMSN1*^{WT} or *SAMSN1*^{T198A}. Lyn was cotransfected in HEK293Ts with indicated SAMSN1 genotype and Lyn was precipitated 48 hours after transfection. There is no appreciable difference in Lyn phosphorylation in the presence of SAMSN1^{WT} or SAMSN1$^{T198A.}$ Representative of 3 repeat experiments.

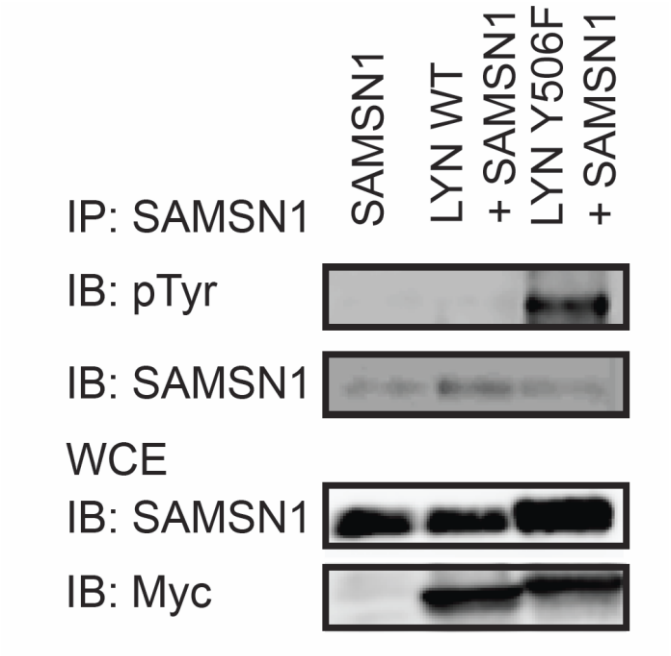


Figure 17. α-phosphotyrosine blotting of SAMSN1 immunoprecipitated 48 hours after cotransfection of HEK293Ts with LYN. Precipitated LYN is phosphorylated by SAMSN1 demonstrating that SAMSN1 is a substrate of LYN. Representative of 3 repeat experiments

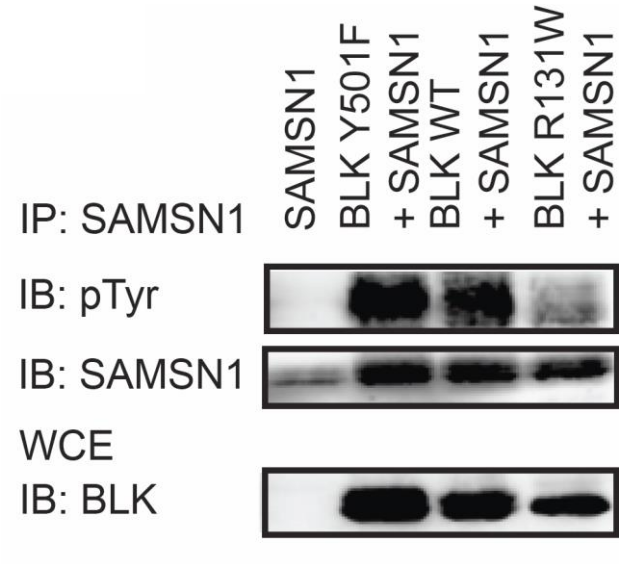


Figure 18. α-phosphotyrosine blotting of immunoprecipitated SAMSN1 after co-transfection with *BLK*^{WT} or *BLK*^{R131W}. 2ug of BLK and SAMSN1 were transfected in HEK293Ts and cells were lysed at 48hours. SAMSN1 was immunoprecipitated and probed with α-phosphotyrosine demonstrating that SAMSN1 is a substrate of BLK and BLK^{R131W} is unable to phosphorylate SAMSN1. Representative of 2 repeat experiments

3.3.4 BANK1W40C alters cellular localisation

BANK1 is known to localize with BLK and exon 2 deletions alter BANK1 localization in the cytoplasm [299]. Transfection of HEK293 cells with $BANK^{WT}$ or $BANK1^{W40C}$ in the presence of TRAF6 revealed that both $BANK1^{WT}$ and $BANK1^{W40C}$ accumulated in large intracytoplasmic aggregates (Fig. 19) but these aggregates were significantly decreased in $BANK1^{W40C}$ transfected cells (Fig. 20). These aggregates were found to be p62+ sequestrosomes (Fig. 21) and contain TRAF6, thought to be inactive in these compartments. It is thus possible that these sequestrosomes also contain inactive forms of BANK1, given that for BANK1 to be active it needs to localize in proximity to the membrane where the Src/BANK1 signalosome forms [299]. Furthermore, an important role of BANK1 may be to sequester TRAF6 in these compartments, upon certain types of BCR/TLR signalling or in cells during particular stages of development.

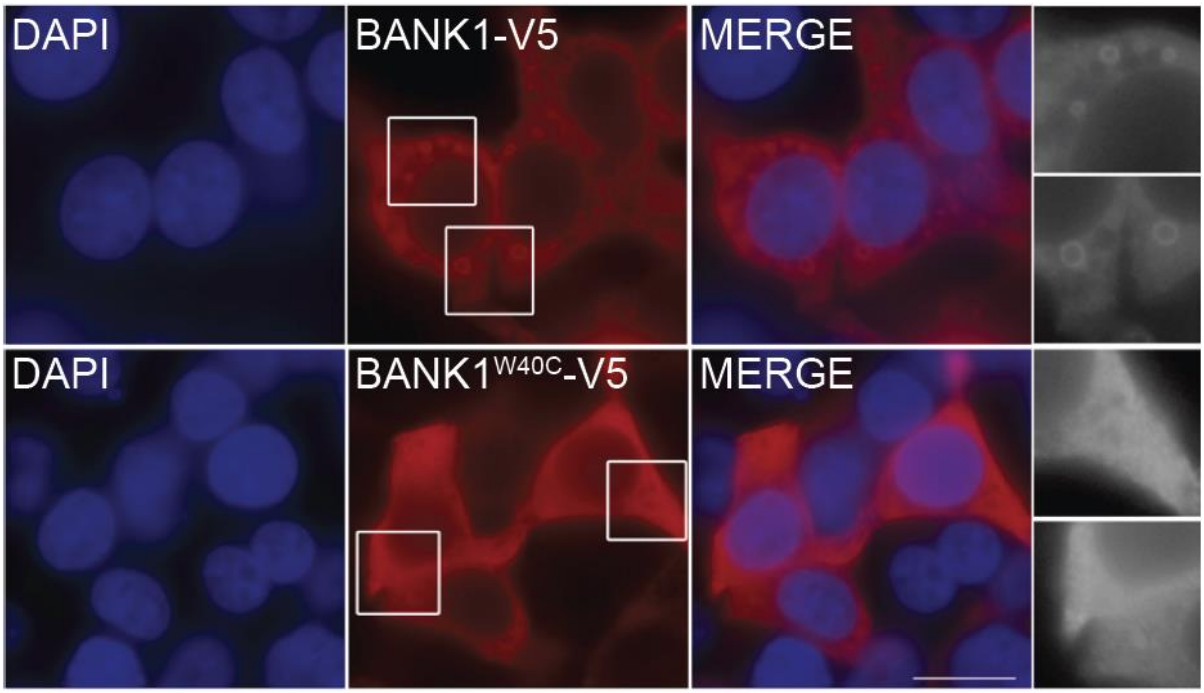


Figure 19. Representative blots of intracellular localization of BANK1^{WT} and BANK1^{W40C} assessed blind to genotype at 72hrs after cotransfection with TRAF6 into HEK293T cells demonstrating vesicle formation or cytoplasmic patterns, respectively (Performed by V. Athanasopoulos). Representative of 3 repeat experiments

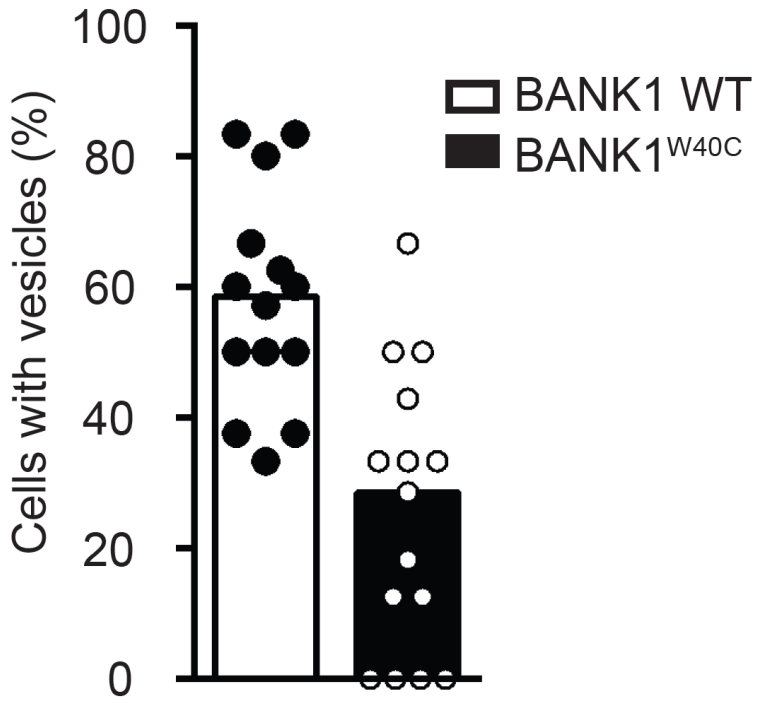


Figure 20. Quantification of cytoplasmic vesicle formation in HEK293T transfected with 3ug of *BANK1*^{WT} or *BANK1*^{W40C} DNA. Measurement of vescicles was performed blind to genotype at 72hours after transfection. Performed by V. Athansopoulos. Date representative of 3 separate experiments. P<0.001.

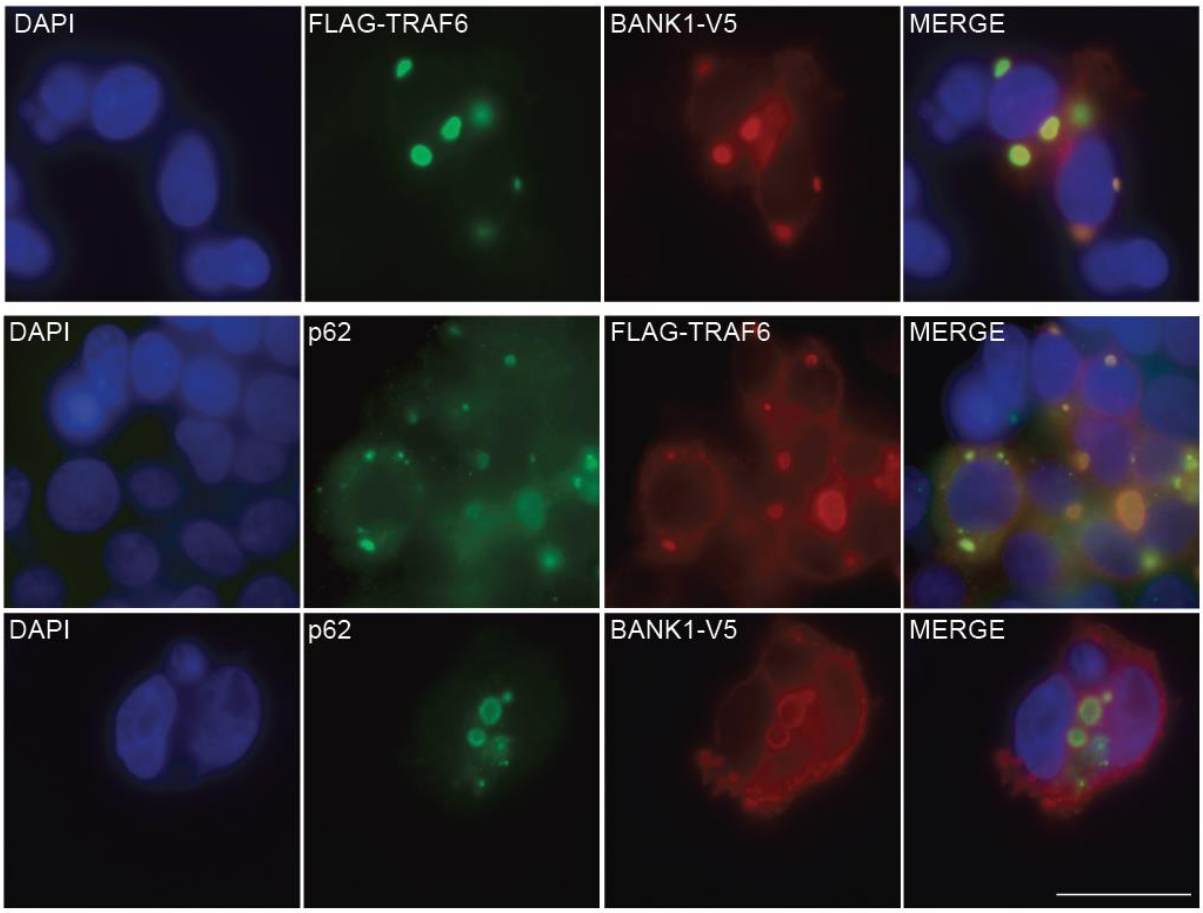
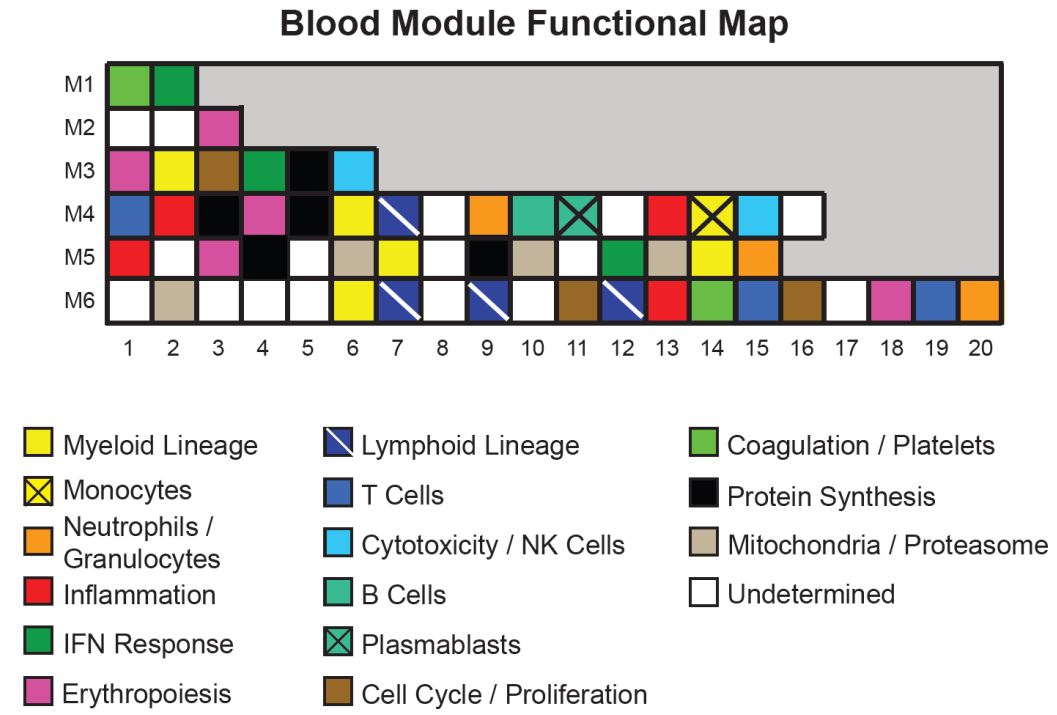


Figure 21. Representative images of intracellular colocalization of BANK1^{WT} with TRAF6 and p62 in cotransfected HEK293T cells (Performed by V. Athanasopoulos) Representative of 2 repeat experiments.

3.4 Rare gene variants alter cellular physiology

3.4.1 PBMCs from the *BANK1*^{W40C} SLE patients have unique transcriptome

To gain insight into the effect of the *BANK1*^{W40C} SNV on global leukocyte function, we performed transcriptome analysis of peripheral blood mononuclear cells (PBMCs) from the probands. Frozen PBMCs were sent to collaborators in the laboratory of V. Pascual, Baylor Medical Institute, U.S., who performed RNA microarrays and analysed transcriptomes with reference to a large cohort of unselected paediatric and adult SLE patients (Fig. 22 and Fig 23). Strikingly, PBMCS from *BANK1*^{W40C} patients were notable for the absence of type-1 interferon signatures (Fig. 23). Interestingly, genes that were prominently associated with probands with *BANK1*^{W40C} correlated with plasma cell signatures suggesting a contribution of the variant to increased plasma cell responses. An important caveat to this observation is that the PBMCs from both the referent SLE cohort were not analysed and differences in lymphocyte frequency and treatment responses may affect the transcriptome response. Prominent type-1 interferon signatures are characteristic of the majority of SLE patients[329] (Fig. 24). RNA analysis demonstrated significant upregulation of IL-6, IL-10 and genes regulated by NF-kB (Fig. 25).



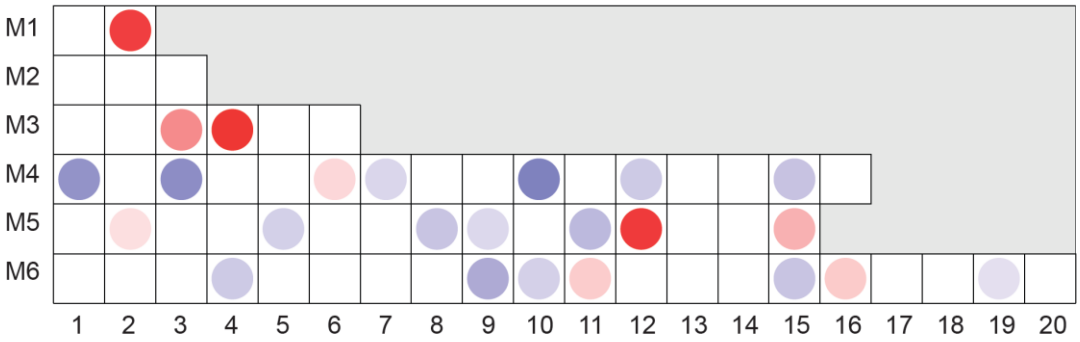


Figure 22. Identification of three type-1 IFN response modules (i= M1.2; ii= M3.4; iii = M5.12 Supp.) in transcriptomes from unselected SLE patients. The top figure indicates the designed map which groups gene profiles into patterns of indicated cellular responses/functions. The bottom figure represents an example of the gene expression map from an unselected SLE patient showing the common upregulation of IFN responses in red. (Blood Module Functional Map designed and provided by V. Pascual)

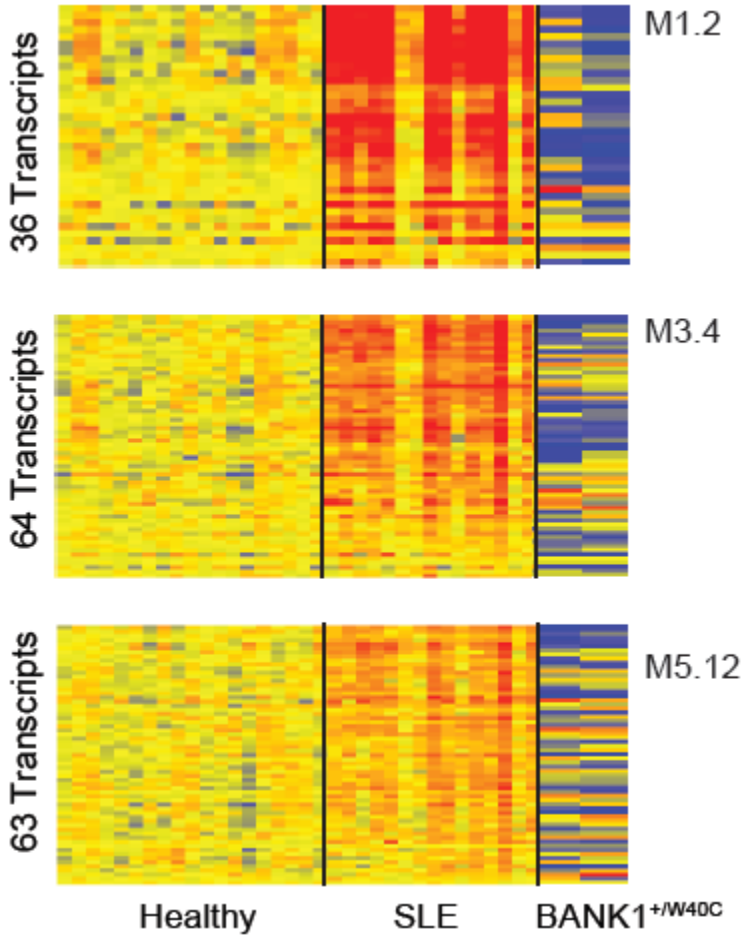


Figure 23. Expression of three type-1 IFN response modules identified in Figure 22 (i= M1.2; ii= M3.4; iii = M5.12.) are abundant in a large majority of early onset SLE patients compared with PBMCs from *BANK1*^{W40C} probands B and C. (Performed by V. Pascual)

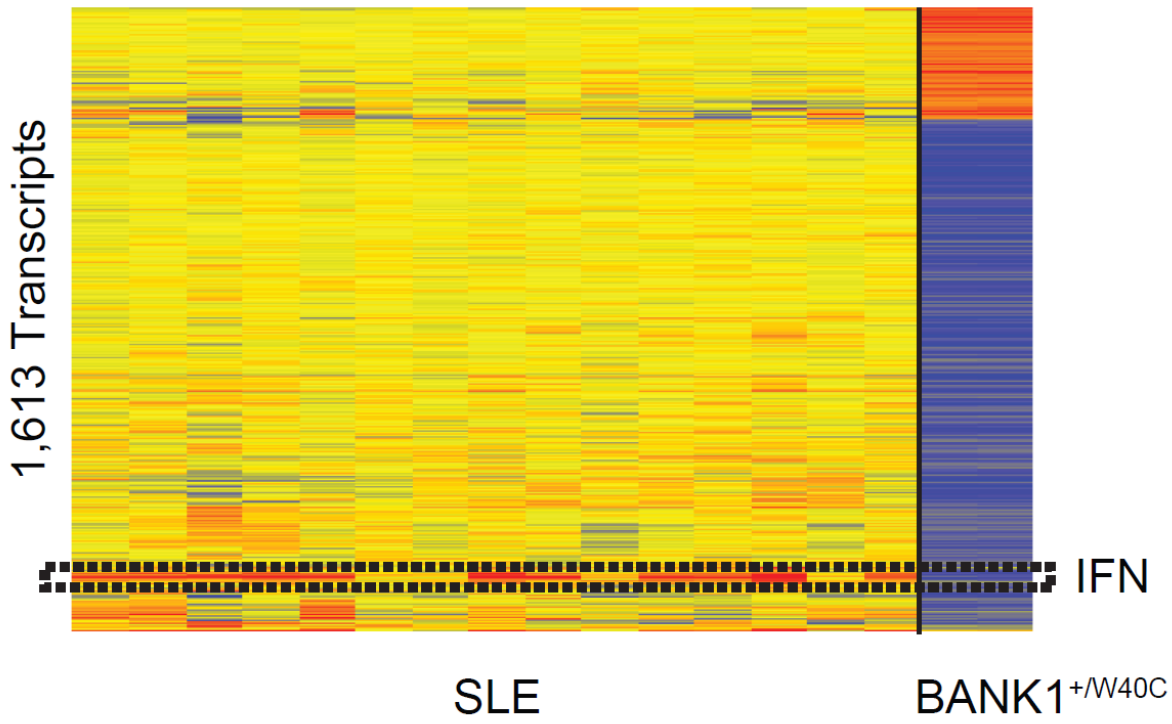


Figure 24. Hierarchical cluster analysis of type-1 IFN genes demonstrates downregulation of type-1 IFN responses in *BANK1*^{W40C} probands' PBMCs global transcriptome profile and indentification of a cluster of upregulated genes associated with plasma blast reponses common to *BANK1*^{W40C} probands. (Performed by V. Pascual)

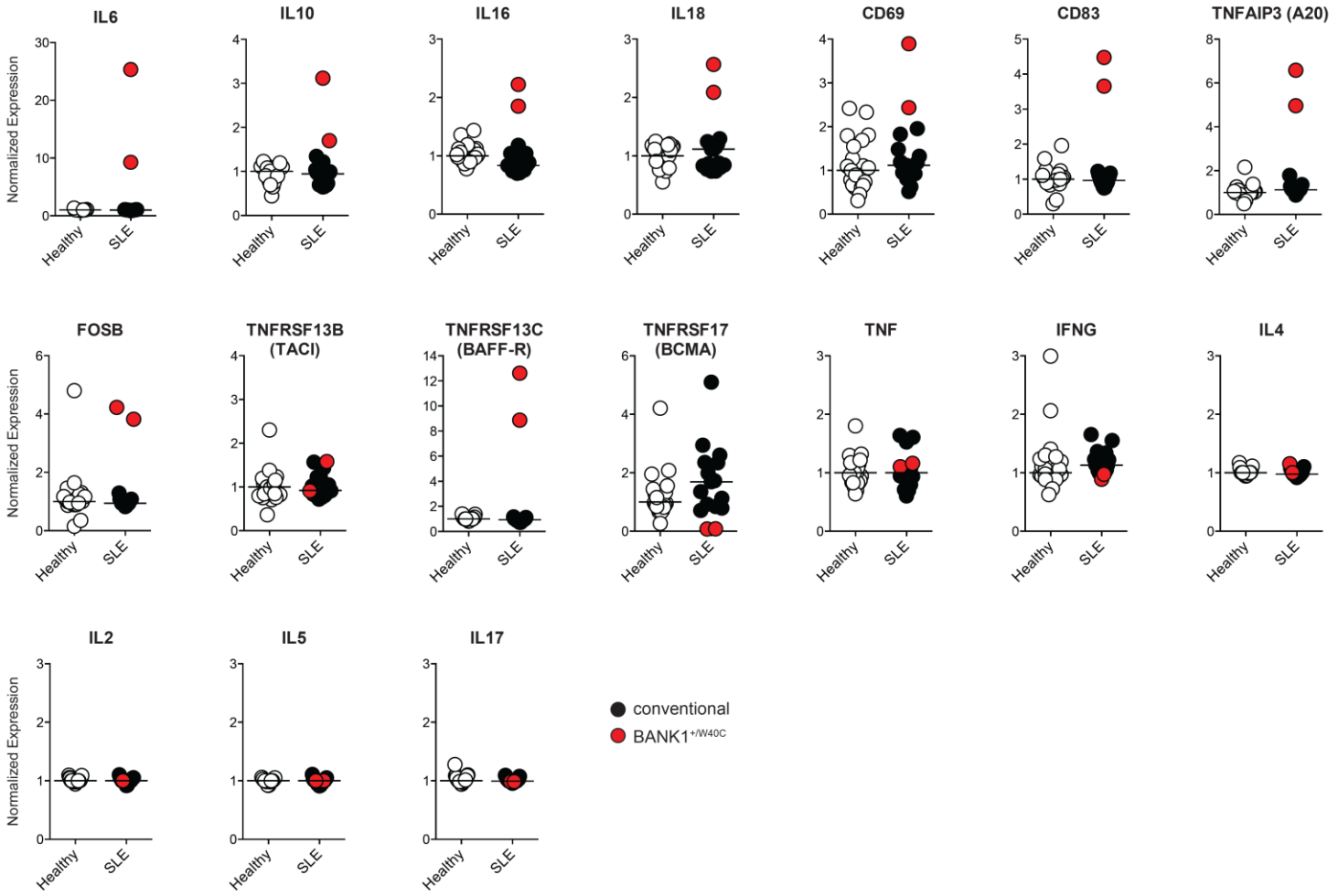


Figure 25. Upregulation of NF-κB-associated genes in *BANK*^{W40C} PBMCs compared with unselected SLE patients and heathly controls.

(Performed by V. Pascual)

3.4.2 Rare variants lead to increased IL-6 and IL-10 production

In order to investigate B cell-autonomous effects of the mutations, lymphoblastoid B cell lines (LCLs) were generated from PBMCs from probands A, B and C and from healthy controls by collaborators in the laboratory of R. Khanna, Queensland Institute of Medical Research, Australia. LCLs were derived from proband B cells given the initial difficulties obtaining blood from affected individuals and the differences in treatment between the probands. Persistent activation with EBV will result in consistent non-canonical NFkB activation and the potential to therefore enhance and TLR response. Given the actions of *BANK1* and *IRAK2* in TLR signalling and *SAMSN1* and *BLK* in BCR signalling, we tested the effect of TLR7/8, TLR9 and BCR stimulation on IL-6 production, since this cytokine had previously been shown to be regulated by BANK1[320] and the probands with the $BANK1^{W40C}$ variant had increased IL-6 mRNA levels (Fig 25). All LCLs derived from probands carrying the $BANK1^{W40C}$ SNV had significantly increased IL-6 production at 72 hours after stimulation with TLR7/8 agonist R848, a trend to increased IL-6 production after TLR9 agonist CpG, but not with LPS or BCR stimulation (Fig. 26). These LCLs also showed a trend towards higher IL-10 production (Fig. 26). IL-6 and IL-10 contribute to plasma cell differentiation and both cytokines have been found to be elevated in the serum of SLE patients[330].

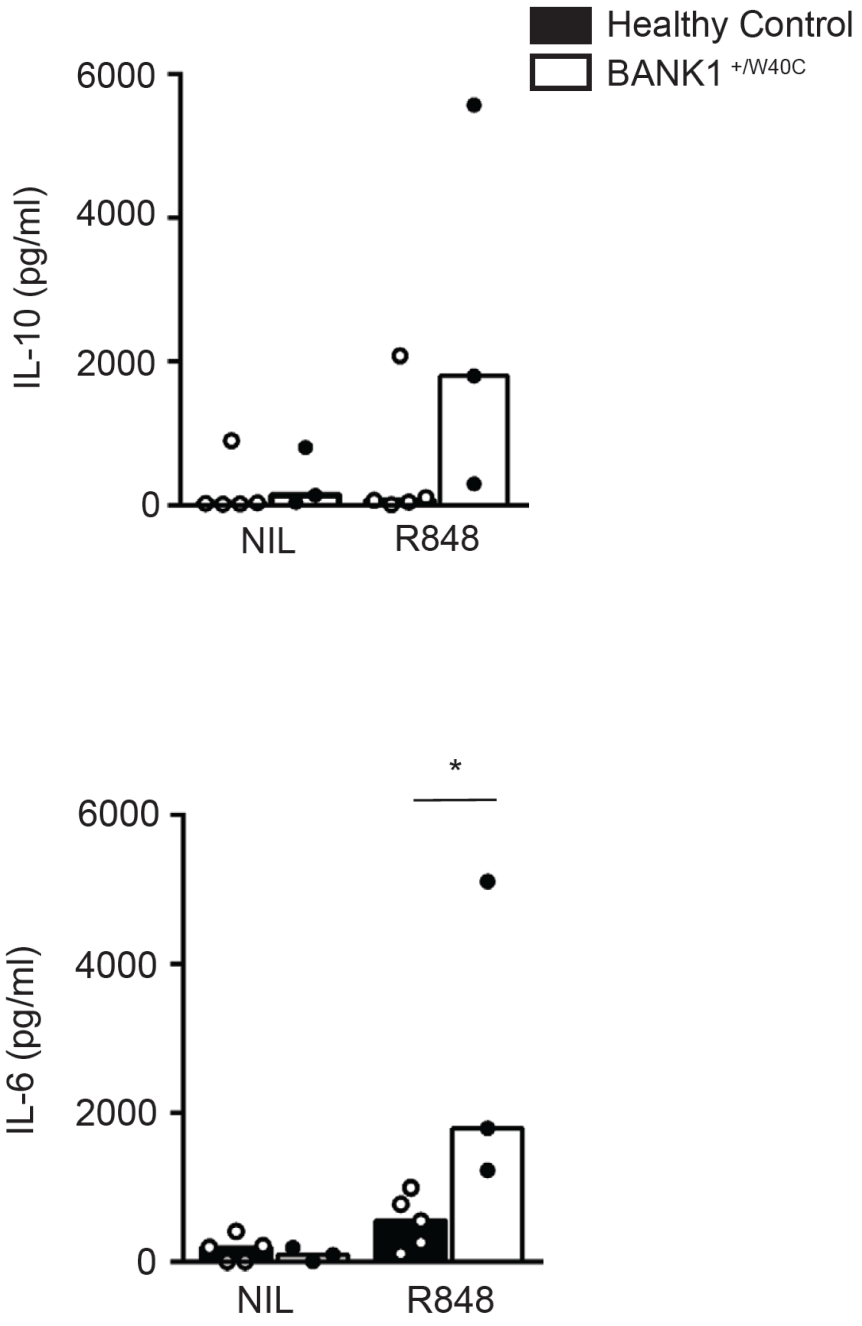


Figure 26. IL-6 (a) and IL-10 (b) secretion by 2.5x 10^5 LCLs derived from *BANK1*^{W40C} probands compared with healthy controls 72 hours after stimulation with R848. Each point represents LCLs derived from a single BANK1 proband. Representive of 3 replicated experiments. *=p<0.05

TLR-ligation results in NF-κB expression and production of proinflammatory cytokines including IL-6. Both identified mutations in *IRAK2* are gain of function variants and the proband PBMCs exhibited elevated levels of IL-6 mRNA transcription. Therefore, we hypothesized that the *IRAK2* mutations would enhance TLR-mediated IL-6 production similar to that observed in the LCLs. HEK293T cells expressing TLR9 were transfected with wild type *IRAK2, IRAK2*^{S47Y} and *IRAK2*^{T508M} and stimulated with CpG. As expected, at 72 hours post stimulation IL-6 production was increased by both variants compared with wild type IRAK2 (Fig. 27).

Next we tested whether the *SAMSN1* SNV could contribute to the TLR mediated IL-6 and IL-10 response observed in the probands' LCLs. For this, we introduced the heterozygous *SAMSN1*^{T198A} variant into the Ramos cell lines using CRISPR-Cas9 DNA editing. Surprisingly, this *SAMSN1*$^{T198A/+}$ B cell line exhibited a significant increase in IL-10 production in response to CpG stimulation, but not R848 (Fig. 28). Thus, SAMSN1$^{T198A/+}$ confers a selective increased IL-10 response to CpG stimulation.

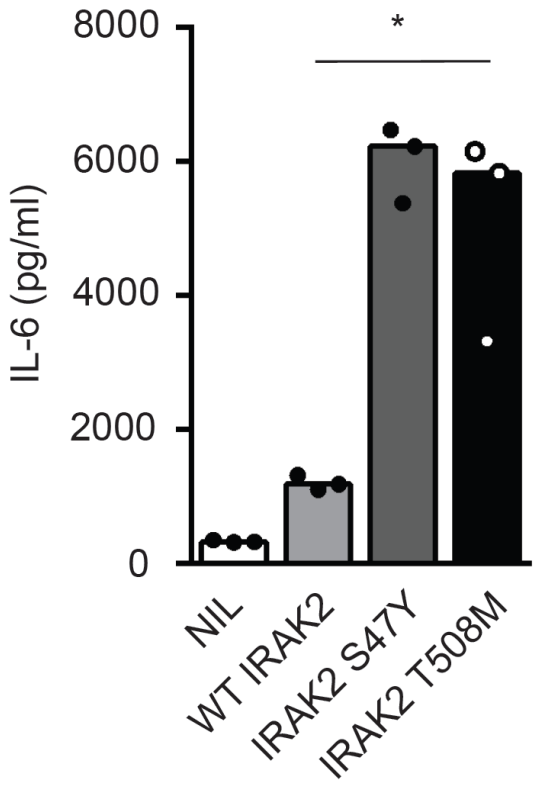


Figure 27. IL-6 production is increased in TLR9-expressing HEK293Ts transfected with *IRAK2* variants. 2.5 x 10^5 HEK293Ts were transfected with indicated plasmids and 24 hours later stimulated with CpG. Supernatant was collect 48 hours post transfection. Representative of 2 separate experiments.

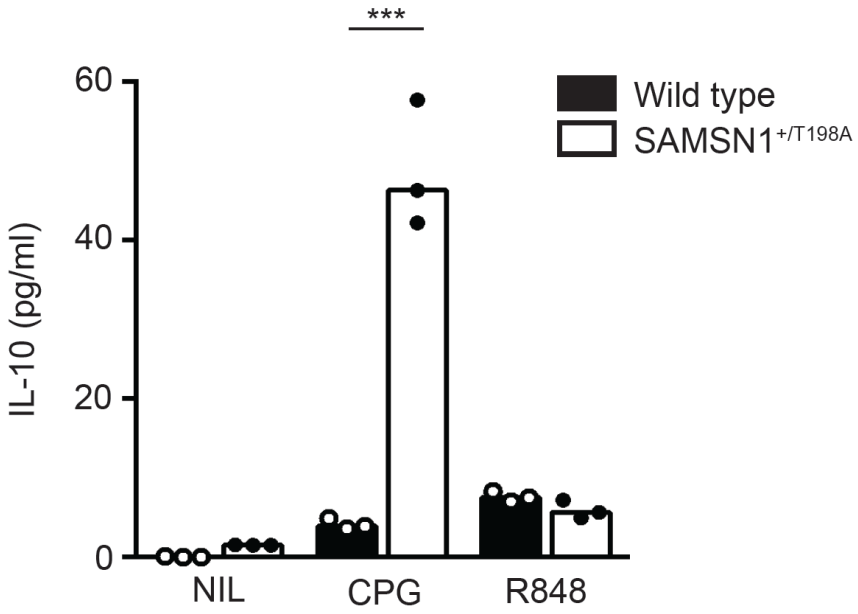


Figure 28. IL-10 production by CRISPR-Cas9 engineered *SAMSN1*$^{T198A/+}$ or WT human B cells (Ramos) 72 hours after CpG and R848 stimulation. Representative of 2 repeat experiments. ***=p<0.001

3.5 Mice with synonymous human rare variants develop lupus

To test the *in vivo* effects of the *BANK1*, *BLK* and *SAMSN1* variants, we created C57BL/6 mice bearing the *BANK1*^{W40C}, *BLK*^{R131W} and *SAMSN1*^{T198A} variants using CRISPR-Cas9 editing. Immunophenotyping of these mouse strains revealed no differences in B or T cell subsets (Fig. 29).

We next investigated the presence of antinuclear antibodies (ANAs). Strikingly, serum from 16 week old *BANK1*$^{W40C/+}$, *SAMSN1*$^{T198A/+}$, and *BANK1*$^{W40C/+}$*BLK*$^{R131W/+}$ mice contained anti-nuclear antibodies (ANAs) whereas serum from WT littermates did not (Fig. 30). Kidneys from the *BANK1*$^{W40C/+}$ and *BANK1*$^{W40C/+}$*BLK*$^{R131W/+}$ CRISPR-Cas9 engineered mice also were tested by I. Papa, JCSMR, ANU, for nephritis and exhibited IgG and IgA deposition (Fig. 31). Taken together, generation of orthologous SNV in mice demonstrates that the rare heterozygous mutations found in human SLE patients induce abnormal B cell responses similar to those observed in patient-derived B cells and also cause end-organ (kidney) involvement.

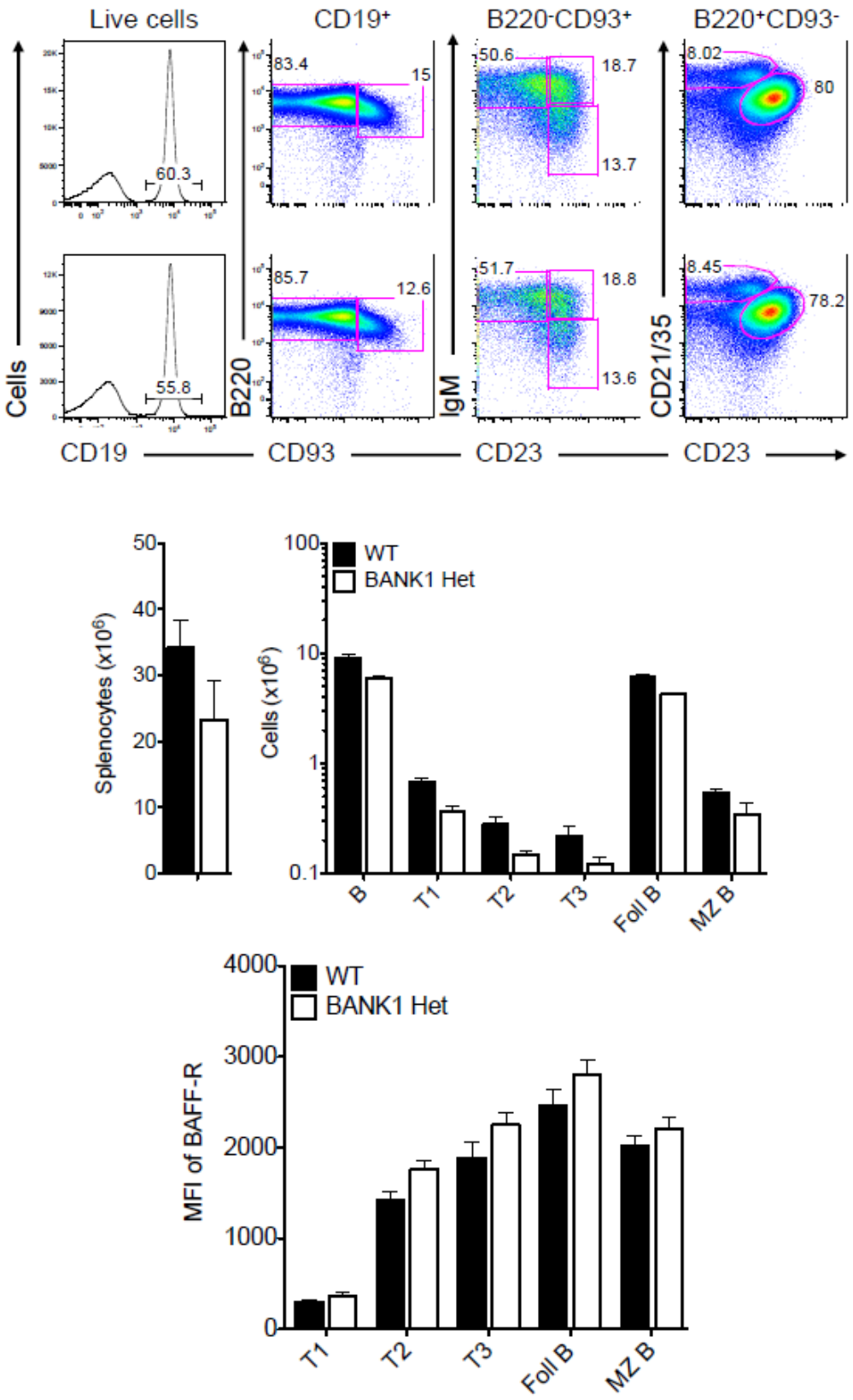


Figure 29. Phenotyping of splenocyte B cell populations in 2 month old WT and *Bank1*$^{+/W40C}$ mice. Performed by M Yabas. Data representative of 2 separate experiments.

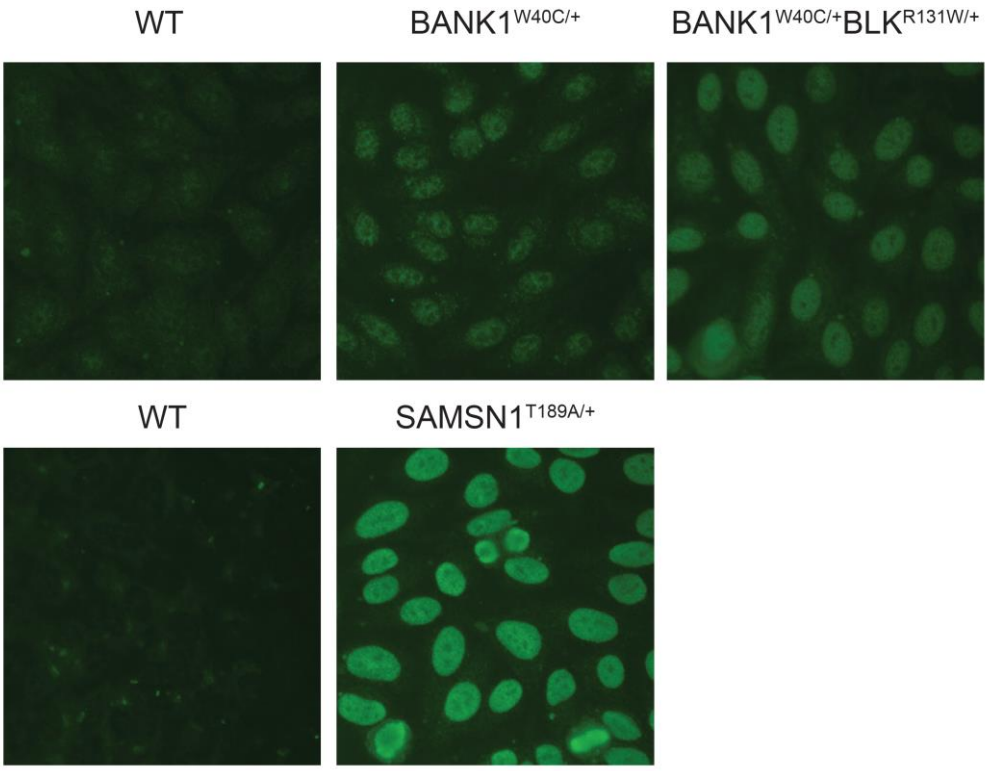


Figure 30. Antinuclear antibodies (ANAs) determined by Hep-2 immunofluorescence at a serum dilution of 1:40 from 16 week-old *Bank1*$^{W40C/+}$, *Bank1*$^{W40C/+}$*Blk*$^{R131W/+}$ and *Samsn1*$^{T198A/+}$ mice compared to wildtype littermate controls as indicated. Representative of 4 mice for each, except for Bank Performed by I. Papa.

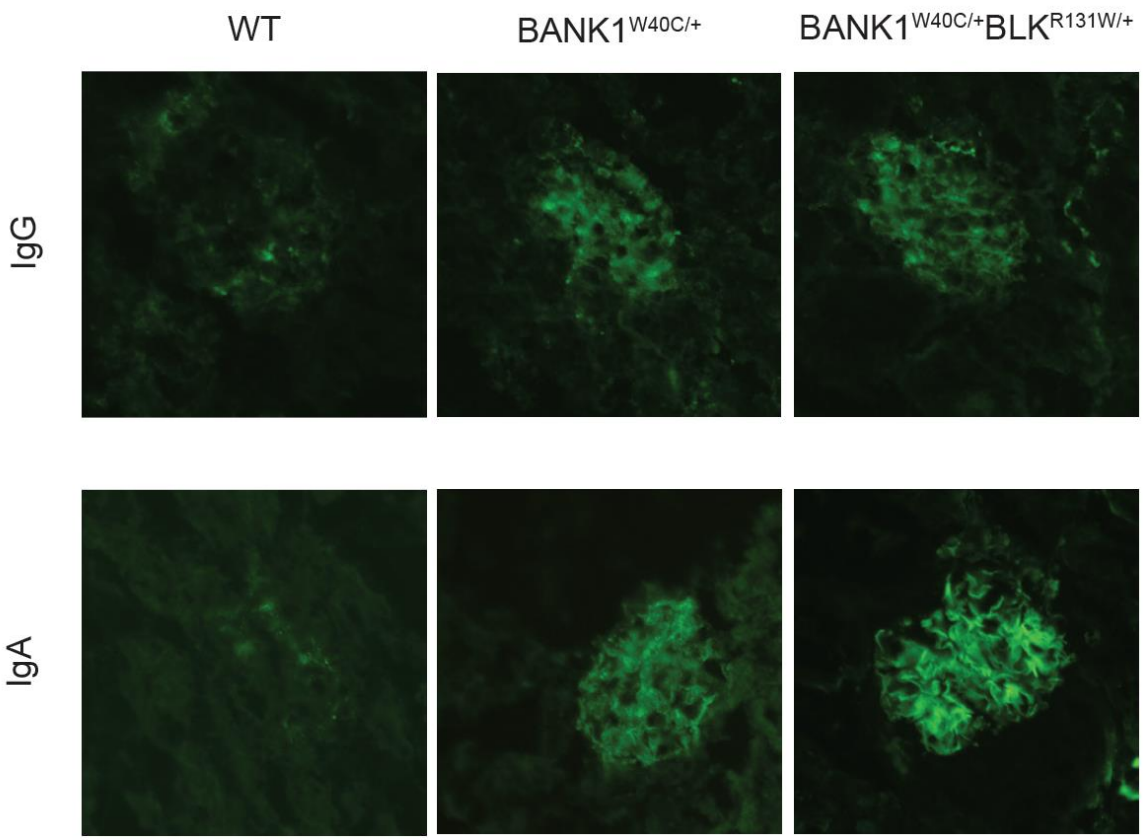


Figure 31. IgG and IgA deposition in renal glomeruli from 16 week-old *Bank1*$^{W40C/+}$ and *Bank1*$^{W40C/+}$*Blk*$^{R131W/+}$ determined by direct immunofluorescence of frozen kidney sections. Representative of 3 *Bank1*$^{W40C/+}$ mice and 2 *Bank1*$^{W40C/+}$*Blk*$^{R131W/+}$. Performed by I. Papa

Chapter 4: Copy number variation in *Vangl1* and lupus nephritis

4.1 Copy Number Variation in *Vangl1* associates with nephritis in SLE

To determine the role of CNV in SLE, the Vinuesa lab undertook Comparative genomic hybridisation (CGH) using Affymetrix 5.0 SNP chip arrays in a small cohort of SLE patients (n=55) and healthy controls (n=11). The Affymetrix 5.0 array has ~500,000 SNP probes and ~420,000 CNV probes which allow for a locus-by-locus measure of CNV. Array data was analysed using R statistical language package CRNA[331] by M. Bahlo and A. Moldovan. All 66 arrays passed QC checks. After filtering a total of 982 CNVs were retained for all 66 samples. Several CNVs were detected in more than one individual by Sevcan Mercan, including three individuals whose CNVs covered *VANGL1.* Sevcan validated these CNVs by real-time using Taqman probes designed at the CNV site, and examined them in the larger APOSLE cohort. In this larger validation study only the association between the CNV within *VANGL1* and SLE nephritis was found to be statistically significant (x^2=24.2, p=<0.0001). Importantly, this association occurred specifically between zero copies or a double deletion of the *VANGL1* CNV and SLE nephritis, but not SLE in general. The initial array analysis suggested all three SLE patients had the same double deletion of an estimated 3.17 kb of this gene spanning chr1:116,030,911-116,034,081 (NCBI36/hg18 assembly), within intron 8. This finding was supported by only two SNPs on the array, given the small size of the CNV. To confirm these findings Sevcan performed qPCR targeting the CNV region identified in intron 8 of *VANGL1* in a larger cohort of SLE patients (n=315) (Fig 32). This assay replicated the association of CNV with SLE nephritis as well as the frequent occurrence of the CNV in this cohort. Furthermore, the correlation between 0, 1 and 2 or more Vangl1 copies implied a gene-dose protective effect for *Vangl1*. The qPCR was repeated in a large Portuguese (n=605) cohort reproducing the correlation between CNV in *Vangl1* and nephritis in SLE patients, albeit to a lower magnitude (Fig 33). Odds ratio for nephritis comparing 0 CNV versus 1 or more CNV was 1.37 (95%Ci 0.87-2.19). The

Portuguese cohort comprised unselected SLE patients recruited by collaborator M. Alarcon-Riquelme as part of the BioLUPUS consortium.

To determine the size of CNV we performed whole genome sequencing in patients with SLE nephritis and identified CNV of variable size in different patients (Fig 34). Public databases report a relatively common occurrence of this CNV in intron 7 of *VANGL1* (Fig 35) and high expression of this gene in human CD34+ and CD105+ cells, which may include endothelial cells, T cells and monocytes[332].

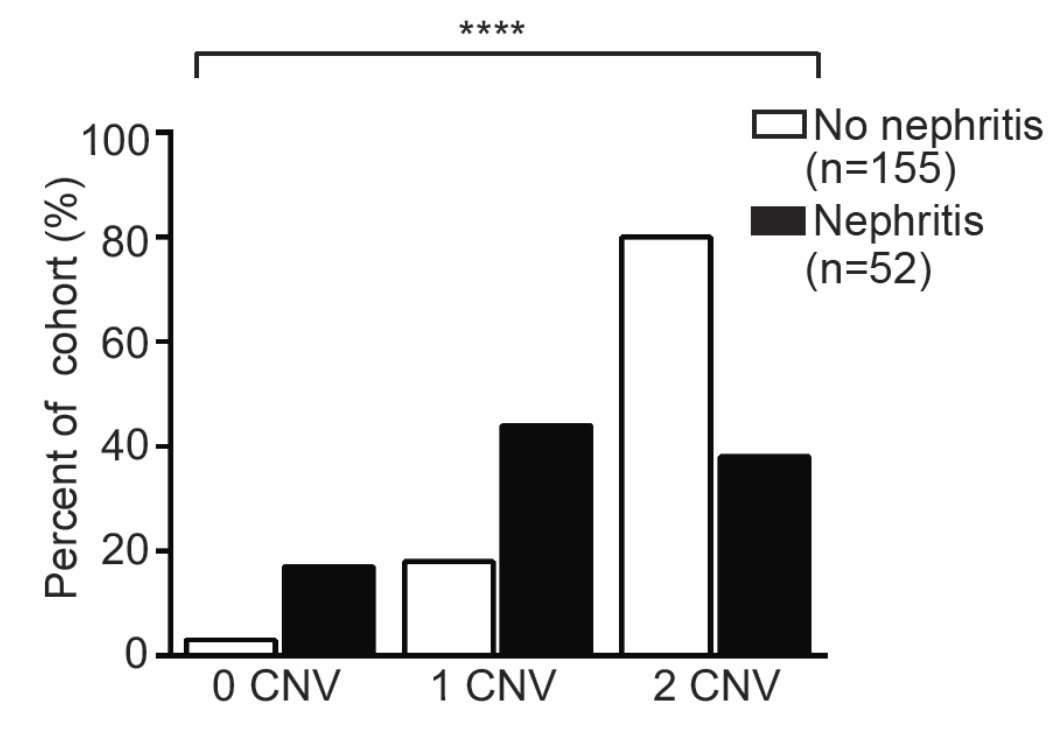


Figure 32. Association of CNV in *VANGL1* with SLE nephritis. (p=<0.0001)

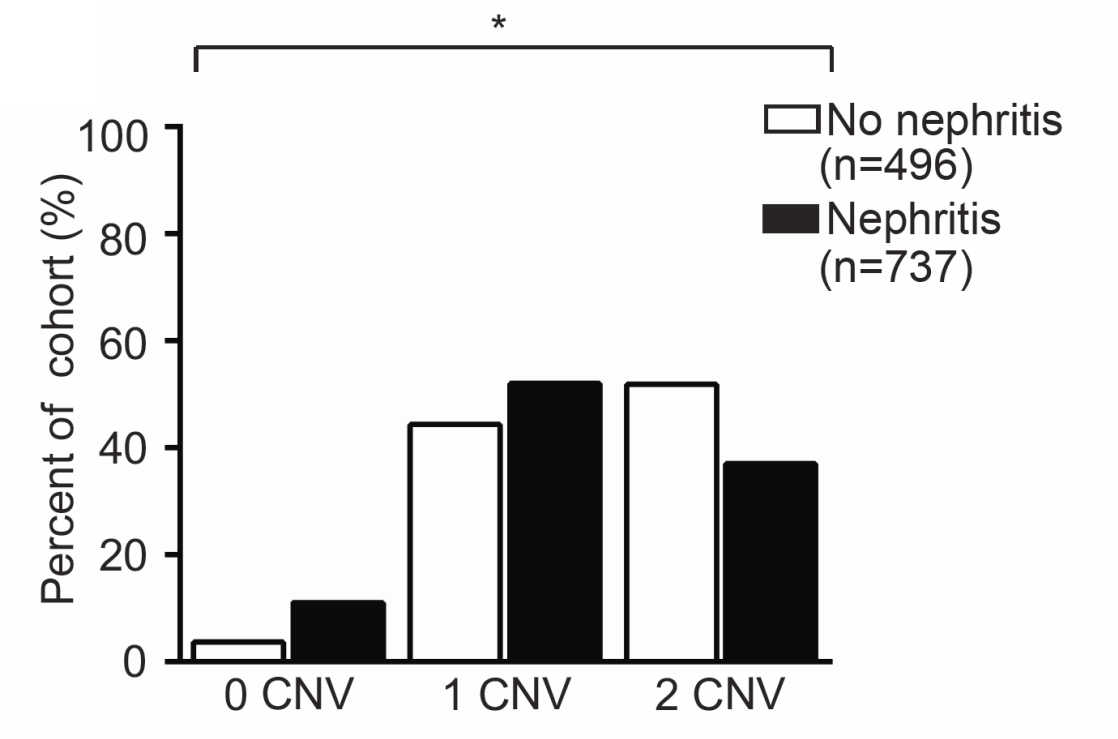


Figure 33. Replication of association with SLE nephritis in a Portuguese cohort (p=0.04).

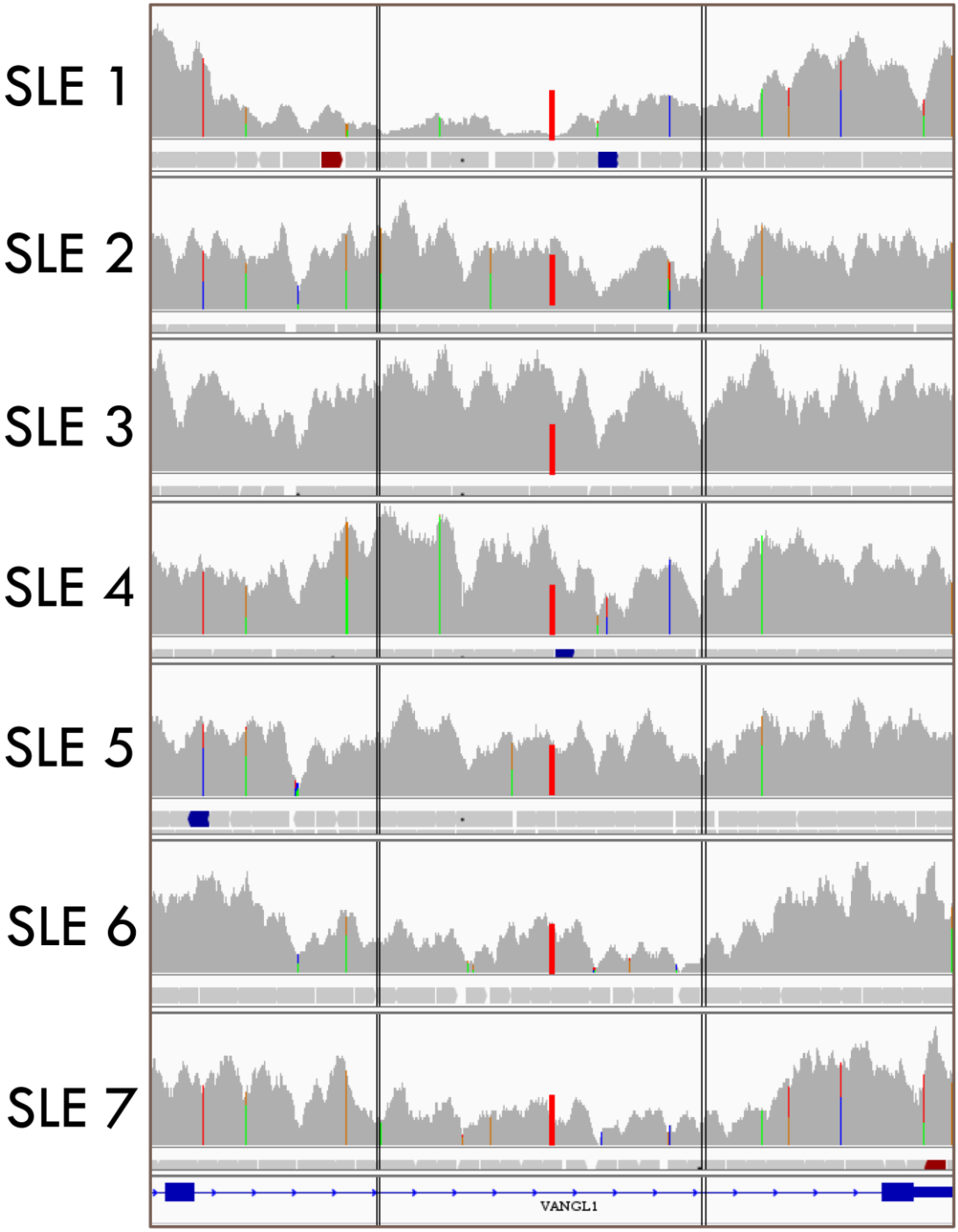


Fig 34. Whole genome sequencing of randomly selected patients with SLE nephritis demonstrates CNV in intron 7. The presence of nephritis is identified by treating physicians. Each peak represents a series of unique sequences generated during WGS. The relative heights of the reads indicates coverage of a given region. Deletion of this region can be confirmed by relative coverage within the deleted are compared to coverage in surrounding region.

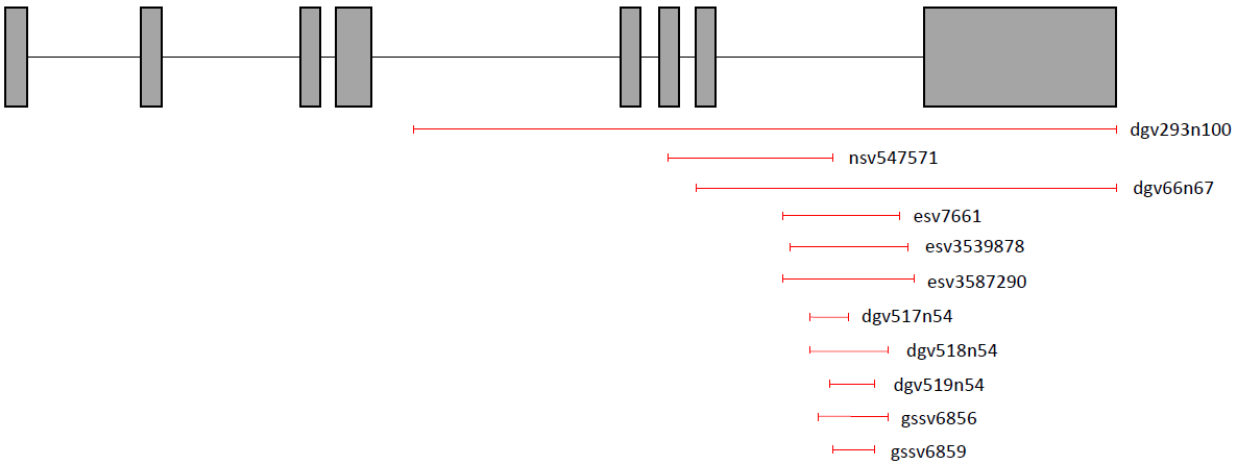


Figure 35. Schematic of CNV deletions in*VANGL1.* (Database of Genomic Variants)

Intronic deletions may cause alterations in splicing or removal of transcription enhancer elements. 4 protein coding splice variants are known to exist, although it unknown if these splicing variants have differential organ expression. I first tested if this CNV altered splicing by sequencing full length cDNA generated from blood from healthy controls vs 0 CNV donors. Sanger sequencing of cDNA from patients with 0 CNV revealed normal full length transcripts of *VANGL1*. We next asked whether the CNV influenced *VANGL1* expression in PBMCs from SLE patients. For this, we performed qPCR on PBMCs from SLE patients with or without the CNV but found no difference in *VANGL1* expression between those that had been identified with 0 CNV and 2 CNV (Fig. 36). Furthermore, there was no observable difference between healthy controls and SLE patients with or without nephritis (Fig. 36) although limited numbers of patients makes false negatives possible. Therefore, CNV in *VANGL1* is associated with the predisposition to nephritis in SLE patients, but does not alter gene expression in PBMCs. A remaining possibility is that kidney specific expression or splicing is affected, but this could not be tested due to lack of biological resources.

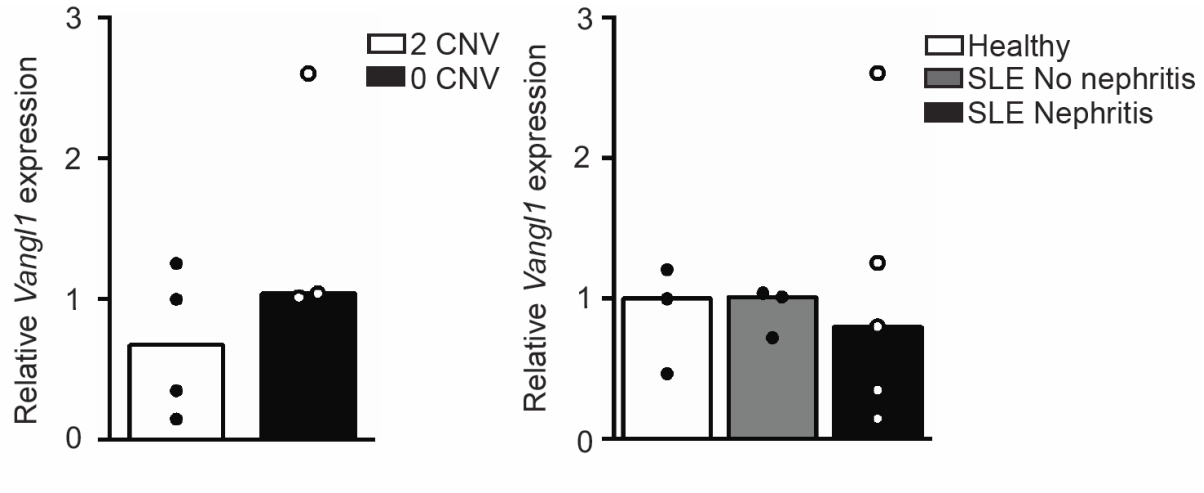


Figure 36. Differences in mRNA expression of *VANGL1* in PBMCs comparing SLE patients with or without 0 CNV or comparing those with or without nephritis. Healthy controls n=5, SLE nephritis n=3, SLE without nephritis n=5. 2 CNV n=4, 0 CNV n=3. Each point represents one individual.

4.2 *Vangl1*$^{+/-}$ mice develop spontaneous deposition of immunoglobulin in the kidney

Vangl1 paralog *Vangl2*-deficient mice have reduced glomerular numbers and impaired kidney morphogenesis. Recent work demonstrated a role for *Vangl2* in directing podocyte repair and genetic deficiency was associated with impaired recovery after acute glomerular injury and a predisposition to focal segmental glomerulosclerosis[333]. We hypothesised that given the important role of *Vangl2* in kidney development and repair, *Vangl1* CNV may play overlapping roles and also predispose to SLE nephritis in a kidney-intrinsic manner. Using immunohistochemistry we stained kidney sections from 10 week old adult C57/B6 mice. Similar to the observations made on Vangl2, Vangl1 was detected largely within tubules but not within the glomeruli (Fig. 37). This likely represents the transient glomerular expression of *Vangl1* during development and repair. To determine if *Vangl1* had a prominent role in glomerular development, akin to *Vangl2*, we stained sections of D18 foetal kidneys but did not detect Vangl1 in the glomeruli of these sections although it was detected in neural tissue, as expected (Fig 38).

To test the consequences of *Vangl1* deficiency in vivo we obtained mice with targeted frameshift null allele from exon 4 of *Vangl1* (KOMP repository) and found *Vangl1*$^{-/-}$ mice were embryonic lethal between D15 and D18 due to neural tube defects (Fig. 37). We examined kidneys from *Vangl1*$^{+/-}$ mice and observed they developed spontaneous deposition of IgA and IgG (Fig. 39), but not IgM, C3 or C4, within the glomeruli. Interestingly, despite universal immunoglobulin (Ig) deposition (performed by I. Papa) observed by immunoflourescence, by light microscopy *Vangl1*$^{+/-}$ glomeruli were normal (Fig 41). Nevertheless, electron microscopy performed by M. Koina (The Canberra Hospital Pathology Department) demonstrated significant deposition within, and mild expansion of, the mesangium in addition to cast-like material within the tubules (Fig 42). To ascertain the clinical consequences of Ig deposition, serum creatinine and proteinuria were measured at 6 and 9 months of age and no difference between wild-type or *Vangl1*$^{+/-}$ mice was detected

(Fig 43). Therefore, *Vangl1*$^{+/-}$ mice have spontaneous mesangial deposition of Ig with no evidence of inflammation or kidney function impairment.

3.8 *Vangl1*$^{+/-}$ immunoglobulin deposition occurs in a kidney intrinsic manner

To determine the potential source of immunoglobulin deposition, we first asked whether antibody deposition it was associated with increased systemic production of Ig or was a manifestation of systemic autoimmunity. Flow cytometric examination of *Vangl1*$^{+/-}$ mouse splenocytes did not demonstrate significant differences in lymphocyte populations, including all B cell subsets (Fig 44). At 20 weeks of age there was no evidence of autoimmunity as measured by ANAs (Fig 45) or difference in any class of Ig produced by *Vangl1*$^{+/-}$ mice (Fig. 46).

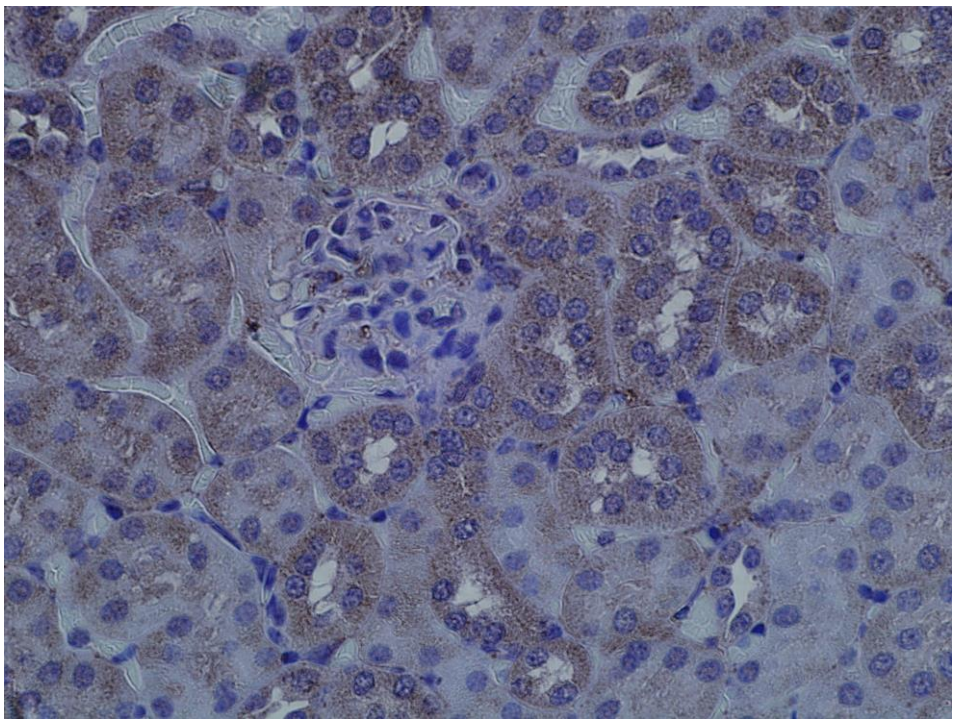
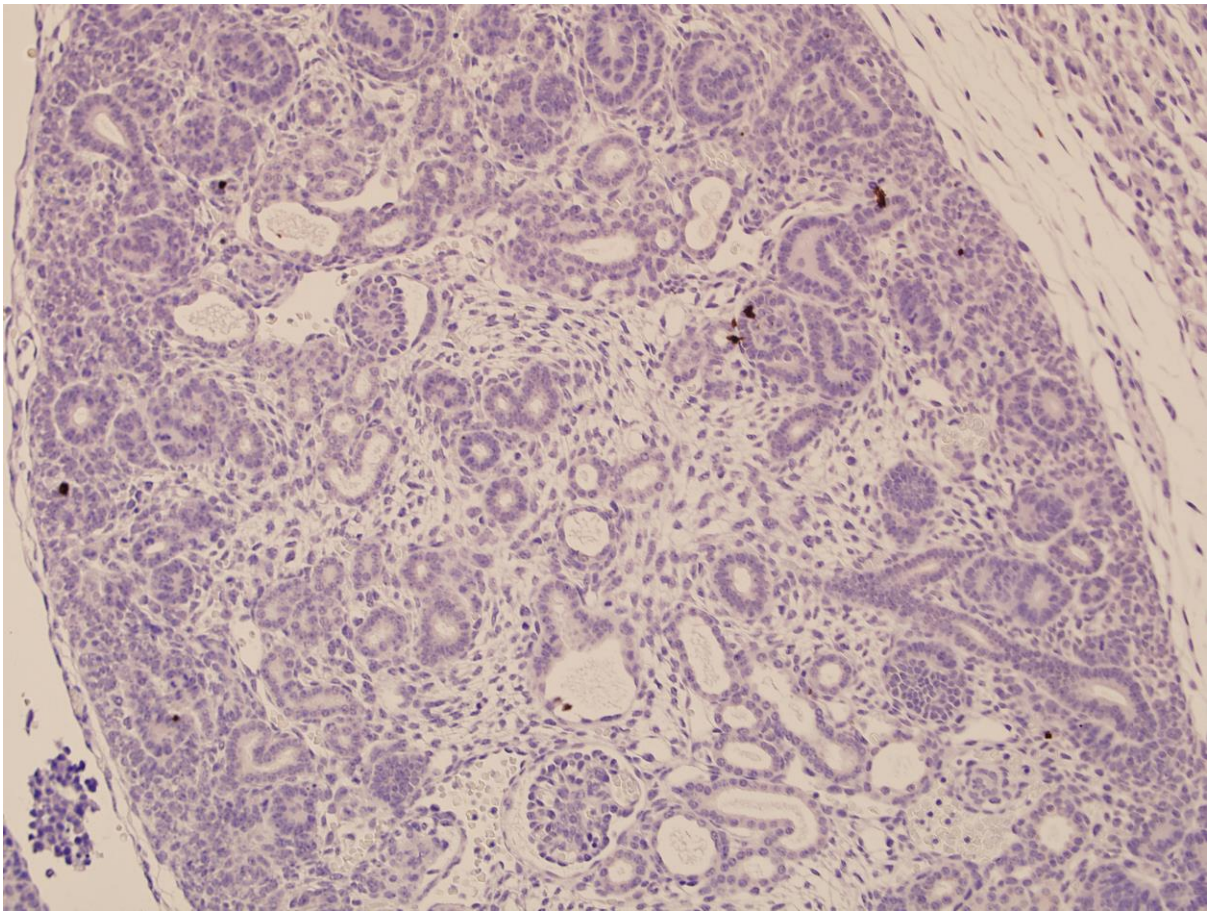


Fig 37. Staining of 3 month old WT mouse kidneys demonstrate Vangl1 expression in tubules. Brown= Vangl1. Representative of 4 mice.



38. Staining for Vangl1 in kidneys obtained from D18 WT mouse foetus demonstrates no Vangl1 expression. Brown= Vangl1. Representative of 4 mice.

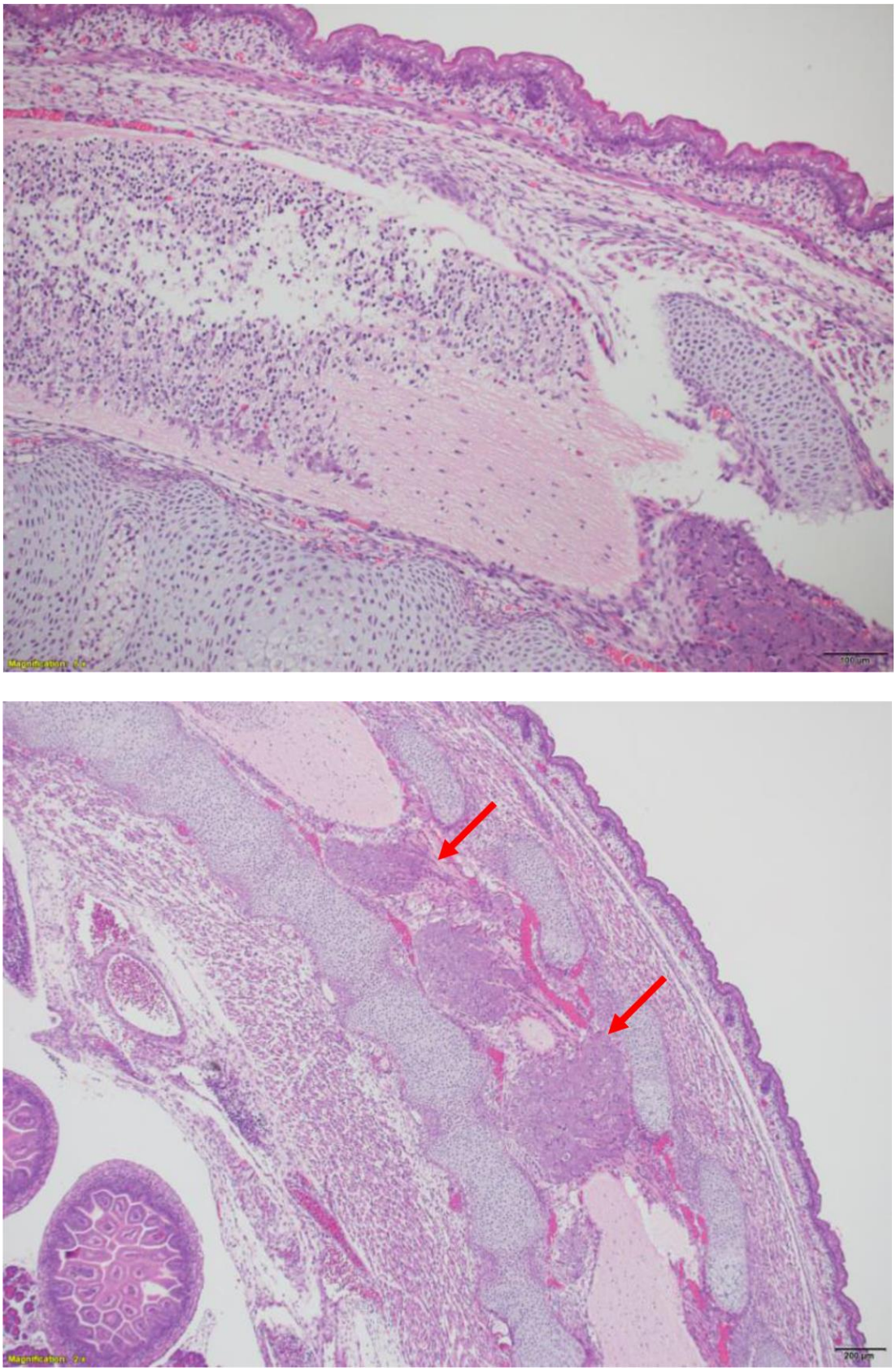


Figure 39. H&E stains of neural tube defects found in D15 *Vangl1*$^{-/-}$ mouse foetus. Representative of 2 homozygous foetuses.

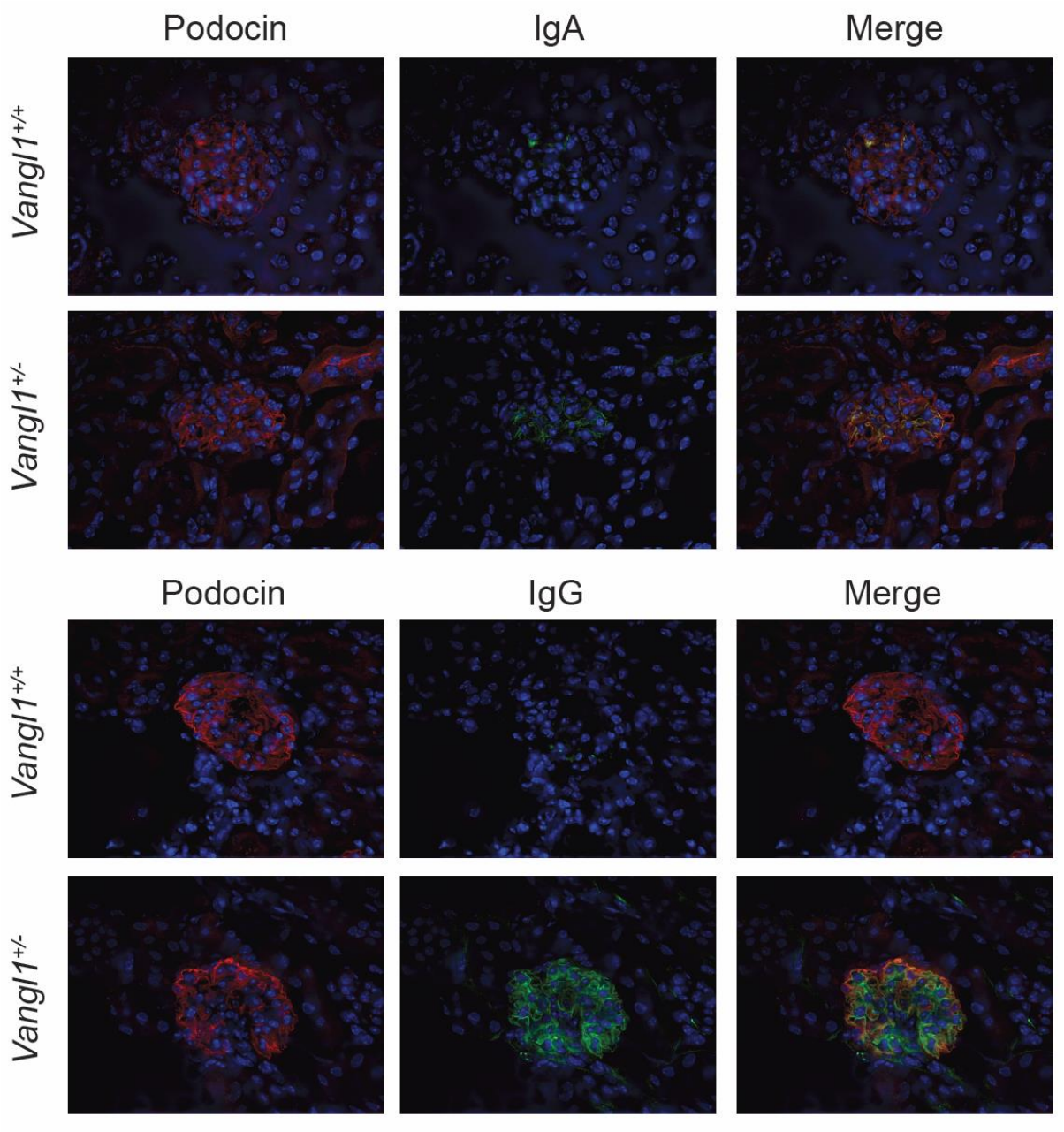


Figure 40. Immunoflourescence detecting trace IgA and IgG in 2 month old *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice. Representative of 5 mice per group. Representative of 3 repeat experiments

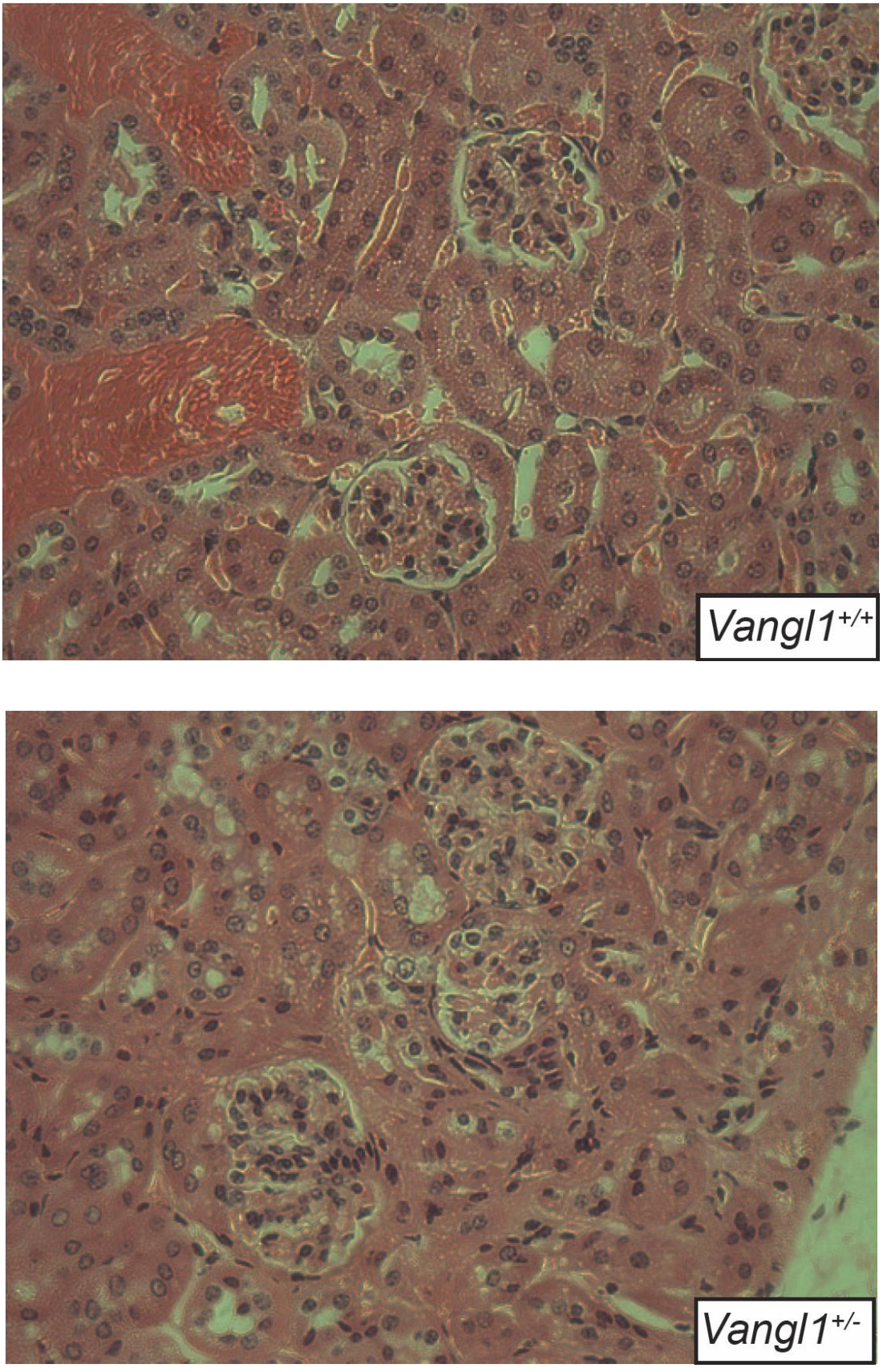
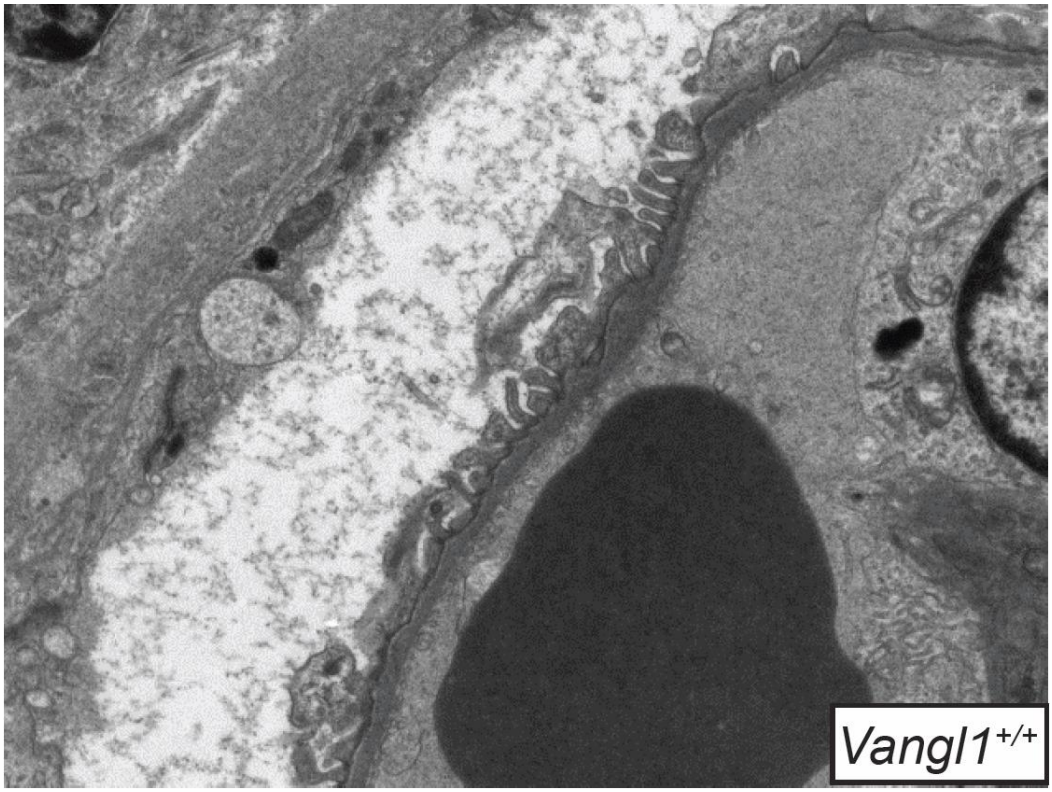


Figure 41. Light microscopy of glomeruli in *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice, aged 2 months. Representative of 5 mice per group.



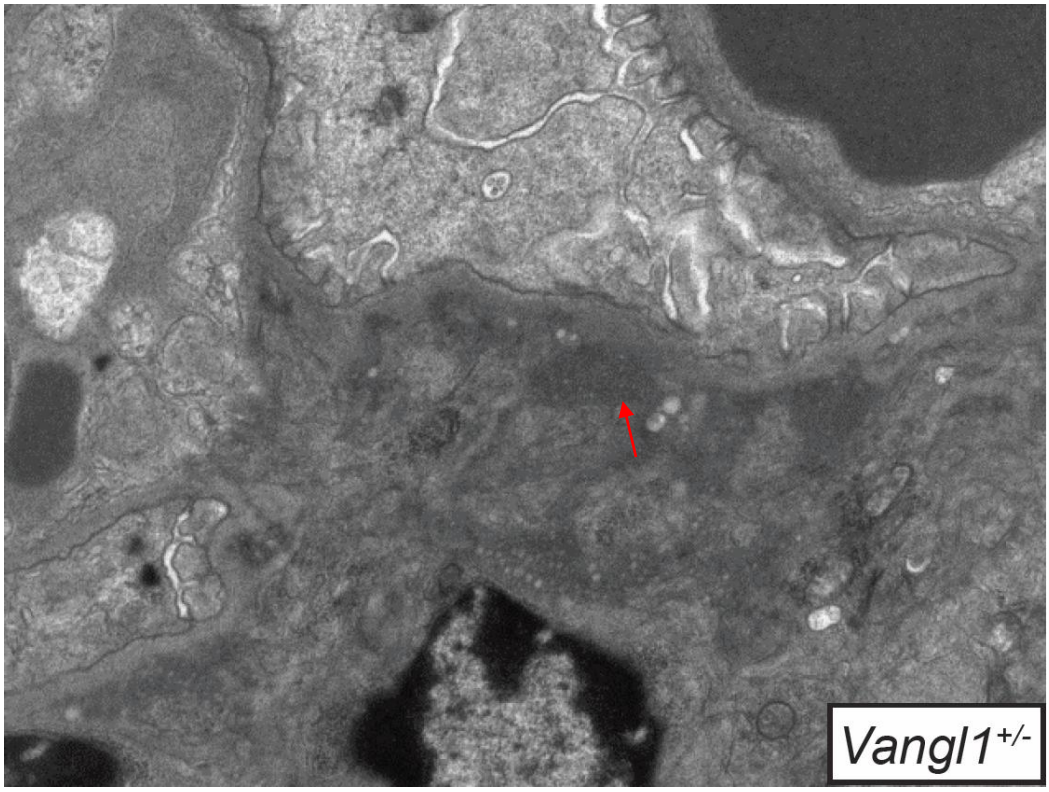


Figure 42. Electron microscopy of *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice kidneys aged 2 months. Red arrows indicate electron dense deposits in the mesangium. (By M. Koina) Representative of 3 mice per group.

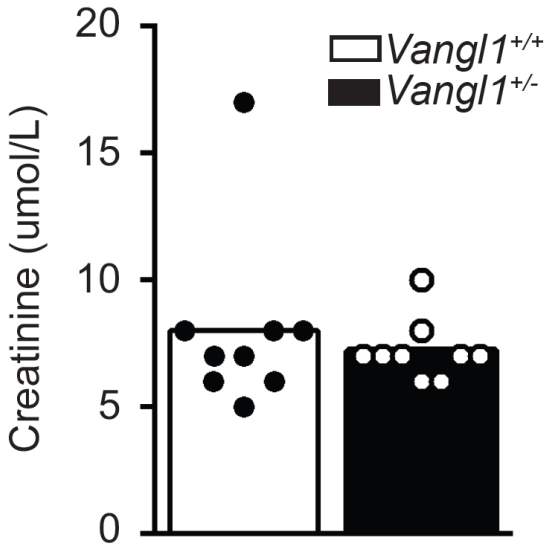
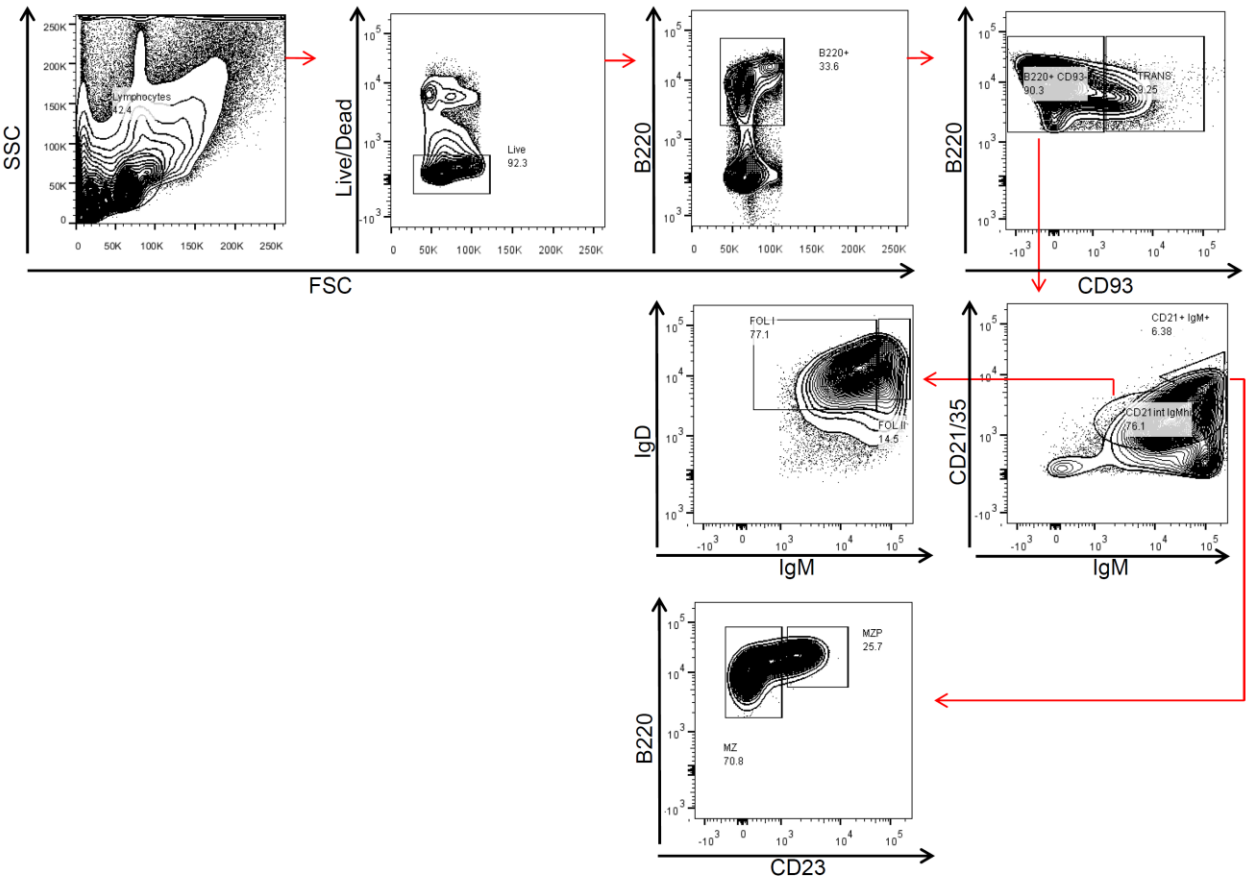


Figure 43. Serum creatinine from 3 month old *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice shows no significant difference.



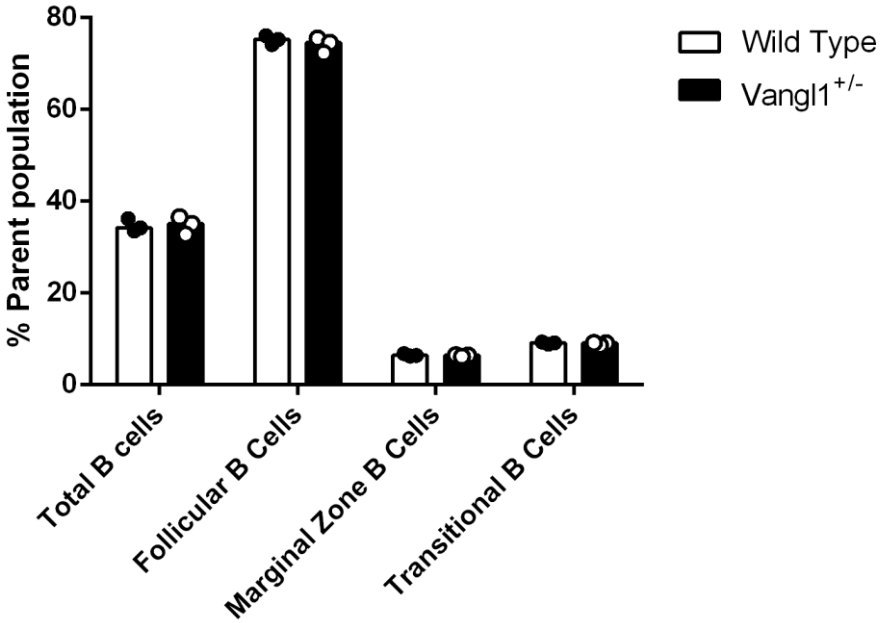


Fig 44. Flow cytometry gating strategy and comparison demonstrates normal B cell populations in 3 month old *Vangl1*$^{+/-}$ mice. 3 mice per group.

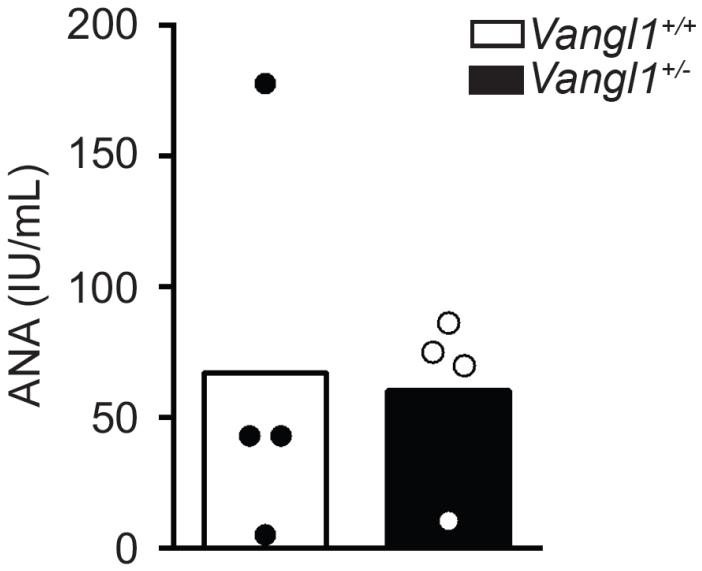


Figure 45. Antinuclear antibodies from 20 week old *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice

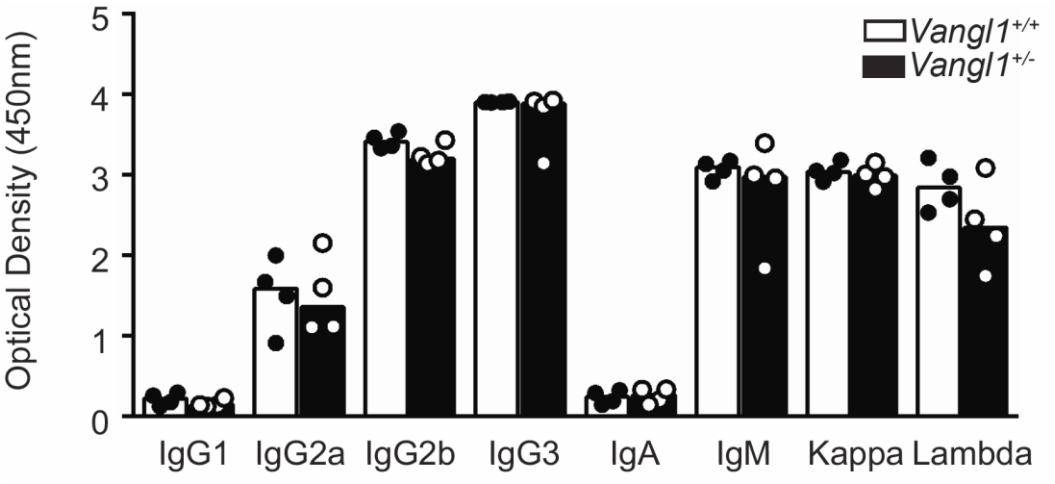


Fig 46. Serum Immunoglobulin levels from 20 week old *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice

In view of no evidence of increased systemic production of Ig, the known role of *Vangl2* in the kidney morphogenesis and podocyte repair, as well as our demonstration of Vangl1 expression in the kidney, we hypothesised that Ig deposition in the kidney in *Vangl1*$^{+/-}$ is due to kidney-intrinsic predisposition to Ig deposition. To test this, we crossed *Vangl1*$^{+/-}$ mice to *CD79a*$^{ken/ken}$ mice that lack mature B cells and thus circulating Ig [334]. Serum from age- and sex-matched *Vangl1*$^{+/-}$ or *Vangl1*$^{+/+}$ mice was injected daily for 5 days into either *Vangl1*$^{+/-}$*CD79a*$^{ken/ken}$ or *Vangl1*$^{+/+}$*CD79a*$^{ken/ken}$ mice. On day 6, the four groups of mice were sacrificed and kidneys were examined for Ig deposition. Serum from either *Vangl1*$^{+/-}$ or *Vangl1*$^{+/+}$ mice was deposited in *Vangl1*$^{+/-}$*CD79a*$^{ken/ken}$ mice whereas *Vangl1*$^{+/+}$*CD79a*$^{ken/ken}$ mice were resistant to IgG deposition regardless of whether the serum came from *Vangl1*$^{+/-}$ or *Vangl1*$^{+/+}$ mice (Fig 47). This result demonstrates that susceptibility to Ig deposition in *Vangl1*$^{+/-}$ is kidney-intrinsic and excludes qualitative abnormalities in *Vangl1*$^{+/-}$ IgG causing Ig deposition. IgA was not detected in any recipient kidneys, likely due to a sensitivity issue (too low circulating IgA to achieve sufficient levels and deposition in recipients). To determine the effect of an autoimmune background on Vangl1 deficiency, we crossed the *Vangl1*$^{+/-}$ mice to *Fas*^{lpr} mice and measured serum creatinine and albuminuria as a marker of nephritis. However, across all time points no significant difference between the *Vangl1*$^{+/-}$ and *Fas*^{lpr} *Vangl1*$^{+/-}$ mice was detected (Fig 48 and Fig 49) suggesting that on the *Fas*^{lpr} background *Vangl1* deficiency did not exacerbate nephritis. All experiments with the exception of the two CNV arrays, electron microscopy, and immunoflourescence were performed by myself.

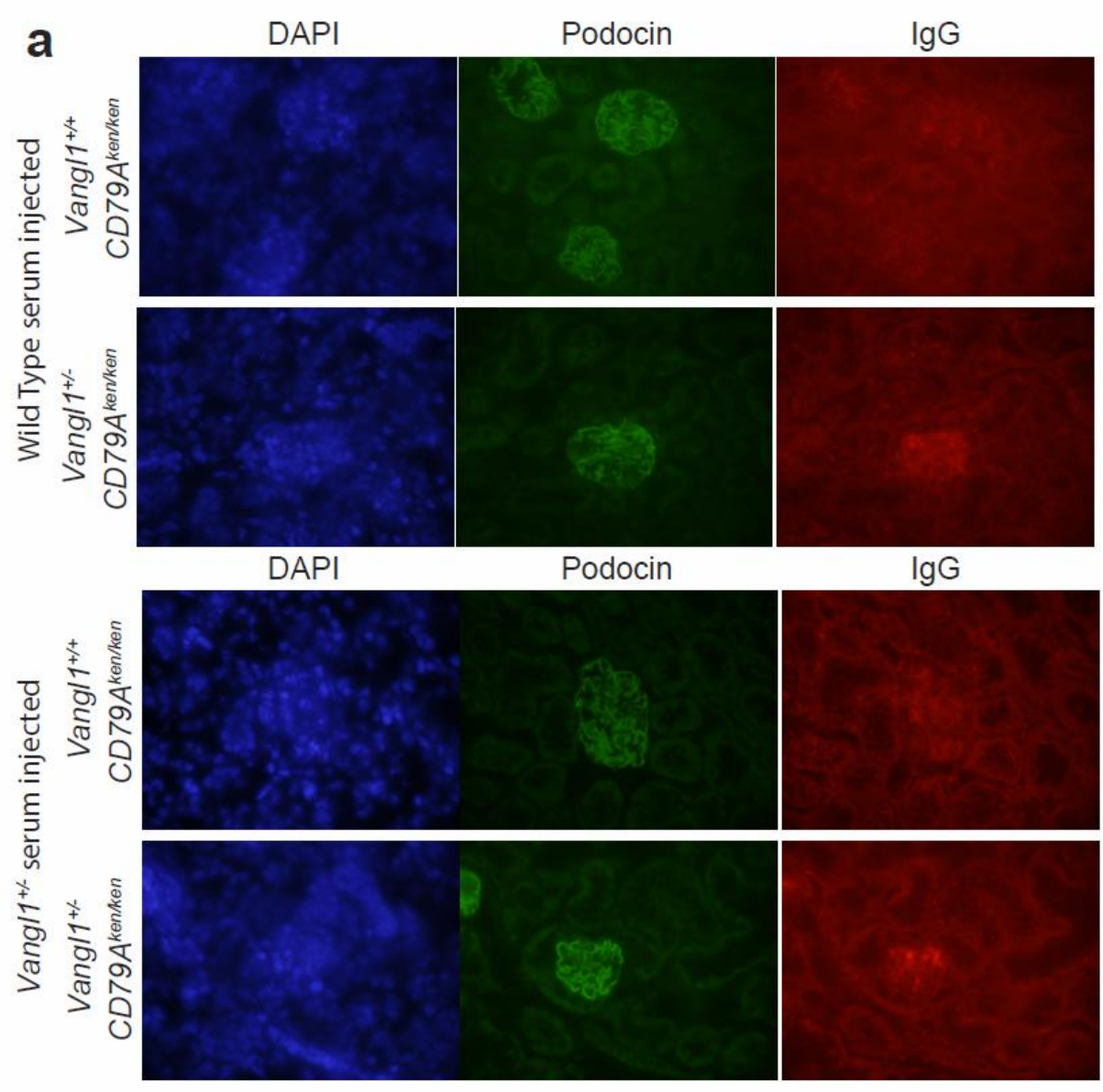


Figure 47. Immunoflourescence of IgG in B cell deficient *Vangl*$^{+/-}$ and *Vangl1*$^{+/+}$ mice injected daily with 100ul of mouse serum for 5 days. Representative of 3 mice per group.

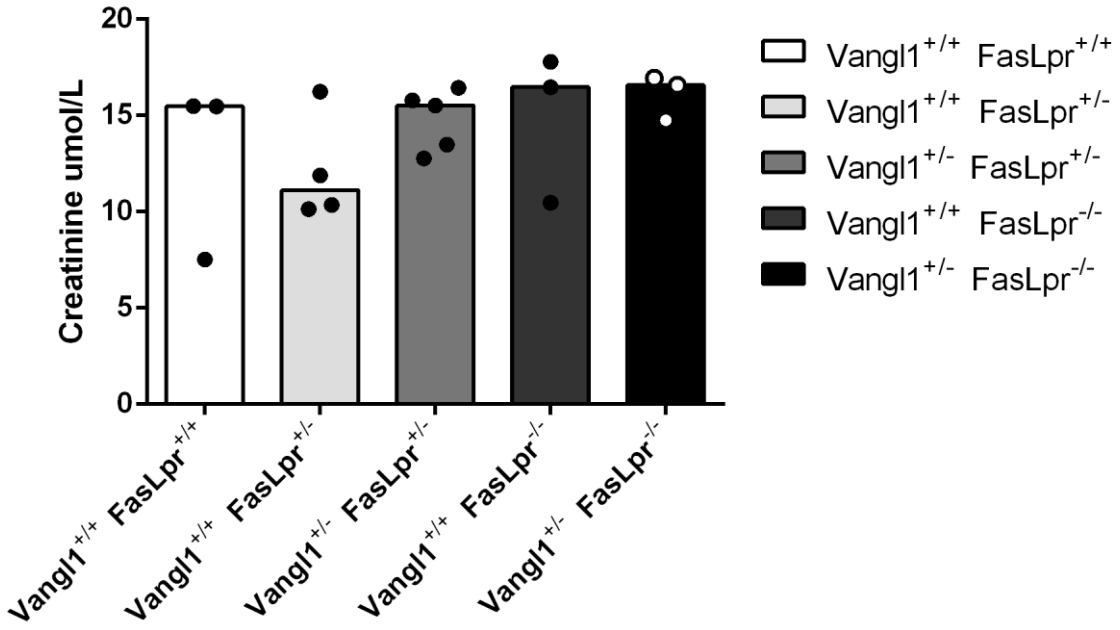


Figure 48. Creatinine measurements of 4 month old *Vangl1*$^{+/-}$ Fas.*lpr* mice. No significant differences observed between genotypes.

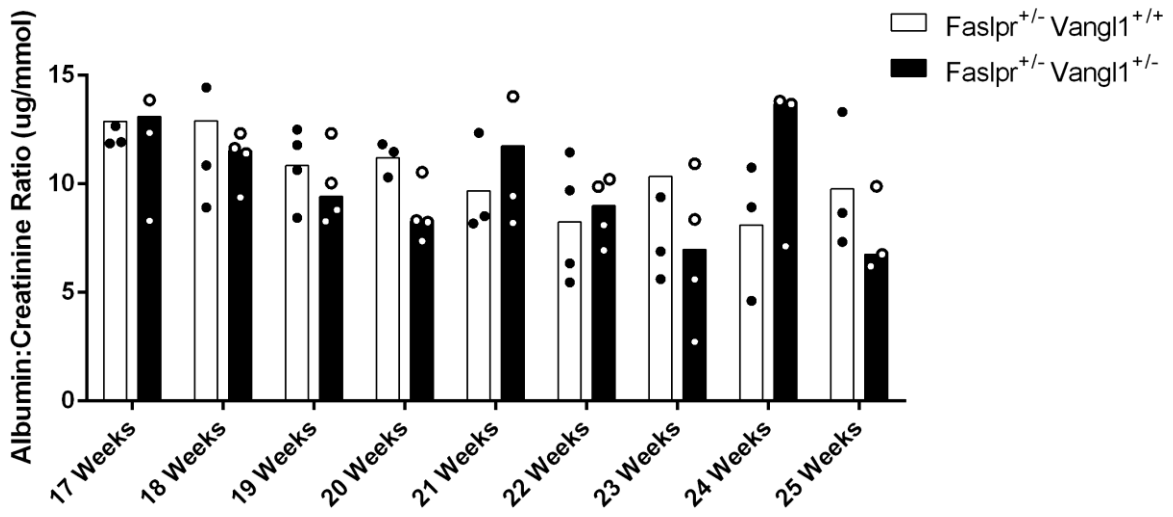


Fig 49. Albumin:creatinine ratios for *Fas*lpr$^{+/-}$ *Vangl1* mice of indicated age.

Chapter 5: Discussion

Chapter 5: Discussion

5.1 Overview

This thesis demonstrates several mechanisms through which genetic variation predisposes towards complex autoimmunity. Firstly, it provides evidence for how complex disease can be dissected using WES, identifying rare and novel genetic variants and, based on analysis of their epistatic interactions, their functional consequences. Whereas the prevailing hypothesis suggests that genetic risk in SLE and other complex autoimmune diseases is the result of a multitude of common, weak variants combining to break tolerance, this thesis demonstrates that single mutations in the heterozygous state have the capacity to induce autoimmunity. Specifically, this thesis demonstrates how variants in the proximal B cell signalling pathway and endosomal TLR pathway can disrupt B cell physiology and contribute to SLE.

The second part of this thesis provides evidence for genetic mechanisms that predispose towards organ involvement. It reports the contribution of a CNV in *VANGL1*, specifically a large deletion in intron 7, towards nephritis in SLE. Although particular autoantibodies have been associated with organ specificity, such as with anti-smith and anti-double stranded DNA antibodies and their association with nephritis in SLE, to date no organ-intrinsic mechanisms have been identified. *Vangl1*, expressed during mouse kidney organogenesis, suggests that a kidney-intrinsic defect that likely occurs during development predisposes to autoimmunity. We therefore add to the growing evidence of the importance of CNV in human disease. This thesis provides evidence for how organ-intrinsic defects may predispose to their involvement in disease.

Together, this work makes several novel contributions to the genetic theory of SLE. First, this thesis demonstrates that rare genetic variants can strongly predispose or overtly induce autoimmunity. Second, that by scoring these variants and identifying similar gene expression profiles or similar phenotypes in gene knockout models we can identify and test

novel interactions. In this fashion, WES and WGS in complex autoimmune diseases can be a vehicle for immune gene function discovery similar to ethyl-nitrosurea mutagenesis programs. Thirdly, this thesis suggests that rare variants may explain a proportion of the genetic risk unidentified to date and may also confer some of the risk haplotypes identified by GWAS. Finally, it is the first demonstration to date of how structural variants in genes expressed in end-organs may explain or influence organ damage in complex autoimmunity with implications for treatment and transplantation.

5.2 Contribution of *BANK1*^{W40C}

This study proposes a method by which bioinformatic data from diverse public databases can be aggregated to select out variants of low interest and therefore enrich for SNV that may contribute to SLE pathogenesis. In initial pedigrees *BANK1*^{W40C} is identified as a critical variant underlying genetic risk in a minority (2-6%) of SLE patients. Identification of *BANK1*^{W40C} as a core risk variant led to the identification of several possible interacting mutations. Interestingly, when determining the prevalence of *BLK*^{R131W} and *IRAK2*^{S47Y} variants identified by WES, unlike the *BANK1*^{W40C}, these were not observed at higher frequencies. This suggests that although these variants contribute to SLE pathogenesis as demonstrated by this work, *BANK1*^{W40C} appears to play a more central role. *BANK1*^{W40C} was found at significantly higher rates in our APOSLE cohort compared to dbSNP reported frequencies. Interestingly, *BANK1*^{W40C} has been reported in all racial backgrounds except those of Asian descent. None of our probands were of Asian descent, and given a significant proportion of the screened APOSLE cohort are of Asian descent, the risk of developing SLE associated with *BANK1*^{W40C} may be higher in cohorts of European or European-American ancestry. Performing exome sequencing on a group of SLE individuals carrying the *BANK1*^{W40C} variant, the scoring algorithm identified secondary mutations likely to contribute to disease. In addition to bioinformatic variables that suggested these second mutations in *SAMSN1*, *BLK*, and *IRAK2* could be important interactors, the reported murine phenotypes

from the respective models of knockout suggested they may interact with *BANK1* on a physiological level. Through further evaluation of these mutations, this study then discovers several novel interactions amongst the identified variants (SAMSN1 and BLK) and between some variants (i.e. IRAK2) and BANK1. Thus, *BANK1* therefore unifies otherwise apparently unrelated genes and their variants.

The natural function of *BANK1* remains unclear, yet it can be hypothesised that *BANK1*^{W40C} is the central variant due to its function as a scaffolding protein that facilitates interaction between BCR and TLR signalling proteins. As a central scaffolding protein, bringing interacting partners in proximity to each other, it is conceptually vulnerable to mutations disrupting multiple pathways, i.e. TLR, BCR, and co-stimulatory paths such as CD40. This observation makes *BANK1* a strong candidate as a central player in autoimmunity as previous work has demonstrated how anergy relies on uncoupling BCR and TLR signals [128]. We clearly link BANK1 to both the BCR and TLR signalling pathways, yet how BANK1 can influence these two pathways to repress autoimmunity requires further investigation. Our demonstration that BANK1 over-expresison induces formation of p62+ sequestrosomes containing TRAF6 suggests this may be a mechanism by which BANK1 can turn off NF-κB signals in B cells upon BCR+/- TLR signals. The ability to sequester TRAF6 may extend to other TRAF family members including NF-kB inhibitory TRAF family members such as TRAF3, known to inhibit NF-kB-mediated IL-10 and IL-6 production and B cell survival downstream of BAFF-R stimulation [335].

5.3 Contribution of *BLK*^{R131W}

Both Src family members *LYN* and *BLK* have been repeatedly implicated in development of SLE and autoimmunity in general by GWAS [298,336]. Similar to the circumstances of *BANK1*, the haplotype implicated by the GWAS identified variant in the *C8orf13-BLK* intergenic region may include the rare variant *BLK*^{R131W} [298]. This may

therefore also support the hypothesis that common variants used by GWAS studies detect rare deleterious mutations within areas of linkage disequilibrium. A further similarity between *BLK* and *BANK1* is their GWAS association in several antibody-mediated and genetically complex autoimmune diseases such as rheumatoid arthritis and systemic sclerosis [337-339] suggesting a contribution to autoimmunity in general and not just SLE.

Several Src family members exist in B cells, *Lyn*, *Fyn*, *Blk*, and *Src*. There appears to be a relative hierarchy of importance amongst these family members, as *Lyn* deficient mice have clear B cell and T cell abnormalities which result in a SLE-like phenotype [340]. Both *Fyn* and *Src* deficiency appear to have mild autoimmune phenotypes with predominantly kidney involvement[341], and combined Src-family member deficient mice have phenotypes more exaggerated than single Src-member deficient mice [341]. $Blk^{-/-}$ mice were reported to have only a modestly abnormal immunophenotype [342] attributed to redundancy with other Src family members, although this has recently been challenged [343]. Arginine residues within the SH2 domains of all Src family members interact with phosphorylated C-terminal tyrosine residues. The interaction between different arginine-tyrosine pairs results in the Src protein to adopt either a closed or open conformation. In the open conformation, the kinase domain is exposed that permits substrate phosphorylation whereas in closed conformations this kinase domain is concealed. In the case of BLK, BANK1 is one such substrate and the BLK^{R131W} variant, due to loss of the arginine required to hold BLK in an open conformation, has a virtually abolished ability to phosphorylate BANK1, a known requirement for BANK1 to interact with partners such as PLCG2 [299]. It is therefore unsurprising that the $BANK1^{W40C/+}$ and $BLK^{R131W/+}$ may have epistatic interactions. The question remains of how the kinase-inactive BLK and its inability to phosphorylate BANK1 contributes to the development of autoimmunity. Given that we speculate mutant BANK1 does nto efficiently localise and perhaps direct TRAF proteins to p62+ sequestrosomes, it is possible that BLK-induced BANK1 phosphorylation is critical for this subcellular localization or TRAF-family interactions.

5.4 Contribution of *IRAK2*^{S47Y} and *IRAK2*^{T508M}

The MyD88-associated *IRAK* family has been strongly implicated in autoimmunity and SLE. A common variant of *IRAK1*, which is a direct interactor with *IRAK2*, has been shown to segregate with SLE and mutations of *IRAK1* cause autoimmunity in the *sle1* mouse model. IRAK1, along with IRAK2 and IRAK4, are recruited to MyD88 wherein IRAK members undergo extensive auto-phosphorylation. Activation within this complex leads to NF-κB activation through TRAF6. We found compound heterozygosity for *IRAK2* in proband A, with both mutations exhibiting an enhanced capacity to interact with other IRAK members as well as TRAF6. This results in increased NF-kB-dependent transcripts and production of the proinflammatory cytokine IL-6. Elevated IL-6 is a common finding in SLE and other autoimmune diseases [330,344,345] and contributes to the TLR-mediated IL-6 production observed in the proband's LCLs. Furthermore, the proband's pedigree suggests the effect of the mutations may predispose to SLE in a dose dependent manner.

IRAK2 is expressed in B cells. In mice, in which expression in well characterized in public databases, IRAK2 expression is high in follicular and marginal zone B cells, with low amounts expressed in germinal center B cells (http://www.immgen.org). Unlike *BANK1* or *Blk*, the *IRAK2* genes are also expressed in myeloid populations. Therefore, besides possible direct epistatic interaction with BANK1 in B cells *IRAK2* may also contribute to the development of autoimmunity via myeloid cell dysregulation: Monocyte or pDC TLR stimulation in the presence of mutant IRAK2 may also lead to excessive NF-κB activation with commensurate increases in IL-6 production and antigen presentation. Myeloid-derived IL-6 production has been reported to be important in mediating autoimmunity associated with *Lyn* deficiency [346] and may similarly exacerbate *BANK1*^{W40C} autoimmunity. It is important to note that while every affected family member in pedigree A carried the BANK1 mutation, only 2/3 carried the IRAK2 allele, suggesting that in at least two patients this allele may exacerbate disease, but as shown in the case of the other two pedigrees, its presence is not

obligatory for SLE development in the presence of BANK1^{W40C}. Genome sequencing of the younger family member that does not carry the IRAK mutation and has been recently diagnosed with Sjogren's Sd at age 11 may reveal yet another hit in the BCR-TLR pathway. Assessing all pedigrees, a pattern emerges: BANK1^{W40C} is sufficient to predispose to production of ANAs, but all individuals with a severe autoimmune syndrome in each of the pedigrees (APS, SLE, SS, RA) have a second rare damaging mutation in a BCR/TLR signalling gene.

5.5 Contribution of SAMSN1^{T198A}

SAMSN1 is a recently described gene identified as a potential regulator of Lyn [327]. Although it has been implicated in BCR signalling as a repressor of Lyn, unlike other genes identified by this study it has not been associated with SLE. It was considered of particular interest as *Samsn1*$^{-/-}$ mouse B cells had increased proliferation and activation [327] reminiscent of the initial reports of the *Bank1*$^{-/-}$ mice and we hypothesised that deficiency of two genes which induce similar phenotypes of B cell hyperactivity may, even if functioning in independent pathways, result in additive or synergistic effects on B cell physiology.

Whilst investigating the contribution of *SAMSN1*, we have made several novel observations. First, SAMSN1 is a novel substrate of BLK and Lyn, similar to BANK1. Whether it therefore has a role in negative feedback of Lyn is an area of further investigation. Second, *SAMSN1* has a significant role in TLR9-mediated IL-10 responses. The high levels of IL-10 in *SAMSN1*$^{T198A/+}$ Ramos cell lines in response to CpG stimulation is consistent with findings of elevated IL-10 in SLE patients and mouse models [211,330]. *SAMSN1*$^{T198A/+}$ may therefore be an example of why elevated IL-10 may also be observed in healthy relatives of SLE patients, representing a partial predisposition to antibody-mediated autoimmune disease [347].

Two important questions arise from the observations found with *SAMSN1*^{T198A}. Firstly, what is the role of SAMSN1 in B cell physiology? Herein we have implicated it directly in both BCR signalling as well as TLR signalling. The novel discovery of SAMSN1 as a substrate of Src family members suggests it, like BANK1, may play a dual role or regulate the convergence or cross-talk between these two pathways. The second main question that presents is how SAMSN1 regulates CpG-induced IL-10 responses. Both will require further investigation.

5.7 Unifed theory of polymorphisms identified in this thesis

This thesis identifies *BANK1*^{W40C} as a variant that forms the genetic basis of a subset of SLE patients. The natural role of BANK1, and the contribution of the *BANK1*^{W40C} allele remains to be elucidated, yet this thesis implicates the central role of BANK1 between the other implicated SNVs. BANK1 appears to link both TLR and BCR signalling pathways; it and SAMSN1 are substrates of BLK which is activated in response to BCR crosslinking. However, BANK1 also interacts directly with IRAK2 and LCLs derived from the probands' PBMCs have increased responses to endosomal TLR stimulation. The interaction between TLR and BCR signalling has been considered important in the maintenance of B cell anergy as exemplified in the HEL/TgHEL mouse model of B cell anergy, yet the mechanism linking these pathways is unclear. Here, I hypothesise that the BANK1 is a natural fulcrum between these two mechanisms of B cell activation and that the *BANK1*^{W40C} mutation alters the ability of B cells to regulate these two responses. Therefore, loss or gain of function mutations in either of these pathways will skew B cell responses. Indeed based on the aggregate effects of these variants it would appear that the *BANK1*^{W40C} mutations impair BCR signalling (loss of function *BLK* SNV) whilst promoting TLR responses (i.e. gain of function *IRAK2* SNV). A summary of the proposed signalling pathway is represented in Figure 50.

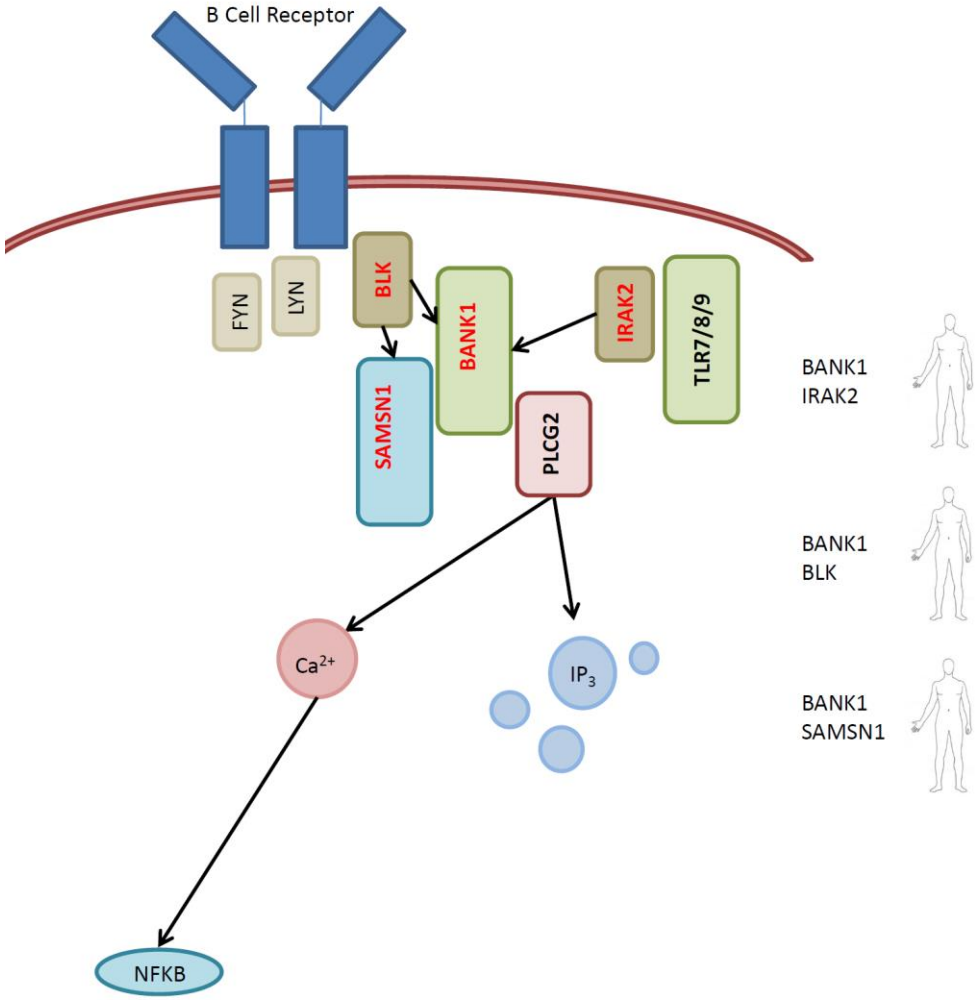


Figure 50. Proposed unification of BANK1 signalling pathway and importance in B cell activation by BCR or TLR.

5.6 Implications for the genetic theory of SLE and the value of WES/WGS

GWAS has identified a growing cohort of genes associated with SLE and other autoimmune diseases. These variants are by necessity relatively common and the majority of experimentally tested variants have not been shown to be significantly deleterious to protein function. In comparison, using WES to identify rare or novel variants under evolutionary pressure we have demonstrated that rare mutations that could not be identified by GWAS have potent deleterious effects. *BANK1*^{W40C}, in our cohort, characterises a subset of SLE in non-Asian populations which, either alone, or when combined with a rare damaging mutation in an interacting partner, results in loss of B cell tolerance and contributes to complex autoimmunity. Further investigation about the existence of epistatic interactions between the gene variants identified in this thesis may provide deeper insights into the basis of SLE and complex autoimmunity. Our results demonstrate the ability for WES and WGS to identify causative mutations and forms the basis for personalising treatment for SLE.

Of the two major theories for genetic risk in autoimmunity, the weak common variant hypothesis or potent rare variant hypothesis, it has been argued that the GWAS identified risk SNVs are not the variants which confer risk. Rather, the SNV in GWAS represent haplotypes containing rare, potent variants that confer risk. Thus, the association detected by GWAS may actually be that of rare variants in disequilibrium with the identified variant. Nevertheless, the opposite argument has also been made: A recent report in Nature concluded the contribution of rare variants to autoimmunity will be negligible, after failing to identify rare variants in 25 GWAS genes sequenced in 24 892 individuals with autoimmune disease and 17 019 healthy individuals [282]. Of note, SLE was not included amongst the autoimmune diseases, and neither BANK1 nor BLK were sequenced. To this debate on the genetic architecture of SLE, this thesis offers evidence supporting the idea of rare variants being the ‘risk signal’ identified by GWAS. Both *BANK1*^{W40C} and *BLK*^{R131W} have been

previously identified as risk alleles in both SLE and other complex autoimmune diseases. That these rare variants with demonstrated pathogenicity are in close genetic proximity to the identified variants supports this argument. However, it is not conclusive and also does not preclude an additional role of other uncommon or common variants in disease pathogenesis. Indeed, it is likely that some common variants do contribute to risk, although this risk is likely to be less than that conferred by rare or novel variants similar to those presented in this study.

5.7 Implications for classification and treatment of SLE

In addition to the contribution to understanding the genetic architecture of complex autoimmunity, these findings have direct implications for SLE. First, a formal diagnosis of SLE is based on meeting 4 of the 11 American College of Rheumatology criteria [14]. However, it has been well characterised through the literature that the clinical manifestations and underlying pathogenic mechanisms of SLE are diverse. The majority of clinical trials, diagnostic and prognostic studies are therefore restricted by this definition of SLE. By identifying molecular mechanisms causing SLE we may therefore reclassify SLE not as a clinical syndrome, but potentially by the genetic lesions or leukocyte or mechanistic pathways affected (i.e DNA degradation, complement activation).

The natural progression to refining SLE from a clinical phenotype to a cellular/genetic classification is the ability to better target therapy. Current non-specific anti-proliferative therapy and general immunosuppressives such as corticosteroids have a high side effect profile and in many cases do not adequately control disease. As a model of a B cell-mediated SLE, *BANK1*^{W40C}-related SLE may be more susceptible to B cell depletion therapy such as rituximab, which is only used as salvage therapy for refractory lupus in current regimes.

5.8 VANGL1 and kidney damage

Whilst SLE is classically associated with the capacity to involve most organs, we provide the first demonstration of genetic predisposition to involvement of a specific organ in SLE. *VANGL1* and *VANGL2* are important PCP genes that have been implicated in human neural tube defects [348], and *Vangl2* in murine kidney development and repair responses to injury [333]. Although loss of *Vangl2* in mouse models results in fatal neural tube defects, conditional loss of *Vangl2* in podocytes results in impaired glomerular repair and greater injury. In several SLE cohorts we identified a CNV in intron 7 of *VANGL1* which associates with nephritis in a gene-dose protective manner. Interestingly, the CNV identified does not represent a single deletion but several deletions of differing sizes in the kilobase range.

To determine the contribution of *VANGL1* CNV to nephritis we examined *Vangl1*$^{+/-}$ mice. Although not immediately comparable with the intronic CNV in the SLE cohorts, *Vangl1* deficient mice permitted examination of the effect of disrupting *Vangl1*. *Vangl1*-deficient mice developed spontaneous IgA and IgG deposition in their mesangium, but not IgM or complement deposits. We hypothesise that this pattern of Ig deposition may be due to disturbed glomerular endothelial expression of Vangl1 permitting passive diffusion of immunoglobulin across the endothelium of glomerular capillaries. This hypothesis stems from public databases of gene expression suggesting *Vangl1* is expressed in endothelial cells. This would explain why monomeric immunoglobulins IgA and IgG, but not pentameric IgM, are detected in the mesangium of *Vangl1*$^{+/-}$ mice. Consistent with this hypothesis and despite deposition of monomeric immunoglobulins, *Vangl1*$^{+/-}$ mice did not deposit complement or show any evidence of inflammation or glomerular injury. It is also likely that this is due to the deposited immunoglobulins not being auto-reactive. Generally, auto-reactive antibodies such as those found in SLE are known to be effective at activating

complement due to their ability to form large antigen-antibody complexes (immune complexes, IC), and inducing an inflammatory reaction.

We propose that the requirement for autoreactive antibody to cause pathology may prevent purifying selection of the relatively common CNV within *Vangl1* from an evolutionary perspective. To specifically test this hypothesis, we crossed *Vangl1* deficient mice onto a lupus-prone mouse model, the *Fas*^{lpr} mouse. Although the systemic autoimmunity in *Fas*$^{lpr/lpr}$ mice appears to induce nephritis regardless of *Vangl1* deficiency, possibly by overwhelming innate resistance kidney resistance to autoantibody deposition, there was a significant difference in renal function comparing *Fas*$^{lpr/+}$ *Vangl1*$^{+/-}$ and *Fas*$^{lpr/+}$ *Vangl1*$^{+/+}$ at 12 weeks of age although WT littermates had creatinine measurements comparable to the *Fas*$^{lpr/+}$ *Vangl1*$^{+/-}$.

5.9 Implications for drug therapy and transplantation

Genetic predisposition to glomerulonephritis (GN) has important implications for the management of patients with autoimmune GN. The first is primary prevention: by identifying patients with CNV in *VANGL1* who also have SLE, we may better predict onset and therefore intervene earlier in patients. Earlier detection and intervention will help minimise accumulation of kidney damage which adversely affects life quality and mortality. From a therapeutic perspective, genetic risk may impact on transplantation practices. Where traditionally live related donors are considered the gold standard for transplantation, in instances of genetic predisposition to GN due to 0 copies in the *VANGL1* intron 7 CNV, it may be that live unrelated donors are superior to related donors who may also carry genetic predisposition to nephritis.

5.10 Limitations of this thesis

Although this thesis strongly implicates rare variants in the development of SLE, best characterised by the phenotypes observed in the mouse carrying CRISPR-Cas9 edited orthologues of the *BANK1*, *BLK*, and *SAMSN1* human mutations, there are significant components to pathogenesis and the process of identifying variants which limit this study.

Coverage with either WES or WGS is imperfect with approximately a 10% and 5% respective false negative rate. It is thus possible that failure to cover genes will result in the inability to identify novel targets. Indeed, the scoring algorithm developed herein utilises imperfect tools such as Polyphen2 and known mouse phenotypes to identify probable targets. The weighting given to genes known to have a role in the immune system, while it can help identify obvious candidates, it is also a potential pitfall, as genes that may be important but who have no known function will therefore be missed. It should be noted however, than in establishing a method for proof of causation this was an accepted deficiency – to both discover novel functions (or functions of unknown genes) as well as their contribution to autoimmunity was beyond the scope of this exploratory work. Nevertheless, our lab has recently identified a novel gene that we predict causes SLE in a multiplex family, so it is not impossible to uncover such variants, particularly in large pedigrees. This is a process as a whole that will require continued development and refinement.

It is also well established that environmental, gender, epigenetic and infectious influences all predispose to the development of SLE. What this thesis proposes is that these rare variants strongly predispose but do not explain absolutely the pathogenesis of complex autoimmunity. Indeed, common variants may also still exert significant genetic risks and be in epistasis with rare coding variants as well as structural, non-coding, and epigenetic variants. The environmental influences likewise will modify the risks associated with these genetic variants. It is highly likely that as work to identify these variants increases, variants

with great epigenetic susceptibility will be identified as well as variants likely to have more fundamental deficits that will be highly penetrant.

In identifying and demonstrating the contribution of rare variants, this thesis does not entirely define the mechanisms through which these variants break tolerance. Although this thesis suggests a common pathway and relates the variants to each other, it does not explain how they combine to break B cell tolerance. That all the identified variants interact with each other suggests they represent a common pathway predisposing to autoimmunity. The newly generated mouse models will be invaluable in understanding lupus pathogenesis caused by the human mutations.

The *Vangl1*$^{-/-}$ mouse model used in this thesis does not recapitulate the *VANGL1* CNV identified SLE patients and differ in fundamental ways: The gene-trapped knockout are embryonic lethal in the homozygous state due to neural tube defects whereas patients homozygous for the identified CNV are commonly observed. Data from the Database of Genomic Variants reports multiple deletions of varying size in intron 7 of *VANGL1*, but not in any other intron or exon. This suggests that *VANGL1* tolerates deletions in intron 7, but not in any exon or other intron. Therefore the intron 7 CNV found in SLE patients with nephritis may have a specific functional defect beyond gene knockout, potentially as loss of a kidney-specific gene promoter within the intron. It is also plausible that the differences observed between *Vangl1* deficient mice and humans relate to differing functions of *Vangl1* in neural and kidney tissue in mice compared with humans.

Several important lines of further investigation follow from the identification of the CNV in *VANGL1*. It is unclear how intronic CNVs in *VANGL1* translate to kidney-specific injury in the absence of neural tube defects. It is possible that different exon usage and therefore isoforms are expressed at the different tissues or during development. To date, the 4-coding transcripts described for human VANGL1 appear to share the same pattern of

intron 7-splicing (Ensembl.org) but libraries of all alternatively spliced-variants expressed across different tissues are still incomplete. The relatively high frequency of CNVs in intron 7 of *VANGL1* would suggest limited selectivity against these CNVs, perhaps even balancing selection where the heterozygous deletion may confer some selective advantage for a hitherto unknown trait. Although many antibody-mediated kidney diseases can be associated with specific antibodies and epitopes, the kidney intrinsic defect from *Vangl1* deficiency may predispose to any form of antibody-mediated kidney disease. The precise mechanism through which *Vangl1* deficiency permits this antibody deposition in such a kidney-intrinsic manner requires further elucidation, but it is possibly related to the defective podocyte repair mechanism described for Vangl2 [333]. In summary, we have described how a common CNV in *VANGL1* predisposes towards nephritis in a kidney-intrinsic manner. This offers novel insights into how SLE may develop predilection for specific tissues and highlights the emerging roles of VANGL1 and other PCP genes in kidney injury and repair.

5.11 Future directions

5.11.1 How does *BANK1*^{W40C} affect tolerance and its role in autoimmunity and B cell signalling

Identification of *BANK1*^{W40C} as a central variant in the predisposition to SLE will form the basis of several lines of investigation. Linking BCR and TLR signalling implies it may have an important role in regulation of NF-κB in the manner reported by Rui [128]. The capacity to sequester TRAF6 into sequestersomes and the elevated IL-6 production observed implicates BANK1 in NF-κB signalling. Crossing the *BANK1*^{W40C} strain with Cblb^{-}

HyHel10^{Tg} will allow testing of whether BCR stimulation with autoantigen is capable of repressing TLR9 mediated autoimmunity in the presence of *BANK1*^{W40C}.

Extension of stimulation studies should be performed with controls of BANK1$^{-/-}$ B cells to determine the polarity of the variant. Breeding to *BANK1*^{W40C} homozygosity and stimulation through BCR and TLR receptors with appropriate negative controls illustrate whether the variant is gain or loss of function. It would be useful to determine if the interaction with TRAF6 extends to other TRAF family members such as TRAF3. The BANK1 variant will need to be crossed with all identified variants to determine if epistatic interactions occur and whether the autoimmune and cellular phenotype observed is altered by the second variant's effect in BCR or TLR signalling.

5.11.2 What role does SAMSN1 in TLR and BCR signalling?

Similar to *BANK1*^{W40C}, further exploration of the role of SAMSN1 will be crucial. Foremost will be to cross *SAMSN1*^{T198A} to the *BANK1*^{W40C} mice to determine potential epistatic interactions between the variants, both in autoimmune phenotype and B cell responses to BCR and TLR signalling. The nature of the interaction between LYN, BLK and SAMSN1 will require further exploration, determining if *SAMSN1*$^{-/-}$ mice or *SAMSN1*^{T198A} mice have constitutive activation of LYN, and whether the SAV may alter the LYN-/- phenotype. Replication of the CpG-mediated IL-10 phenotype observed in CRISPR-Cas9 edited *SAMSN1*^{T198A} Ramos cell lines will need to be replicated in the *SAMSN1*^{T198A} murine B cells and the mechanisms of this specific response explored further. One potential explanation is that SAMSN1 may be a specific scavenging receptor for CpG as its expression profile with some similarity to that of DEC205, a scavenging receptor which may play a role in recognition and endocytosis of self-antigen and contribute to peripheral tolerance. Replication of TLR9 stimulation experiments in the *SAMSN1*^{T198A} mice should be

performed alongside a *SAMSN1*$^{-/-}$ control to determine also the polarity of the *SAMSN1*^{T198A} variant.

5.11.3 How does *BLK*^{R131W} alter BCR signalling and does *BLK*^{R131W} exert epistasis with *BANK1*^{W40C}?

BLK$^{-/-}$ mice develop expanded MZ B cell populations and glomerulonephritis. This thesis demonstrates *BLK*^{R131W} as a loss of function variant. The mechanism through which BLK deficiency contributes to B cell autoimmunity remains to be elucidated. Several hypothesis may explain these observations. BLK, in combination with other Src family members, alters BCR signalling and may fail to dampen autoantigen receptor engagement adequately, thereby causing impairing necessary signals for receptor editing, deletion or anergy. The MZ phenotype observed in *Blk*$^{-/-}$ mice supports the role of BCR signalling abnormalities. Its mechanism of action may also be mediated through impaired BANK1 signalling as evidenced by reduced phosphorylation of BANK1 observed in this thesis.

5.11.4 How does deletion of VANGL1 result in immunoglobulin deposition? Does VANGL1 affect other antibody mediated kidney disease?

Although this thesis offers evidence supporting a kidney intrinsic mechanism of antibody deposition, the mechanism through which this occurs is unclear. Based on gene expression profiles suggesting expression of VANGL1 in vascular endothelium (CD34+ and CD105+ populations) and absence of a discernible peripheral autoimmune phenotype, it is possible that deficiency of VANGL1 results from abnormal development of endothelium permitting passage of antibodies into the kidney mesangium.

If the lesion represents a predisposition to immunoglobulin, it would then not necessarily represent a predisposition to SLE but to any antibody mediated kidney disease.

Further lines of investigation would involve determining if the copy number variation is highly represented in other autoimmune glomerulonephritis conditions such as IgA nephropathy and membranous glomerulonephritis. Similarly, epidemiological data of transplantation outcomes, comparing recurrence of autoimmune glomerulonephritis between live unrelated and live related donors, may support a kidney intrinsic predisposition to nephritis. Such an observation would have significant transplant policy and treatment outcomes.

5.12 Conclusion

This thesis has used WES to identify rare gene variants in a pathways that links BCR with TLR signalling and can predispose to complex autoimmunity. This approach demonstrates how WES/WGS can provide novel insights into gene function and map novel pathways in human autoimmune disease. These findings support the hypothesis that complex autoimmunity may result from as little as one or two rare variants with strongly deleterious effects that act alone or together to alter B cell responses. This thesis also demonstrates that genetic mechanisms exist that may explain some of the organ-specificity in SLE.

Chapter 6:Bibliography

1. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ: **The 1982 revised criteria for the classification of systemic lupus erythematosus**. *Arthritis Rheum* 1982, **25**:1271-1277.
2. Danchenko N, Satia JA, Anthony MS: **Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden**. *Lupus* 2006, **15**:308-318.
3. Robak E, Robak T: **Monoclonal antibodies in the treatment of systemic lupus erythematosus**. *Curr Drug Targets* 2009, **10**:26-37.
4. Furie R, Petri M, Zamani O, Cervera R, Wallace DJ, Tegzova D, Sanchez-Guerrero J, Schwarting A, Merrill JT, Chatham WW, et al.: **A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus**. *Arthritis Rheum* 2011, **63**:3918-3930.
5. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, Jimenez RE, Li EK, Thomas M, Kim HY, Leon MG, et al.: **Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial**. *Lancet* 2011, **377**:721-731.
6. Smith CD, Cyr M: **The history of lupus erythematosus. From Hippocrates to Osler**. *Rheum Dis Clin North Am* 1988, **14**:1-14.
7. Cazenave PLAe, Schedel HE, Biett, Burgess TH, Bulkley HD: *Manual of diseases of the skin* edn 2d American. New York,: S. S. & W. Wood; 1852.
8. Kaposi M, Fox GH, Johnston JC: *Pathology and treatment of diseases of the skin, for practitioners and students*. New York,: W. Wood & company; 1895.
9. Osler W: **Classics: On the visceral complications of erythema exudativum multiforme. William Osler, M.D., F.R.C.P. Lond**. *Am J Med Sci* 1976, **271**:106-117.
10. Cerovec M, Anic B, Padjen I, Cikes N: **Prevalence of the American College of Rheumatology classification criteria in a group of 162 systemic lupus erythematosus patients from Croatia**. *Croat Med J* 2012, **53**:149-154.
11. Manger K, Manger B, Repp R, Geisselbrecht M, Geiger A, Pfahlberg A, Harrer T, Kalden JR: **Definition of risk factors for death, end stage renal disease, and thromboembolic events in a monocentric cohort of 338 patients with systemic lupus erythematosus**. *Ann Rheum Dis* 2002, **61**:1065-1070.
12. Bujan S, Ordi-Ros J, Paredes J, Mauri M, Matas L, Cortes J, Vilardell M: **Contribution of the initial features of systemic lupus erythematosus to the clinical evolution and survival of a cohort of Mediterranean patients**. *Ann Rheum Dis* 2003, **62**:859-865.
13. Hochberg MC: **Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus**. *Arthritis Rheum* 1997, **40**:1725.
14. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, Bruce IN, Isenberg D, Wallace DJ, Nived O, et al.: **Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus**. *Arthritis Rheum* 2012, **64**:2677-2686.
15. Vilar MJ, Sato EI: **Estimating the incidence of systemic lupus erythematosus in a tropical region (Natal, Brazil)**. *Lupus* 2002, **11**:528-532.
16. McCarty DJ, Manzi S, Medsger TA, Jr., Ramsey-Goldman R, LaPorte RE, Kwoh CK: **Incidence of systemic lupus erythematosus. Race and gender differences**. *Arthritis Rheum* 1995, **38**:1260-1270.
17. Uramoto KM, Michet CJ, Jr., Thumboo J, Sunku J, O'Fallon WM, Gabriel SE: **Trends in the incidence and mortality of systemic lupus erythematosus, 1950-1992**. *Arthritis Rheum* 1999, **42**:46-50.
18. Johnson AE, Gordon C, Palmer RG, Bacon PA: **The prevalence and incidence of systemic lupus erythematosus in Birmingham, England. Relationship to ethnicity and country of birth**. *Arthritis Rheum* 1995, **38**:551-558.
19. Borchers AT, Naguwa SM, Shoenfeld Y, Gershwin ME: **The geoepidemiology of systemic lupus erythematosus**. *Autoimmun Rev* 2010, **9**:A277-287.

20. Borchers AT, Keen CL, Shoenfeld Y, Gershwin ME: **Surviving the butterfly and the wolf: mortality trends in systemic lupus erythematosus**. *Autoimmun Rev* 2004, **3**:423-453.
21. Kasitanon N, Magder LS, Petri M: **Predictors of survival in systemic lupus erythematosus**. *Medicine (Baltimore)* 2006, **85**:147-156.
22. Pons-Estel BA, Catoggio LJ, Cardiel MH, Soriano ER, Gentiletti S, Villa AR, Abadi I, Caeiro F, Alvarellos A, Alarcon-Segovia D, et al.: **The GLADEL multinational Latin American prospective inception cohort of 1,214 patients with systemic lupus erythematosus: ethnic and disease heterogeneity among "Hispanics"**. *Medicine (Baltimore)* 2004, **83**:1-17.
23. Houman MH, Smiti-Khanfir M, Ben Ghorbell I, Miled M: **Systemic lupus erythematosus in Tunisia: demographic and clinical analysis of 100 patients**. *Lupus* 2004, **13**:204-211.
24. Rabbani MA, Habib HB, Islam M, Ahmad B, Majid S, Saeed W, Shah SM, Ahmad A: **Survival analysis and prognostic indicators of systemic lupus erythematosus in Pakistani patients**. *Lupus* 2009, **18**:848-855.
25. Wadee S, Tikly M, Hopley M: **Causes and predictors of death in South Africans with systemic lupus erythematosus**. *Rheumatology (Oxford)* 2007, **46**:1487-1491.
26. Kaslow RA: **High rate of death caused by systemic lupus erythematosus among U. S. residents of Asian descent**. *Arthritis Rheum* 1982, **25**:414-418.
27. Krishnan E, Hubert HB: **Ethnicity and mortality from systemic lupus erythematosus in the US**. *Ann Rheum Dis* 2006, **65**:1500-1505.
28. Urowitz MB, Gladman DD: **Evolving spectrum of mortality and morbidity in SLE**. *Lupus* 1999, **8**:253-255.
29. Appel GB, Contreras G, Dooley MA, Ginzler EM, Isenberg D, Jayne D, Li LS, Mysler E, Sanchez-Guerrero J, Solomons N, et al.: **Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis**. *J Am Soc Nephrol* 2009, **20**:1103-1112.
30. Davidson A: **The rationale for BAFF inhibition in systemic lupus erythematosus**. *Curr Rheumatol Rep* 2012, **14**:295-302.
31. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, Maciuca R, Zhang D, Garg JP, Brunetta P, et al.: **Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study**. *Arthritis Rheum* 2012, **64**:1215-1226.
32. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J: **T cell antigen receptor recognition of antigen-presenting molecules**. *Annu Rev Immunol* 2015, **33**:169-200.
33. Heath WR, Carbone FR: **Cross-presentation in viral immunity and self-tolerance**. *Nat Rev Immunol* 2001, **1**:126-134.
34. Klein L, Kyewski B, Allen PM, Hogquist KA: **Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see)**. *Nat Rev Immunol* 2014, **14**:377-391.
35. Zhu J, Yamane H, Paul WE: **Differentiation of effector CD4 T cell populations (*)**. *Annu Rev Immunol* 2010, **28**:445-489.
36. Tobon GJ, Izquierdo JH, Canas CA: **B lymphocytes: development, tolerance, and their role in autoimmunity-focus on systemic lupus erythematosus**. *Autoimmune Dis* 2013, **2013**:827254.
37. Kaminski DA, Wei C, Qian Y, Rosenberg AF, Sanz I: **Advances in human B cell phenotypic profiling**. *Front Immunol* 2012, **3**:302.
38. Wirths S, Lanzavecchia A: **ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells**. *Eur J Immunol* 2005, **35**:3433-3441.
39. Harwood NE, Batista FD: **Early events in B cell activation**. *Annu Rev Immunol* 2010, **28**:185-210.
40. Kurosaki T: **Regulation of BCR signaling**. *Mol Immunol* 2011, **48**:1287-1291.
41. Pascual V, Liu YJ, Magalski A, de Bouteiller O, Banchereau J, Capra JD: **Analysis of somatic mutation in five B cell subsets of human tonsil**. *J Exp Med* 1994, **180**:329-339.
42. Nemazee D, Buerki K: **Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras**. *Proc Natl Acad Sci U S A* 1989, **86**:8039-8043.

43. Goodnow CC, Crosbie J, Jorgensen H, Brink RA, Basten A: **Induction of self-tolerance in mature peripheral B lymphocytes**. *Nature* 1989, **342**:385-391.
44. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC: **Predominant autoantibody production by early human B cell precursors**. *Science* 2003, **301**:1374-1377.
45. Lesley R, Xu Y, Kalled SL, Hess DM, Schwab SR, Shu HB, Cyster JG: **Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF**. *Immunity* 2004, **20**:441-453.
46. Cook MC, Basten A, Fazekas de St Groth B: **Rescue of self-reactive B cells by provision of T cell help in vivo**. *Eur J Immunol* 1998, **28**:2549-2558.
47. Sabouri Z, Schofield P, Horikawa K, Spierings E, Kipling D, Randall KL, Langley D, Roome B, Vazquez-Lombardi R, Rouet R, et al.: **Redemption of autoantibodies on anergic B cells by variable-region glycosylation and mutation away from self-reactivity**. *Proc Natl Acad Sci U S A* 2014, **111**:E2567-2575.
48. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, Schwartzberg PL, Cook MC, Walters GD, Vinuesa CG: **Follicular helper T cells are required for systemic autoimmunity**. *J Exp Med* 2009, **206**:561-576.
49. Zubler RH: **Naive and memory B cells in T-cell-dependent and T-independent responses**. *Springer Semin Immunopathol* 2001, **23**:405-419.
50. Akira S, Takeda K: **Toll-like receptor signalling**. *Nat Rev Immunol* 2004, **4**:499-511.
51. Hua Z, Hou B: **TLR signaling in B-cell development and activation**. *Cell Mol Immunol* 2013, **10**:103-106.
52. Jiang SH, Shen N, Vinuesa CG: **Posttranscriptional T cell gene regulation to limit Tfh cells and autoimmunity**. *Curr Opin Immunol* 2015, **37**:21-27.
53. Ricklin D, Hajishengallis G, Yang K, Lambris JD: **Complement: a key system for immune surveillance and homeostasis**. *Nat Immunol* 2010, **11**:785-797.
54. Harboe M, Mollnes TE: **The alternative complement pathway revisited**. *J Cell Mol Med* 2008, **12**:1074-1084.
55. Rutkowski MJ, Sughrue ME, Kane AJ, Mills SA, Parsa AT: **Cancer and the complement cascade**. *Mol Cancer Res* 2010, **8**:1453-1465.
56. Illei GG, Tackey E, Lapteva L, Lipsky PE: **Biomarkers in systemic lupus erythematosus: II. Markers of disease activity**. *Arthritis Rheum* 2004, **50**:2048-2065.
57. Alper CA, Rosen FS: **Alper CA, Rosen FS: Studies of the in vivo behavior of human C'3 in normal subjects and patients**. *J Clin Invest* 1967, **46**:2021-2034.
58. Hopkins P, Belmont HM, Buyon J, Philips M, Weissmann G, Abramson SB: **Increased levels of plasma anaphylatoxins in systemic lupus erythematosus predict flares of the disease and may elicit vascular injury in lupus cerebritis**. *Arthritis Rheum* 1988, **31**:632-641.
59. Manzi S, Rairie JE, Carpenter AB, Kelly RH, Jagarlapudi SP, Sereika SM, Medsger TA, Jr., Ramsey-Goldman R: **Sensitivity and specificity of plasma and urine complement split products as indicators of lupus disease activity**. *Arthritis Rheum* 1996, **39**:1178-1188.
60. Porcel JM, Ordi J, Castro-Salomo A, Vilardell M, Rodrigo MJ, Gene T, Warburton F, Kraus M, Vergani D: **The value of complement activation products in the assessment of systemic lupus erythematosus flares**. *Clin Immunol Immunopathol* 1995, **74**:283-288.
61. Nagy G, Brozik M, Tornoci L, Gergely P: **Diagnostic value of combined evaluation of neopterin and anti-DNA antibody levels for assessment of disease activity in systemic lupus erythematosus**. *Clin Exp Rheumatol* 2000, **18**:699-705.
62. Truedsson L, Bengtsson AA, Sturfelt G: **Complement deficiencies and systemic lupus erythematosus**. *Autoimmunity* 2007, **40**:560-566.
63. Zhao J, Wu H, Khosravi M, Cui H, Qian X, Kelly JA, Kaufman KM, Langefeld CD, Williams AH, Comeau ME, et al.: **Association of genetic variants in complement factor H and factor H-related genes with systemic lupus erythematosus susceptibility**. *PLoS Genet* 2011, **7**:e1002079.

64. Arkwright PD, Riley P, Hughes SM, Alachkar H, Wynn RF: **Successful cure of C1q deficiency in human subjects treated with hematopoietic stem cell transplantation**. *Journal of Allergy and Clinical Immunology* 2014, **133**:265-267.
65. Clarke EV, Weist BM, Walsh CM, Tenner AJ: **Complement protein C1q bound to apoptotic cells suppresses human macrophage and dendritic cell-mediated Th17 and Th1 T cell subset proliferation**. *J Leukoc Biol* 2015, **97**:147-160.
66. Benoit ME, Clarke EV, Morgado P, Fraser DA, Tenner AJ: **Complement protein C1q directs macrophage polarization and limits inflammasome activity during the uptake of apoptotic cells**. *J Immunol* 2012, **188**:5682-5693.
67. Marto N, Bertolaccini ML, Calabuig E, Hughes GR, Khamashta MA: **Anti-C1q antibodies in nephritis: correlation between titres and renal disease activity and positive predictive value in systemic lupus erythematosus**. *Ann Rheum Dis* 2005, **64**:444-448.
68. Yin Y, Wu X, Shan G, Zhang X: **Diagnostic value of serum anti-C1q antibodies in patients with lupus nephritis: a meta-analysis**. *Lupus* 2012, **21**:1088-1097.
69. Jonsson G, Sjoholm AG, Truedsson L, Bengtsson AA, Braconier JH, Sturfelt G: **Rheumatological manifestations, organ damage and autoimmunity in hereditary C2 deficiency**. *Rheumatology (Oxford)* 2007, **46**:1133-1139.
70. Sullivan KE, Petri MA, Schmeckpeper BJ, McLean RH, Winkelstein JA: **Prevalence of a mutation causing C2 deficiency in systemic lupus erythematosus**. *J Rheumatol* 1994, **21**:1128-1133.
71. Boteva L, Morris DL, Cortes-Hernandez J, Martin J, Vyse TJ, Fernando MM: **Genetically determined partial complement C4 deficiency states are not independent risk factors for SLE in UK and Spanish populations**. *Am J Hum Genet* 2012, **90**:445-456.
72. Truedsson L, Sturfelt G, Johansen P, Nived O, Thuresson B: **Sharing of MHC haplotypes among patients with systemic lupus erythematosus from unrelated Caucasian multicase families: disease association with the extended haplotype [HLA-B8, SC01, DR17]**. *J Rheumatol* 1995, **22**:1852-1861.
73. Christiansen FT, Dawkins RL, Uko G, McCluskey J, Kay PH, Zilko PJ: **Complement allotyping in SLE: association with C4A null**. *Aust N Z J Med* 1983, **13**:483-488.
74. Howard PF, Hochberg MC, Bias WB, Arnett FC, Jr., McLean RH: **Relationship between C4 null genes, HLA-D region antigens, and genetic susceptibility to systemic lupus erythematosus in Caucasian and black Americans**. *Am J Med* 1986, **81**:187-193.
75. Skarsvag S: **The importance of C4A null genes in Norwegian patients with systemic lupus erythematosus**. *Scand J Immunol* 1995, **42**:572-576.
76. Awdeh ZL, Raum DD, Glass D, Agnello V, Schur PH, Johnston RB, Jr., Gelfand EW, Ballow M, Yunis E, Alper CA: **Complement-human histocompatibility antigen haplotypes in C2 deficiency**. *J Clin Invest* 1981, **67**:581-583.
77. Nath SK, Han S, Kim-Howard X, Kelly JA, Viswanathan P, Gilkeson GS, Chen W, Zhu C, McEver RP, Kimberly RP, et al.: **A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus**. *Nat Genet* 2008, **40**:152-154.
78. Carroll MC: **A protective role for innate immunity in systemic lupus erythematosus**. *Nat Rev Immunol* 2004, **4**:825-831.
79. Chatterjee P, Agyemang AF, Alimzhanov MB, Degn S, Tsiftsoglou SA, Alicot E, Jones SA, Ma M, Carroll MC: **Complement C4 maintains peripheral B-cell tolerance in a myeloid cell dependent manner**. *Eur J Immunol* 2013, **43**:2441-2450.
80. Iida K, Mornaghi R, Nussenzweig V: **Complement receptor (CR1) deficiency in erythrocytes from patients with systemic lupus erythematosus**. *J Exp Med* 1982, **155**:1427-1438.
81. Walport MJ, Ross GD, Mackworth-Young C, Watson JV, Hogg N, Lachmann PJ: **Family studies of erythrocyte complement receptor type 1 levels: reduced levels in patients with SLE are acquired, not inherited**. *Clin Exp Immunol* 1985, **59**:547-554.
82. Townsend MJ, Monroe JG, Chan AC: **B-cell targeted therapies in human autoimmune diseases: an updated perspective**. *Immunol Rev* 2010, **237**:264-283.

83. Meffre E: **The establishment of early B cell tolerance in humans: lessons from primary immunodeficiency diseases**. *Ann N Y Acad Sci* 2011, **1246**:1-10.
84. Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA, Pritchard-Briscoe H, Wotherspoon JS, Loblay RH, Raphael K, et al.: **Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice**. *Nature* 1988, **334**:676-682.
85. Burnet FM: **A modification of Jerne's theory of antibody production using the concept of clonal selection (Reprinted from Australian Journal of Science, vol 20, 1957)**. *Nature Immunology* 2007, **8**:1024-1026.
86. Nemazee DA, Burki K: **Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes**. *Nature* 1989, **337**:562-566.
87. Hartley SB, Crosbie J, Brink R, Kantor AB, Basten A, Goodnow CC: **Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens**. *Nature* 1991, **353**:765-769.
88. Gay D, Saunders T, Camper S, Weigert M: **Receptor editing: an approach by autoreactive B cells to escape tolerance**. *J Exp Med* 1993, **177**:999-1008.
89. Radic MZ, Erikson J, Litwin S, Weigert M: **B lymphocytes may escape tolerance by revising their antigen receptors**. *J Exp Med* 1993, **177**:1165-1173.
90. Tiegs SL, Russell DM, Nemazee D: **Receptor editing in self-reactive bone marrow B cells**. *J Exp Med* 1993, **177**:1009-1020.
91. Pelanda R, Schwers S, Sonoda E, Torres RM, Nemazee D, Rajewsky K: **Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification**. *Immunity* 1997, **7**:765-775.
92. Hippen KL, Schram BR, Tze LE, Pape KA, Jenkins MK, Behrens TW: **In vivo assessment of the relative contributions of deletion, anergy, and editing to B cell self-tolerance**. *J Immunol* 2005, **175**:909-916.
93. Halverson R, Torres RM, Pelanda R: **Receptor editing is the main mechanism of B cell tolerance toward membrane antigens**. *Nat Immunol* 2004, **5**:645-650.
94. Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, Ambrose C, Lawton P, Bixler S, Acha-Orbea H, et al.: **BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth**. *J Exp Med* 1999, **189**:1747-1756.
95. Shu HB, Hu WH, Johnson H: **TALL-1 is a novel member of the TNF family that is down-regulated by mitogens**. *J Leukoc Biol* 1999, **65**:680-683.
96. Mukhopadhyay A, Ni J, Zhai Y, Yu GL, Aggarwal BB: **Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase**. *J Biol Chem* 1999, **274**:15978-15981.
97. Grewal IS, Flavell RA: **CD40 and CD154 in cell-mediated immunity**. *Annu Rev Immunol* 1998, **16**:111-135.
98. Stoddart A, Fleming HE, Paige CJ: **The role of the preBCR, the interleukin-7 receptor, and homotypic interactions during B-cell development**. *Immunol Rev* 2000, **175**:47-58.
99. Nardelli B, Belvedere O, Roschke V, Moore PA, Olsen HS, Migone TS, Sosnovtseva S, Carrell JA, Feng P, Giri JG, et al.: **Synthesis and release of B-lymphocyte stimulator from myeloid cells**. *Blood* 2001, **97**:198-204.
100. Hahne M, Kataoka T, Schroter M, Hofmann K, Irmler M, Bodmer JL, Schneider P, Bornand T, Holler N, French LE, et al.: **APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth**. *J Exp Med* 1998, **188**:1185-1190.
101. Kelly K, Manos E, Jensen G, Nadauld L, Jones DA: **APRIL/TRDL-1, a tumor necrosis factor-like ligand, stimulates cell death**. *Cancer Res* 2000, **60**:1021-1027.
102. Mackay F, Browning JL: **BAFF: a fundamental survival factor for B cells**. *Nat Rev Immunol* 2002, **2**:465-475.

103. Laabi Y, Gras MP, Carbonnel F, Brouet JC, Berger R, Larsen CJ, Tsapis A: **A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma**. *EMBO J* 1992, **11**:3897-3904.
104. Madry C, Laabi Y, Callebaut I, Roussel J, Hatzoglou A, Le Coniat M, Mornon JP, Berger R, Tsapis A: **The characterization of murine BCMA gene defines it as a new member of the tumor necrosis factor receptor superfamily**. *Int Immunol* 1998, **10**:1693-1702.
105. Wu Y, Bressette D, Carrell JA, Kaufman T, Feng P, Taylor K, Gan Y, Cho YH, Garcia AD, Gollatz E, et al.: **Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLyS**. *J Biol Chem* 2000, **275**:35478-35485.
106. Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, Hession C, Schneider P, Sizing ID, Mullen C, et al.: **BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF**. *Science* 2001, **293**:2108-2111.
107. Yan M, Brady JR, Chan B, Lee WP, Hsu B, Harless S, Cancro M, Grewal IS, Dixit VM: **Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency**. *Curr Biol* 2001, **11**:1547-1552.
108. Loder F, Mutschler B, Ray RJ, Paige CJ, Sideras P, Torres R, Lamers MC, Carsetti R: **B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals**. *J Exp Med* 1999, **190**:75-89.
109. Allman DM, Ferguson SE, Lentz VM, Cancro MP: **Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells**. *J Immunol* 1993, **151**:4431-4444.
110. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, Frew E, Scott ML: **An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway**. *Science* 2001, **293**:2111-2114.
111. Gross JA, Dillon SR, Mudri S, Johnston J, Littau A, Roque R, Rixon M, Schou O, Foley KP, Haugen H, et al.: **TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. impaired B cell maturation in mice lacking BLyS**. *Immunity* 2001, **15**:289-302.
112. Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S, Harris AW: **Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease**. *Proc Natl Acad Sci U S A* 1991, **88**:8661-8665.
113. Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, Xu W, Parrish-Novak J, Foster D, Lofton-Day C, et al.: **TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease**. *Nature* 2000, **404**:995-999.
114. Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, Tschopp J, Browning JL: **Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations**. *J Exp Med* 1999, **190**:1697-1710.
115. Do RK, Hatada E, Lee H, Tourigny MR, Hilbert D, Chen-Kiang S: **Attenuation of apoptosis underlies B lymphocyte stimulator enhancement of humoral immune response**. *J Exp Med* 2000, **192**:953-964.
116. Batten M, Groom J, Cachero TG, Qian F, Schneider P, Tschopp J, Browning JL, Mackay F: **BAFF mediates survival of peripheral immature B lymphocytes**. *J Exp Med* 2000, **192**:1453-1466.
117. Moore PA, Belvedere O, Orr A, Pieri K, LaFleur DW, Feng P, Soppet D, Charters M, Gentz R, Parmelee D, et al.: **BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator**. *Science* 1999, **285**:260-263.
118. Khare SD, Sarosi I, Xia XZ, McCabe S, Miner K, Solovyev I, Hawkins N, Kelley M, Chang D, Van G, et al.: **Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice**. *Proc Natl Acad Sci U S A* 2000, **97**:3370-3375.
119. Groom JR, Fletcher CA, Walters SN, Grey ST, Watt SV, Sweet MJ, Smyth MJ, Mackay CR, Mackay F: **BAFF and MyD88 signals promote a lupuslike disease independent of T cells**. *J Exp Med* 2007, **204**:1959-1971.

120. Amano M, Baumgarth N, Dick MD, Brossay L, Kronenberg M, Herzenberg LA, Strober S: **CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms**. *J Immunol* 1998, **161**:1710-1717.
121. Grimaldi CM, Michael DJ, Diamond B: **Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus**. *J Immunol* 2001, **167**:1886-1890.
122. Zhang J, Roschke V, Baker KP, Wang Z, Alarcon GS, Fessler BJ, Bastian H, Kimberly RP, Zhou T: **Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus**. *J Immunol* 2001, **166**:6-10.
123. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, Tschopp J, Cachero TG, Batten M, Wheway J, et al.: **Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome**. *J Clin Invest* 2002, **109**:59-68.
124. Yurasov S, Tiller T, Tsuiji M, Velinzon K, Pascual V, Wardemann H, Nussenzweig MC: **Persistent expression of autoantibodies in SLE patients in remission**. *J Exp Med* 2006, **203**:2255-2261.
125. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, Nussenzweig MC: **Defective B cell tolerance checkpoints in systemic lupus erythematosus**. *J Exp Med* 2005, **201**:703-711.
126. Taylor KE, Chung SA, Graham RR, Ortmann WA, Lee AT, Langefeld CD, Jacob CO, Kamboh MI, Alarcon-Riquelme ME, Tsao BP, et al.: **Risk alleles for systemic lupus erythematosus in a large case-control collection and associations with clinical subphenotypes**. *PLoS Genet* 2011, **7**:e1001311.
127. Isnardi I, Ng YS, Srdanovic I, Motaghedi R, Rudchenko S, von Bernuth H, Zhang SY, Puel A, Jouanguy E, Picard C, et al.: **IRAK-4- and MyD88-dependent pathways are essential for the removal of developing autoreactive B cells in humans**. *Immunity* 2008, **29**:746-757.
128. Rui L, Vinuesa CG, Blasioli J, Goodnow CC: **Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling**. *Nat Immunol* 2003, **4**:594-600.
129. Dorner T, Jacobi AM, Lipsky PE: **B cells in autoimmunity**. *Arthritis Res Ther* 2009, **11**:247.
130. Tipton CM, Fucile CF, Darce J, Chida A, Ichikawa T, Gregoretti I, Schieferl S, Hom J, Jenks S, Feldman RJ, et al.: **Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus**. *Nat Immunol* 2015, **16**:755-765.
131. Cappione A, 3rd, Anolik JH, Pugh-Bernard A, Barnard J, Dutcher P, Silverman G, Sanz I: **Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus**. *J Clin Invest* 2005, **115**:3205-3216.
132. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, Lipsky PE, Radbruch A, Dorner T: **Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus**. *J Immunol* 2000, **165**:5970-5979.
133. Zhang J, Jacobi AM, Wang T, Diamond B: **Pathogenic autoantibodies in systemic lupus erythematosus are derived from both self-reactive and non-self-reactive B cells**. *Mol Med* 2008, **14**:675-681.
134. Sanz I, Kelly P, Williams C, Scholl S, Tucker P, Capra JD: **The smaller human VH gene families display remarkably little polymorphism**. *EMBO J* 1989, **8**:3741-3748.
135. Pugh-Bernard AE, Silverman GJ, Cappione AJ, Villano ME, Ryan DH, Insel RA, Sanz I: **Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance**. *J Clin Invest* 2001, **108**:1061-1070.
136. Anolik J, Sanz I: **B cells in human and murine systemic lupus erythematosus**. *Curr Opin Rheumatol* 2004, **16**:505-512.
137. Jacobi AM, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, Hansen A, Burmester GR, Diamond B, Lipsky PE, et al.: **Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95**. *Arthritis Rheum* 2008, **58**:1762-1773.

138. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, Lee EH, Milner EC, Sanz I: **A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus**. *J Immunol* 2007, **178**:6624-6633.
139. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, Warnatz K: **A new CD21low B cell population in the peripheral blood of patients with SLE**. *Clin Immunol* 2004, **113**:161-171.
140. Griffin DO, Rothstein TL: **A small CD11b(+) human B1 cell subpopulation stimulates T cells and is expanded in lupus**. *J Exp Med* 2011, **208**:2591-2598.
141. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE: **Identification and characterization of circulating human transitional B cells**. *Blood* 2005, **105**:4390-4398.
142. Jacobi AM, Odendahl M, Reiter K, Bruns A, Burmester GR, Radbruch A, Valet G, Lipsky PE, Dorner T: **Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus**. *Arthritis Rheum* 2003, **48**:1332-1342.
143. Dorner T, Lipsky PE: **Molecular basis of immunoglobulin variable region gene usage in systemic autoimmunity**. *Clin Exp Med* 2005, **4**:159-169.
144. Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D, Radbruch A, Hiepe F, Manz RA: **Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice**. *J Exp Med* 2004, **199**:1577-1584.
145. Illei GG, Shirota Y, Yarboro CH, Daruwalla J, Tackey E, Takada K, Fleisher T, Balow JE, Lipsky PE: **Tocilizumab in systemic lupus erythematosus: data on safety, preliminary efficacy, and impact on circulating plasma cells from an open-label phase I dosage-escalation study**. *Arthritis Rheum* 2010, **62**:542-552.
146. Heymann W, Hackel DB, Harwood S, Wilson SG, Hunter JL: **Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions. 1951**. *J Am Soc Nephrol* 2000, **11**:183-188.
147. Nimmerjahn F, Ravetch JV: **Fc-receptors as regulators of immunity**. *Adv Immunol* 2007, **96**:179-204.
148. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV: **Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice**. *Nature* 1996, **379**:346-349.
149. Dorner T, Giesecke C, Lipsky PE: **Mechanisms of B cell autoimmunity in SLE**. *Arthritis Res Ther* 2011, **13**:243.
150. Dorner T, Jacobi AM, Lee J, Lipsky PE: **Abnormalities of B cell subsets in patients with systemic lupus erythematosus**. *J Immunol Methods* 2011, **363**:187-197.
151. Guo W, Smith D, Aviszus K, Detanico T, Heiser RA, Wysocki LJ: **Somatic hypermutation as a generator of antinuclear antibodies in a murine model of systemic autoimmunity**. *J Exp Med* 2010, **207**:2225-2237.
152. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S: **Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function**. *Immunity* 1998, **9**:143-150.
153. Ma J, Xu J, Madaio MP, Peng Q, Zhang J, Grewal IS, Flavell RA, Craft J: **Autoimmune lpr/lpr mice deficient in CD40 ligand: spontaneous Ig class switching with dichotomy of autoantibody responses**. *J Immunol* 1996, **157**:417-426.
154. Komori H, Furukawa H, Mori S, Ito MR, Terada M, Zhang MC, Ishii N, Sakuma N, Nose M, Ono M: **A signal adaptor SLAM-associated protein regulates spontaneous autoimmunity and Fas-dependent lymphoproliferation in MRL-Faslpr lupus mice**. *J Immunol* 2006, **176**:395-400.
155. Hron JD, Caplan L, Gerth AJ, Schwartzberg PL, Peng SL: **SH2D1A regulates T-dependent humoral autoimmunity**. *J Exp Med* 2004, **200**:261-266.

156. Lee SK, Silva DG, Martin JL, Pratama A, Hu X, Chang P-P, Walters G, Vinuesa CG: **Interferon-γ excess leads to pathogenic accumulation of follicular helper T cells and germinal centers**. *Immunity* 2012, **37**:880-892.
157. Ozmen L, Roman D, Fountoulakis M, Schmid G, Ryffel B, Garotta G: **Experimental therapy of systemic lupus erythematosus: the treatment of NZB/W mice with mouse soluble interferon-gamma receptor inhibits the onset of glomerulonephritis**. *Eur J Immunol* 1995, **25**:6-12.
158. Balomenos D, Rumold R, Theofilopoulos AN: **Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice**. *J Clin Invest* 1998, **101**:364-371.
159. Ding BB, Bi E, Chen H, Yu JJ, Ye BH: **IL-21 and CD40L synergistically promote plasma cell differentiation through upregulation of Blimp-1 in human B cells**. *J Immunol* 2013, **190**:1827-1836.
160. Bubier JA, Sproule TJ, Foreman O, Spolski R, Shaffer DJ, Morse HC, 3rd, Leonard WJ, Roopenian DC: **A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice**. *Proc Natl Acad Sci U S A* 2009, **106**:1518-1523.
161. Wu HY, Staines NA: **A deficiency of CD4+CD25+ T cells permits the development of spontaneous lupus-like disease in mice, and can be reversed by induction of mucosal tolerance to histone peptide autoantigen**. *Lupus* 2004, **13**:192-200.
162. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI: **Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells**. *J Immunol* 2006, **177**:1451-1459.
163. Iikuni N, Lourenco EV, Hahn BH, La Cava A: **Cutting edge: Regulatory T cells directly suppress B cells in systemic lupus erythematosus**. *J Immunol* 2009, **183**:1518-1522.
164. Bagavant H, Tung KS: **Failure of CD25+ T cells from lupus-prone mice to suppress lupus glomerulonephritis and sialoadenitis**. *J Immunol* 2005, **175**:944-950.
165. Monk CR, Spachidou M, Rovis F, Leung E, Botto M, Lechler RI, Garden OA: **MRL/Mp CD4+,CD25- T cells show reduced sensitivity to suppression by CD4+,CD25+ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus**. *Arthritis Rheum* 2005, **52**:1180-1184.
166. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, Yu D, Domaschenz H, Whittle B, Lambe T, et al.: **A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity**. *Nature* 2005, **435**:452-458.
167. Moulton VR, Tsokos GC: **Abnormalities of T cell signaling in systemic lupus erythematosus**. *Arthritis Res Ther* 2011, **13**:207.
168. Liu MF, Liu HS, Wang CR, Lei HY: **Expression of CTLA-4 molecule in peripheral blood T lymphocytes from patients with systemic lupus erythematosus**. *J Clin Immunol* 1998, **18**:392-398.
169. Jury EC, Isenberg DA, Mauri C, Ehrenstein MR: **Atorvastatin restores Lck expression and lipid raft-associated signaling in T cells from patients with systemic lupus erythematosus**. *J Immunol* 2006, **177**:7416-7422.
170. Nambiar MP, Fisher CU, Kumar A, Tsokos CG, Warke VG, Tsokos GC: **Forced expression of the Fc receptor gamma-chain renders human T cells hyperresponsive to TCR/CD3 stimulation**. *J Immunol* 2003, **170**:2871-2876.
171. Koshy M, Berger D, Crow MK: **Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes**. *J Clin Invest* 1996, **98**:826-837.
172. Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK: **Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production**. *J Clin Invest* 1996, **97**:2063-2073.
173. Kyttaris VC, Juang YT, Tenbrock K, Weinstein A, Tsokos GC: **Cyclic adenosine 5'-monophosphate response element modulator is responsible for the decreased expression of c-fos and**

activator protein-1 binding in T cells from patients with systemic lupus erythematosus. *J Immunol* 2004, **173**:3557-3563.
174. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA: **CD4+CD25high regulatory cells in human peripheral blood**. *J Immunol* 2001, **167**:1245-1253.
175. Fontenot JD, Gavin MA, Rudensky AY: **Foxp3 programs the development and function of CD4+CD25+ regulatory T cells**. *Nat Immunol* 2003, **4**:330-336.
176. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD: **The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3**. *Nat Genet* 2001, **27**:20-21.
177. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, et al.: **CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells**. *J Exp Med* 2006, **203**:1701-1711.
178. Tran DQ, Ramsey H, Shevach EM: **Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype**. *Blood* 2007, **110**:2983-2990.
179. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW: **Helios expression is a marker of T cell activation and proliferation**. *PLoS One* 2011, **6**:e24226.
180. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, et al.: **Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells**. *J Exp Med* 2006, **203**:1693-1700.
181. Crispin JC, Martinez A, Alcocer-Varela J: **Quantification of regulatory T cells in patients with systemic lupus erythematosus**. *J Autoimmun* 2003, **21**:273-276.
182. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, Nochy D, Debre P, Piette JC, Gorochov G: **Global natural regulatory T cell depletion in active systemic lupus erythematosus**. *J Immunol* 2005, **175**:8392-8400.
183. Bonelli M, von Dalwigk K, Savitskaya A, Smolen JS, Scheinecker C: **Foxp3 expression in CD4+ T cells of patients with systemic lupus erythematosus: a comparative phenotypic analysis**. *Ann Rheum Dis* 2008, **67**:664-671.
184. Yates J, Whittington A, Mitchell P, Lechler RI, Lightstone L, Lombardi G: **Natural regulatory T cells: number and function are normal in the majority of patients with lupus nephritis**. *Clin Exp Immunol* 2008, **153**:44-55.
185. Venigalla RK, Tretter T, Krienke S, Max R, Eckstein V, Blank N, Fiehn C, Ho AD, Lorenz HM: **Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127-/low regulatory T cells in patients with active systemic lupus erythematosus**. *Arthritis Rheum* 2008, **58**:2120-2130.
186. Valencia X, Yarboro C, Illei G, Lipsky PE: **Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus**. *J Immunol* 2007, **178**:2579-2588.
187. Lyssuk EY, Torgashina AV, Soloviev SK, Nassonov EL, Bykovskaia SN: **Reduced number and function of CD4+CD25highFoxP3+ regulatory T cells in patients with systemic lupus erythematosus**. *Adv Exp Med Biol* 2007, **601**:113-119.
188. Barath S, Aleksza M, Tarr T, Sipka S, Szegedi G, Kiss E: **Measurement of natural (CD4+CD25high) and inducible (CD4+IL-10+) regulatory T cells in patients with systemic lupus erythematosus**. *Lupus* 2007, **16**:489-496.
189. Platanias LC: **Introduction: interferon signals: what is classical and what is nonclassical?** *J Interferon Cytokine Res* 2005, **25**:732.
190. Platanias LC: **Mechanisms of type-I- and type-II-interferon-mediated signalling**. *Nat Rev Immunol* 2005, **5**:375-386.
191. Pascual V, Farkas L, Banchereau J: **Systemic lupus erythematosus: all roads lead to type I interferons**. *Curr Opin Immunol* 2006, **18**:676-682.

192. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, Shark KB, Grande WJ, Hughes KM, Kapur V, et al.: **Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus**. *Proc Natl Acad Sci U S A* 2003, **100**:2610-2615.
193. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, et al.: **IRF-7 is the master regulator of type-I interferon-dependent immune responses**. *Nature* 2005, **434**:772-777.
194. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF: **Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo**. *Immunity* 2001, **14**:461-470.
195. Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF: **Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus**. *Arthritis Rheum* 2005, **52**:201-211.
196. Andersen LS, Petersen J, Svenson M, Bendtzen K: **Production of IL-1beta, IL-1 receptor antagonist and IL-10 by mononuclear cells from patients with SLE**. *Autoimmunity* 1999, **30**:235-242.
197. Suzuki H, Takemura H, Kashiwagi H: **Interleukin-1 receptor antagonist in patients with active systemic lupus erythematosus. Enhanced production by monocytes and correlation with disease activity**. *Arthritis Rheum* 1995, **38**:1055-1059.
198. Sturfelt G, Roux-Lombard P, Wollheim FA, Dayer JM: **Low levels of interleukin-1 receptor antagonist coincide with kidney involvement in systemic lupus erythematosus**. *Br J Rheumatol* 1997, **36**:1283-1289.
199. Alcocer-Varela J, Alarcon-Segovia D: **Decreased production of and response to interleukin-2 by cultured lymphocytes from patients with systemic lupus erythematosus**. *J Clin Invest* 1982, **69**:1388-1392.
200. Viallard JF, Pellegrin JL, Ranchin V, Schaeverbeke T, Dehais J, Longy-Boursier M, Ragnaud JM, Leng B, Moreau JF: **Th1 (IL-2, interferon-gamma (IFN-gamma)) and Th2 (IL-10, IL-4) cytokine production by peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE)**. *Clin Exp Immunol* 1999, **115**:189-195.
201. Danieli MG, Paoletti P, Recchioni A, Gabrielli A, Danieli G: **Serum levels of soluble interleukin-2 receptor in patients with systemic lupus erythematosus and systemic idiopathic vasculitis**. *Scand J Rheumatol* 1993, **22**:215-219.
202. Airo P, Braga S, Prati E, Brugnoni D, Bettinzioli M, Gorla R, Cattaneo R: **Evaluation of serum levels of soluble interleukin-2 receptor as an indicator of disease activity in systemic lupus erythematosus**. *Clin Rheumatol* 1992, **11**:97-100.
203. Tokano Y, Murashima A, Takasaki Y, Hashimoto H, Okumura K, Hirose S: **Relation between soluble interleukin 2 receptor and clinical findings in patients with systemic lupus erythematosus**. *Ann Rheum Dis* 1989, **48**:803-809.
204. Swaak AJ, Hintzen RQ, Huysen V, van den Brink HG, Smeenk JT: **Serum levels of soluble forms of T cell activation antigens CD27 and CD25 in systemic lupus erythematosus in relation with lymphocytes count and disease course**. *Clin Rheumatol* 1995, **14**:293-300.
205. ter Borg EJ, Horst G, Limburg PC, Kallenberg CG: **Changes in plasma levels of interleukin-2 receptor in relation to disease exacerbations and levels of anti-dsDNA and complement in systemic lupus erythematosus**. *Clin Exp Immunol* 1990, **82**:21-26.
206. Klashman DJ, Martin RA, Martinez-Maza O, Stevens RH: **In vitro regulation of B cell differentiation by interleukin-6 and soluble CD23 in systemic lupus erythematosus B cell subpopulations and antigen-induced normal B cells**. *Arthritis Rheum* 1991, **34**:276-286.
207. Jones BM, Liu T, Wong RW: **Reduced in vitro production of interferon-gamma, interleukin-4 and interleukin-12 and increased production of interleukin-6, interleukin-10 and tumour necrosis factor-alpha in systemic lupus erythematosus. Weak correlations of cytokine production with disease activity**. *Autoimmunity* 1999, **31**:117-124.

208. Hagiwara E, Gourley MF, Lee S, Klinman DK: **Disease severity in patients with systemic lupus erythematosus correlates with an increased ratio of interleukin-10:interferon-gamma-secreting cells in the peripheral blood**. *Arthritis Rheum* 1996, **39**:379-385.
209. Vitali C, Bencivelli W, Isenberg DA, Smolen JS, Snaith ML, Sciuto M, d'Ascanio A, Bombardieri S: **Disease activity in systemic lupus erythematosus: report of the Consensus Study Group of the European Workshop for Rheumatology Research. I. A descriptive analysis of 704 European lupus patients. European Consensus Study Group for Disease Activity in SLE**. *Clin Exp Rheumatol* 1992, **10**:527-539.
210. Boehme MW, Raeth U, Galle PR, Stremmel W, Scherbaum WA: **Serum thrombomodulin-a reliable marker of disease activity in systemic lupus erythematosus (SLE): advantage over established serological parameters to indicate disease activity**. *Clin Exp Immunol* 2000, **119**:189-195.
211. Grondal G, Gunnarsson I, Ronnelid J, Rogberg S, Klareskog L, Lundberg I: **Cytokine production, serum levels and disease activity in systemic lupus erythematosus**. *Clin Exp Rheumatol* 2000, **18**:565-570.
212. Studnicka-Benke A, Steiner G, Petera P, Smolen JS: **Tumour necrosis factor alpha and its soluble receptors parallel clinical disease and autoimmune activity in systemic lupus erythematosus**. *Br J Rheumatol* 1996, **35**:1067-1074.
213. Peterson E, Robertson AD, Emlen W: **Serum and urinary interleukin-6 in systemic lupus erythematosus**. *Lupus* 1996, **5**:571-575.
214. Yin Z, Bahtiyar G, Zhang N, Liu L, Zhu P, Robert ME, McNiff J, Madaio MP, Craft J: **IL-10 regulates murine lupus**. *J Immunol* 2002, **169**:2148-2155.
215. Ishida H, Muchamuel T, Sakaguchi S, Andrade S, Menon S, Howard M: **Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice**. *J Exp Med* 1994, **179**:305-310.
216. Barcellini W, Rizzardi GP, Borghi MO, Nicoletti F, Fain C, Del Papa N, Meroni PL: **In vitro type-1 and type-2 cytokine production in systemic lupus erythematosus: lack of relationship with clinical disease activity**. *Lupus* 1996, **5**:139-145.
217. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, et al.: **Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function**. *Nature* 2000, **408**:57-63.
218. Wurster AL, Rodgers VL, Satoskar AR, Whitters MJ, Young DA, Collins M, Grusby MJ: **Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells**. *J Exp Med* 2002, **196**:969-977.
219. Fantini MC, Rizzo A, Fina D, Caruso R, Becker C, Neurath MF, Macdonald TT, Pallone F, Monteleone G: **IL-21 regulates experimental colitis by modulating the balance between Treg and Th17 cells**. *Eur J Immunol* 2007, **37**:3155-3163.
220. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK: **IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells**. *Nature* 2007, **448**:484-487.
221. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, et al.: **Essential autocrine regulation by IL-21 in the generation of inflammatory T cells**. *Nature* 2007, **448**:480-483.
222. King C, Tangye SG, Mackay CR: **T follicular helper (TFH) cells in normal and dysregulated immune responses**. *Annu Rev Immunol* 2008, **26**:741-766.
223. Kasaian MT, Whitters MJ, Carter LL, Lowe LD, Jussif JM, Deng B, Johnson KA, Witek JS, Senices M, Konz RF, et al.: **IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity**. *Immunity* 2002, **16**:559-569.
224. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE: **IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells**. *J Immunol* 2005, **175**:7867-7879.

225. Pene J, Gauchat JF, Lecart S, Drouet E, Guglielmi P, Boulay V, Delwail A, Foster D, Lecron JC, Yssel H: **Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells**. *J Immunol* 2004, **172**:5154-5157.
226. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, Vinuesa CG: **IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses**. *J Exp Med* 2010, **207**:353-363.
227. Sawalha AH, Kaufman KM, Kelly JA, Adler AJ, Aberle T, Kilpatrick J, Wakeland EK, Li QZ, Wandstrat AE, Karp DR, et al.: **Genetic association of interleukin-21 polymorphisms with systemic lupus erythematosus**. *Ann Rheum Dis* 2008, **67**:458-461.
228. Webb R, Merrill JT, Kelly JA, Sestak A, Kaufman KM, Langefeld CD, Ziegler J, Kimberly RP, Edberg JC, Ramsey-Goldman R, et al.: **A polymorphism within IL21R confers risk for systemic lupus erythematosus**. *Arthritis Rheum* 2009, **60**:2402-2407.
229. Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, et al.: **Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6**. *J Immunol* 2004, **173**:5361-5371.
230. Wong CK, Ho CY, Li EK, Lam CW: **Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus**. *Lupus* 2000, **9**:589-593.
231. Amerio P, Frezzolini A, Abeni D, Teofoli P, Girardelli CR, De Pita O, Puddu P: **Increased IL-18 in patients with systemic lupus erythematosus: relations with Th-1, Th-2, pro-inflammatory cytokines and disease activity. IL-18 is a marker of disease activity but does not correlate with pro-inflammatory cytokines**. *Clin Exp Rheumatol* 2002, **20**:535-538.
232. Kuroiwa T, Schlimgen R, Illei GG, Boumpas DT: **Monocyte response to Th1 stimulation and effector function toward human mesangial cells are not impaired in patients with lupus nephritis**. *Clin Immunol* 2003, **106**:65-72.
233. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB: **Development of autoantibodies before the clinical onset of systemic lupus erythematosus**. *N Engl J Med* 2003, **349**:1526-1533.
234. ter Borg EJ, Horst G, Hummel EJ, Limburg PC, Kallenberg CG: **Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long-term, prospective study**. *Arthritis Rheum* 1990, **33**:634-643.
235. Ellyard JI, Jerjen R, Martin JL, Lee AY, Field MA, Jiang SH, Cappello J, Naumann SK, Andrews TD, Scott HS, et al.: **Identification of a pathogenic variant in TREX1 in early-onset cerebral systemic lupus erythematosus by Whole-exome sequencing**. *Arthritis Rheumatol* 2014, **66**:3382-3386.
236. Yasutomo K, Horiuchi T, Kagami S, Tsukamoto H, Hashimura C, Urushihara M, Kuroda Y: **Mutation of DNASE1 in people with systemic lupus erythematosus**. *Nat Genet* 2001, **28**:313-314.
237. Gunther C, Kind B, Reijns MA, Berndt N, Martinez-Bueno M, Wolf C, Tungler V, Chara O, Lee YA, Hubner N, et al.: **Defective removal of ribonucleotides from DNA promotes systemic autoimmunity**. *J Clin Invest* 2015, **125**:413-424.
238. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S: **Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848**. *Nat Immunol* 2002, **3**:499.
239. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, et al.: **A Toll-like receptor recognizes bacterial DNA**. *Nature* 2000, **408**:740-745.
240. Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A: **Activation of autoreactive B cells by CpG dsDNA**. *Immunity* 2003, **19**:837-847.
241. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S: **Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication**. *Science* 2006, **312**:1669-1672.

242. Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, Liang C, Bartov G, McDaniel LD, Zhou XJ, et al.: **A Tlr7 translocation accelerates systemic autoimmunity in murine lupus**. *Proc Natl Acad Sci U S A* 2006, **103**:9970-9975.
243. Forabosco P, Gorman JD, Cleveland C, Kelly JA, Fisher SA, Ortmann WA, Johansson C, Johanneson B, Moser KL, Gaffney PM, et al.: **Meta-analysis of genome-wide linkage studies of systemic lupus erythematosus**. *Genes Immun* 2006, **7**:609-614.
244. Fernando MM, Stevens CR, Walsh EC, De Jager PL, Goyette P, Plenge RM, Vyse TJ, Rioux JD: **Defining the role of the MHC in autoimmunity: a review and pooled analysis**. *PLoS Genet* 2008, **4**:e1000024.
245. Tsao BP: **Update on human systemic lupus erythematosus genetics**. *Curr Opin Rheumatol* 2004, **16**:513-521.
246. Pickering MC, Walport MJ: **Links between complement abnormalities and systemic lupus erythematosus**. *Rheumatology (Oxford)* 2000, **39**:133-141.
247. Naves M, Hajeer AH, Teh LS, Davies EJ, Ordi-Ros J, Perez-Pemen P, Vilardel-Tarres M, Thomson W, Worthington J, Ollier WE: **Complement C4B null allele status confers risk for systemic lupus erythematosus in a Spanish population**. *Eur J Immunogenet* 1998, **25**:317-320.
248. Aggarwal R, Sestak AL, D'Sousa A, Dillon SP, Namjou B, Scofield RH: **Complete complement deficiency in a large cohort of familial systemic lupus erythematosus**. *Lupus* 2010, **19**:52-57.
249. Gough SC, Simmonds MJ: **The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action**. *Curr Genomics* 2007, **8**:453-465.
250. Zanelli E, Breedveld FC, de Vries RR: **HLA association with autoimmune disease: a failure to protect?** *Rheumatology (Oxford)* 2000, **39**:1060-1066.
251. Ettinger RA, Papadopoulos GK, Moustakas AK, Nepom GT, Kwok WW: **Allelic variation in key peptide-binding pockets discriminates between closely related diabetes-protective and diabetes-susceptible HLA-DQB1*06 alleles**. *J Immunol* 2006, **176**:1988-1998.
252. Gromme M, Neefjes J: **Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways**. *Mol Immunol* 2002, **39**:181-202.
253. Ezkurdia I, Juan D, Rodriguez JM, Frankish A, Diekhans M, Harrow J, Vazquez J, Valencia A, Tress ML: **Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes**. *Hum Mol Genet* 2014, **23**:5866-5878.
254. Wolfsberg TG, McEntyre J, Schuler GD: **Guide to the draft human genome**. *Nature* 2001, **409**:824-826.
255. Hastings PJ, Lupski JR, Rosenberg SM, Ira G: **Mechanisms of change in gene copy number**. *Nat Rev Genet* 2009, **10**:551-564.
256. Rose AB: **Intron-mediated regulation of gene expression**. *Curr Top Microbiol Immunol* 2008, **326**:277-290.
257. Scrimshaw NS, Murray EB: **The acceptability of milk and milk products in populations with a high prevalence of lactose intolerance**. *Am J Clin Nutr* 1988, **48**:1079-1159.
258. Orr N, Chanock S: **Common genetic variation and human disease**. *Adv Genet* 2008, **62**:1-32.
259. Hughes AL, Packer B, Welch R, Chanock SJ, Yeager M: **High level of functional polymorphism indicates a unique role of natural selection at human immune system loci**. *Immunogenetics* 2005, **57**:821-827.
260. Jacobs PA, Browne C, Gregson N, Joyce C, White H: **Estimates of the frequency of chromosome abnormalities detectable in unselected newborns using moderate levels of banding**. *J Med Genet* 1992, **29**:103-108.
261. Kruglyak L, Nickerson DA: **Variation is the spice of life**. *Nat Genet* 2001, **27**:234-236.
262. Nobrega MA, Zhu Y, Plajzer-Frick I, Afzal V, Rubin EM: **Megabase deletions of gene deserts result in viable mice**. *Nature* 2004, **431**:988-993.

263. Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD: **Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays**. *Science* 2003, **302**:2141-2144.
264. Bejerano G, Pheasant M, Makunin I, Stephen S, Kent WJ, Mattick JS, Haussler D: **Ultraconserved elements in the human genome**. *Science* 2004, **304**:1321-1325.
265. Krawczak M, Ball EV, Fenton I, Stenson PD, Abeysinghe S, Thomas N, Cooper DN: **Human gene mutation database-a biomedical information and research resource**. *Hum Mutat* 2000, **15**:45-51.
266. McCarroll SA, Altshuler DM: **Copy-number variation and association studies of human disease**. *Nat Genet* 2007, **39**:S37-42.
267. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, et al.: **Global variation in copy number in the human genome**. *Nature* 2006, **444**:444-454.
268. Bruder CE, Piotrowski A, Gijsbers AA, Andersson R, Erickson S, Diaz de Stahl T, Menzel U, Sandgren J, von Tell D, Poplawski A, et al.: **Phenotypically concordant and discordant monozygotic twins display different DNA copy-number-variation profiles**. *Am J Hum Genet* 2008, **82**:763-771.
269. Piotrowski A, Bruder CE, Andersson R, Diaz de Stahl T, Menzel U, Sandgren J, Poplawski A, von Tell D, Crasto C, Bogdan A, et al.: **Somatic mosaicism for copy number variation in differentiated human tissues**. *Hum Mutat* 2008, **29**:1118-1124.
270. Beckmann JS, Estivill X, Antonarakis SE: **Copy number variants and genetic traits: closer to the resolution of phenotypic to genotypic variability**. *Nat Rev Genet* 2007, **8**:639-646.
271. Nguyen DQ, Webber C, Ponting CP: **Bias of selection on human copy-number variants**. *PLoS Genet* 2006, **2**:e20.
272. Hollox EJ, Huffmeier U, Zeeuwen PL, Palla R, Lascorz J, Rodijk-Olthuis D, van de Kerkhof PC, Traupe H, de Jongh G, den Heijer M, et al.: **Psoriasis is associated with increased beta-defensin genomic copy number**. *Nat Genet* 2008, **40**:23-25.
273. Fellermann K, Stange DE, Schaeffeler E, Schmalzl H, Wehkamp J, Bevins CL, Reinisch W, Teml A, Schwab M, Lichter P, et al.: **A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon**. *Am J Hum Genet* 2006, **79**:439-448.
274. Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, Mangion J, Roberton-Lowe C, Marshall AJ, Petretto E, et al.: **Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans**. *Nature* 2006, **439**:851-855.
275. Tyler C, Edman JC: **Down syndrome, Turner syndrome, and Klinefelter syndrome: primary care throughout the life span**. *Prim Care* 2004, **31**:627-648, x-xi.
276. Amano H, Amano E, Moll T, Marinkovic D, Ibnou-Zekri N, Martinez-Soria E, Semac I, Wirth T, Nitschke L, Izui S: **The Yaa mutation promoting murine lupus causes defective development of marginal zone B cells**. *J Immunol* 2003, **170**:2293-2301.
277. Sawalha AH, Harley JB, Scofield RH: **Autoimmunity and Klinefelter's syndrome: when men have two X chromosomes**. *J Autoimmun* 2009, **33**:31-34.
278. Bush WS, Moore JH: **Chapter 11: Genome-wide association studies**. *PLoS Comput Biol* 2012, **8**:e1002822.
279. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: **Identification of the cystic fibrosis gene: genetic analysis**. *Science* 1989, **245**:1073-1080.
280. Kazazian HH, Jr., Wong C, Youssoufian H, Scott AF, Phillips DG, Antonarakis SE: **Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man**. *Nature* 1988, **332**:164-166.
281. Ramos PS, Brown EE, Kimberly RP, Langefeld CD: **Genetic factors predisposing to systemic lupus erythematosus and lupus nephritis**. *Semin Nephrol* 2010, **30**:164-176.

282. Hunt KA, Mistry V, Bockett NA, Ahmad T, Ban M, Barker JN, Barrett JC, Blackburn H, Brand O, Burren O, et al.: **Negligible impact of rare autoimmune-locus coding-region variants on missing heritability**. *Nature* 2013, **498**:232-235.
283. Belkadi A, Bolze A, Itan Y, Cobat A, Vincent QB, Antipenko A, Shang L, Boisson B, Casanova JL, Abel L: **Whole-genome sequencing is more powerful than whole-exome sequencing for detecting exome variants**. *Proc Natl Acad Sci U S A* 2015, **112**:5473-5478.
284. Ng PC, Henikoff S: **SIFT: Predicting amino acid changes that affect protein function**. *Nucleic Acids Res* 2003, **31**:3812-3814.
285. Ramensky V, Bork P, Sunyaev S: **Human non-synonymous SNPs: server and survey**. *Nucleic Acids Res* 2002, **30**:3894-3900.
286. Kircher T, Krug A, Stratmann M, Ghazi S, Schales C, Frauenheim M, Turner L, Fahrmann P, Hornig T, Katzev M, et al.: **A rating scale for the assessment of objective and subjective formal Thought and Language Disorder (TALD)**. *Schizophr Res* 2014, **160**:216-221.
287. Flanagan SE, Patch AM, Ellard S: **Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations**. *Genet Test Mol Biomarkers* 2010, **14**:533-537.
288. Grimm DG, Azencott CA, Aicheler F, Gieraths U, MacArthur DG, Samocha KE, Cooper DN, Stenson PD, Daly MJ, Smoller JW, et al.: **The evaluation of tools used to predict the impact of missense variants is hindered by two types of circularity**. *Hum Mutat* 2015, **36**:513-523.
289. Miosge LA, Field MA, Sontani Y, Cho V, Johnson S, Palkova A, Balakishnan B, Liang R, Zhang Y, Lyon S, et al.: **Comparison of predicted and actual consequences of missense mutations**. *Proc Natl Acad Sci U S A* 2015, **112**:E5189-5198.
290. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, Walker A, Mack TM: **A revised estimate of twin concordance in systemic lupus erythematosus**. *Arthritis Rheum* 1992, **35**:311-318.
291. Lawrence JS, Martins CL, Drake GL: **A family survey of lupus erythematosus. 1. Heritability**. *J Rheumatol* 1987, **14**:913-921.
292. Javierre BM, Fernandez AF, Richter J, Al-Shahrour F, Martin-Subero JI, Rodriguez-Ubreva J, Berdasco M, Fraga MF, O'Hanlon TP, Rider LG, et al.: **Changes in the pattern of DNA methylation associate with twin discordance in systemic lupus erythematosus**. *Genome Res* 2010, **20**:170-179.
293. Alarcon-Segovia D, Alarcon-Riquelme ME, Cardiel MH, Caeiro F, Massardo L, Villa AR, Pons-Estel BA, Grupo Latinoamericano de Estudio del Lupus E: **Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort**. *Arthritis Rheum* 2005, **52**:1138-1147.
294. Kaufman KM, Kelly JA, Herring BJ, Adler AJ, Glenn SB, Namjou B, Frank SG, Dawson SL, Bruner GR, James JA, et al.: **Evaluation of the genetic association of the PTPN22 R620W polymorphism in familial and sporadic systemic lupus erythematosus**. *Arthritis Rheum* 2006, **54**:2533-2540.
295. Stetson DB, Ko JS, Heidmann T, Medzhitov R: **Trex1 prevents cell-intrinsic initiation of autoimmunity**. *Cell* 2008, **134**:587-598.
296. Kozyrev SV, Abelson AK, Wojcik J, Zaghlool A, Linga Reddy MV, Sanchez E, Gunnarsson I, Svenungsson E, Sturfelt G, Jonsen A, et al.: **Functional variants in the B-cell gene BANK1 are associated with systemic lupus erythematosus**. *Nat Genet* 2008, **40**:211-216.
297. International Consortium for Systemic Lupus Erythematosus G, Harley JB, Alarcon-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, Moser KL, Tsao BP, Vyse TJ, Langefeld CD, et al.: **Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci**. *Nat Genet* 2008, **40**:204-210.
298. Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S, Lee AT, Chung SA, Ferreira RC, Pant PV, et al.: **Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX**. *N Engl J Med* 2008, **358**:900-909.

299. Castillejo-Lopez C, Delgado-Vega AM, Wojcik J, Kozyrev SV, Thavathiru E, Wu YY, Sanchez E, Pollmann D, Lopez-Egido JR, Fineschi S, et al.: **Genetic and physical interaction of the B-cell systemic lupus erythematosus-associated genes BANK1 and BLK**. *Annals of the Rheumatic Diseases* 2012, **71**:136-142.
300. Moser KL, Kelly JA, Lessard CJ, Harley JB: **Recent insights into the genetic basis of systemic lupus erythematosus**. *Genes Immun* 2009, **10**:373-379.
301. Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ, Ercan-Sencicek AG, DiLullo NM, Parikshak NN, Stein JL, et al.: **De novo mutations revealed by whole-exome sequencing are strongly associated with autism**. *Nature* 2012, **485**:237-241.
302. Epi KC, Epilepsy Phenome/Genome P, Allen AS, Berkovic SF, Cossette P, Delanty N, Dlugos D, Eichler EE, Epstein MP, Glauser T, et al.: **De novo mutations in epileptic encephalopathies**. *Nature* 2013, **501**:217-221.
303. Vidal S, Kono DH, Theofilopoulos AN: **Loci predisposing to autoimmunity in MRL-Fas lpr and C57BL/6-Faslpr mice**. *J Clin Invest* 1998, **101**:696-702.
304. Belot A, Cimaz R: **Monogenic forms of systemic lupus erythematosus: new insights into SLE pathogenesis**. *Pediatr Rheumatol Online J* 2012, **10**:21.
305. Waters H, Konrad P, Walford RL: **The distribution of HL-A histocompatibility factors and genes in patients with systemic lupus erythematosus**. *Tissue Antigens* 1971, **1**:68-73.
306. Fernando MM, Stevens CR, Sabeti PC, Walsh EC, McWhinnie AJ, Shah A, Green T, Rioux JD, Vyse TJ: **Identification of two independent risk factors for lupus within the MHC in United Kingdom families**. *PLoS Genet* 2007, **3**:e192.
307. Brown EE, Edberg JC, Kimberly RP: **Fc receptor genes and the systemic lupus erythematosus diathesis**. *Autoimmunity* 2007, **40**:567-581.
308. Karassa FB, Trikalinos TA, Ioannidis JP, Fc gamma R-SLEm-ai: **The Fc gamma RIIIA-F158 allele is a risk factor for the development of lupus nephritis: a meta-analysis**. *Kidney Int* 2003, **63**:1475-1482.
309. Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, Sturfelt G, Jonsen A, Rantapaa-Dahlqvist S, Moller B, Kere J, et al.: **Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus**. *Am J Hum Genet* 2005, **76**:528-537.
310. Graham RR, Kyogoku C, Sigurdsson S, Vlasova IA, Davies LR, Baechler EC, Plenge RM, Koeuth T, Ortmann WA, Hom G, et al.: **Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus**. *Proc Natl Acad Sci U S A* 2007, **104**:6758-6763.
311. Botto M, Walport MJ: **C1q, autoimmunity and apoptosis**. *Immunobiology* 2002, **205**:395-406.
312. Fielder AH, Walport MJ, Batchelor JR, Rynes RI, Black CM, Dodi IA, Hughes GR: **Family study of the major histocompatibility complex in patients with systemic lupus erythematosus: importance of null alleles of C4A and C4B in determining disease susceptibility**. *Br Med J (Clin Res Ed)* 1983, **286**:425-428.
313. Yang Y, Chung EK, Wu YL, Savelli SL, Nagaraja HN, Zhou B, Hebert M, Jones KN, Shu Y, Kitzmiller K, et al.: **Gene copy-number variation and associated polymorphisms of complement component C4 in human systemic lupus erythematosus (SLE): low copy number is a risk factor for and high copy number is a protective factor against SLE susceptibility in European Americans**. *Am J Hum Genet* 2007, **80**:1037-1054.
314. George J, Motshwene PG, Wang H, Kubarenko AV, Rautanen A, Mills TC, Hill AV, Gay NJ, Weber AN: **Two human MYD88 variants, S34Y and R98C, interfere with MyD88-IRAK4-myddosome assembly**. *The Journal of biological chemistry* 2011, **286**:1341-1353.
315. Keating SE, Maloney GM, Moran EM, Bowie AG: **IRAK-2 participates in multiple toll-like receptor signaling pathways to NFkappaB via activation of TRAF6 ubiquitination**. *J Biol Chem* 2007, **282**:33435-33443.

316. Chaussabel D, Baldwin N: **Democratizing systems immunology with modular transcriptional repertoire analyses**. *Nat Rev Immunol* 2014, **14**:271-280.
317. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al.: **Multiplex genome engineering using CRISPR/Cas systems**. *Science* 2013, **339**:819-823.
318. Jacob CO, Zhu J, Armstrong DL, Yan M, Han J, Zhou XJ, Thomas JA, Reiff A, Myones BL, Ojwang JO, et al.: **Identification of IRAK1 as a risk gene with critical role in the pathogenesis of systemic lupus erythematosus**. *Proc Natl Acad Sci U S A* 2009, **106**:6256-6261.
319. Muzio M, Ni J, Feng P, Dixit VM: **IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling**. *Science* 1997, **278**:1612-1615.
320. Wu YY, Kumar R, Haque MS, Castillejo-Lopez C, Alarcon-Riquelme ME: **BANK1 controls CpG-induced IL-6 secretion via a p38 and MNK1/2/eIF4E translation initiation pathway**. *J Immunol* 2013, **191**:6110-6116.
321. Yokoyama K, Su I, Tezuka T, Yasuda T, Mikoshiba K, Tarakhovsky A, Yamamoto T: **BANK regulates BCR-induced calcium mobilization by promoting tyrosine phosphorylation of IP3 receptor**. *Embo Journal* 2002, **21**:83-92.
322. Bernal-Quiros M, Wu YY, Alarcon-Riquelme ME, Castillejo-Lopez C: **BANK1 and BLK act through phospholipase C gamma 2 in B-cell signaling**. *PLoS One* 2013, **8**:e59842.
323. Delgado-Vega AM, Dozmorov MG, Quiros MB, Wu YY, Martinez-Garcia B, Kozyrev SV, Frostegard J, Truedsson L, de Ramon E, Gonzalez-Escribano MF, et al.: **Fine mapping and conditional analysis identify a new mutation in the autoimmunity susceptibility gene BLK that leads to reduced half-life of the BLK protein**. *Ann Rheum Dis* 2012, **71**:1219-1226.
324. Fujimoto M, Poe JC, Jansen PJ, Sato S, Tedder TF: **CD19 amplifies B lymphocyte signal transduction by regulating Src-family protein tyrosine kinase activation**. *J Immunol* 1999, **162**:7088-7094.
325. Boggon TJ, Eck MJ: **Structure and regulation of Src family kinases**. *Oncogene* 2004, **23**:7918-7927.
326. Oda H, Kumar S, Howley PM: **Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination**. *Proc Natl Acad Sci U S A* 1999, **96**:9557-9562.
327. Wang D, Stewart AK, Zhuang L, Zhu Y, Wang Y, Shi C, Keating A, Slutsky A, Zhang H, Wen XY: **Enhanced adaptive immunity in mice lacking the immunoinhibitory adaptor Hacs1**. *FASEB J* 2010, **24**:947-956.
328. Smith KG, Tarlinton DM, Doody GM, Hibbs ML, Fearon DT: **Inhibition of the B cell by CD22: a requirement for Lyn**. *J Exp Med* 1998, **187**:807-811.
329. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V: **Interferon and granulopoiesis signatures in systemic lupus erythematosus blood**. *J Exp Med* 2003, **197**:711-723.
330. Ripley BJ, Goncalves B, Isenberg DA, Latchman DS, Rahman A: **Raised levels of interleukin 6 in systemic lupus erythematosus correlate with anaemia**. *Ann Rheum Dis* 2005, **64**:849-853.
331. Bengtsson H, Wirapati P, Speed TP: **A single-array preprocessing method for estimating full-resolution raw copy numbers from all Affymetrix genotyping arrays including GenomeWideSNP 5 & 6**. *Bioinformatics* 2009, **25**:2149-2156.
332. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss JW, 3rd, et al.: **BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources**. *Genome Biol* 2009, **10**:R130.
333. Rocque BL, Babayeva S, Li J, Leung V, Nezvitsky L, Cybulsky AV, Gros P, Torban E: **Deficiency of the planar cell polarity protein Vangl2 in podocytes affects glomerular morphogenesis and increases susceptibility to injury**. *J Am Soc Nephrol* 2015, **26**:576-586.
334. Yabas M, Teh CE, Frankenreiter S, Lal D, Roots CM, Whittle B, Andrews DT, Zhang Y, Teoh NC, Sprent J, et al.: **ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes**. *Nat Immunol* 2011, **12**:441-449.

335. Xu LG, Shu HB: **TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production**. *J Immunol* 2002, **169**:6883-6889.
336. Lu R, Vidal GS, Kelly JA, Delgado-Vega AM, Howard XK, Macwana SR, Dominguez N, Klein W, Burrell C, Harley IT, et al.: **Genetic associations of LYN with systemic lupus erythematosus**. *Genes Immun* 2009, **10**:397-403.
337. Genin E, Coustet B, Allanore Y, Ito I, Teruel M, Constantin A, Schaeverbeke T, Ruyssen-Witrand A, Tohma S, Cantagrel A, et al.: **Epistatic interaction between BANK1 and BLK in rheumatoid arthritis: results from a large trans-ethnic meta-analysis**. *PLoS One* 2013, **8**:e61044.
338. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Kawamoto M, Fujimoto M, Takehara K, Sato S, Hara M, et al.: **Association of the FAM167A-BLK region with systemic sclerosis**. *Arthritis Rheum* 2010, **62**:890-895.
339. Ito I, Kawasaki A, Ito S, Kondo Y, Sugihara M, Horikoshi M, Hayashi T, Goto D, Matsumoto I, Tsutsumi A, et al.: **Replication of association between FAM167A(C8orf13)-BLK region and rheumatoid arthritis in a Japanese population**. *Ann Rheum Dis* 2010, **69**:936-937.
340. Hibbs ML, Tarlinton DM, Armes J, Grail D, Hodgson G, Maglitto R, Stacker SA, Dunn AR: **Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease**. *Cell* 1995, **83**:301-311.
341. Yu CC, Yen TS, Lowell CA, DeFranco AL: **Lupus-like kidney disease in mice deficient in the Src family tyrosine kinases Lyn and Fyn**. *Curr Biol* 2001, **11**:34-38.
342. Texido G, Su IH, Mecklenbrauker I, Saijo K, Malek SN, Desiderio S, Rajewsky K, Tarakhovsky A: **The B-cell-specific Src-family kinase Blk is dispensable for B-cell development and activation**. *Mol Cell Biol* 2000, **20**:1227-1233.
343. Samuelson EM, Laird RM, Papillion AM, Tatum AH, Princiotta MF, Hayes SM: **Reduced B lymphoid kinase (Blk) expression enhances proinflammatory cytokine production and induces nephrosis in C57BL/6-lpr/lpr mice**. *PLoS One* 2014, **9**:e92054.
344. Cohick CB, Furst DE, Quagliata S, Corcoran KA, Steere KJ, Yager JG, Lindsley HB: **Analysis of elevated serum interleukin-6 levels in rheumatoid arthritis: correlation with erythrocyte sedimentation rate or C-reactive protein**. *J Lab Clin Med* 1994, **123**:721-727.
345. Grisius MM, Bermudez DK, Fox PC: **Salivary and serum interleukin 6 in primary Sjogren's syndrome**. *J Rheumatol* 1997, **24**:1089-1091.
346. Tsantikos E, Oracki SA, Quilici C, Anderson GP, Tarlinton DM, Hibbs ML: **Autoimmune disease in Lyn-deficient mice is dependent on an inflammatory environment established by IL-6**. *J Immunol* 2010, **184**:1348-1360.
347. Llorente L, Richaud-Patin Y, Couderc J, Alarcon-Segovia D, Ruiz-Soto R, Alcocer-Castillejos N, Alcocer-Varela J, Granados J, Bahena S, Galanaud P, et al.: **Dysregulation of interleukin-10 production in relatives of patients with systemic lupus erythematosus**. *Arthritis Rheum* 1997, **40**:1429-1435.
348. Kibar Z, Torban E, McDearmid JR, Reynolds A, Berghout J, Mathieu M, Kirillova I, De Marco P, Merello E, Hayes JM, et al.: **Mutations in VANGL1 associated with neural-tube defects**. *N Engl J Med* 2007, **356**:1432-1437.